

The Receptors

Gavril W. Pasternak
Editor

The Opiate Receptors

Second Edition



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THE RECEPTORS

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Editor

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Second Edition



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Preface

The growth of the opiate field has been enormous. Early work focused upon the strategic clinical importance of morphine and the attempt to develop non-addicting analogs with fewer side-effects, but the discovery of the receptors and the enkephalins and other endogenous opioid peptides and the recognition of their widespread actions within brain has expanded the field to include investigators in almost all areas of neuroscience and pharmacology. However, this field of research with its vast literature has become progressively more complex. The receptors are no longer limited to opiates, but include many subtypes selective for the opioid peptides. Indeed, they might be better termed opioid, rather than opiate, receptors. Many controversies have emerged and been settled; others remain. Early studies must now be interpreted on the basis of current information. Thousands of papers examining various aspects of opiates and the endogenous opioids present separate pieces of a large puzzle. The goal of this volume is to put the pieces together and attempt to obtain a coherent overview of opiate receptor pharmacology with insights into both the molecular and classical pharmacology of opiates and the opioid peptides. However, many pieces of this immense puzzle remain unknown and will need to be addressed in the future.

The study of opiates and opioid peptides provides a unique research opportunity in the neuropharmacology of drug receptors. The availability of a wide variety of agonist and antagonist ligands has permitted studies not possible in other systems. Second, the close association of opiate drugs with easily measurable pharmacological bioassays and behavioral responses permits the correlation of molecularly defined receptors with pharmacological actions and helps to bridge the gap between molecular and classical pharmacology. In this regard, the opiate system is relatively unique.

Understanding the multiple classes of opiate and opioid peptide receptors at the molecular level and functionally is the major focus of this second edition. Much has happened since the first edition of this volume. The greatest advance has been the cloning of the various classes of opioid receptors. This has opened new areas of investigation and provided greater insight into the biochemical understanding of the receptors and their actions. This second edition has tried to incorporate these new areas and merge them with the earlier studies. Sections of the book cover historical perspectives in the concept of multiple opiate receptors along with a general

overview of the opioid peptides and the molecular and functional characterization of the receptors. Through-out the entire volume, we have attempted to provide an integrated approach that builds on the groundwork set forth in the first edition, pulling together the biochemical, physiological, and pharmacological studies of opiate action. We feel that this volume will be a valuable resource for scientists actively working in the opiate field, as well as others interested in neuroscience and pharmacology in general.

New York, NY

Gavril W. Pasternak

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Chapter 1

You've Come a Long Way Baby!

Solomon H. Snyder

Abstract Current challenges in opiate pharmacology today are remarkably similar to those almost 40 years ago, when opiate receptors were first identified. There are two key problems to be resolved: (1) We need less-addicting opiates. (2) An understanding of addiction is lacking. Characterization of opiate receptors led to insights into the differentiation of agonists and antagonists. As mixed agonist–antagonists are less addicting, there were hopes for more effective, safer drugs – but these hopes have largely gone unrealized. Appreciation of opioid peptides as endogenous ligands for receptors portended promised further insights into addiction – but we still do not understand the fundamentals of the field. Cloning of receptors and creation of knockouts for them may help answer some of these questions.

Keywords Naloxone • Nicotinic receptor • Receptor multiplicity • Pro-opiomelanocortin • Methionine enkephalin

The field of opiate receptors and associated endogenous ligands, memorialized in the second edition of this now classic volume, has indeed “come a long way.” Our sophistication in understanding how opiates act is far greater than it was decades ago. The tools of molecular biology have greatly augmented insights into receptors and their ligands. And yet, some of the fundamental questions have not yet been satisfactorily addressed. What is the nature of the addictive process? Is there a rational approach to the design of less-addicting opiates based on differential influences upon subtypes of opiate receptors? Just what are the unique functions of the different opioid peptides, and which is physiologically associated with which receptors? I have not personally been involved in opiate research for many years and so will not address recent findings in detail. Instead let me attempt to shed

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historical perspective that may facilitate efforts of the new generations of opiate researchers.

In founding the American Philosophical Society, Benjamin Franklin established its mission as “the promotion of new and useful knowledge.” Of all the biomedical sciences, pharmacology best epitomizes this motto. Our goal is to understand the workings of the body, but we always bear in mind the need to apply our understanding to the development of novel therapies.

As a medical student, I adored pharmacology, even learning both the generic and brand names for major drugs. Working with Julie Axelrod at the NIH afforded many lessons on how neurotransmitters act and interface with drugs. When I came to Johns Hopkins for a psychiatry residency and then joined the faculty in pharmacology, my initial focus was upon how psychotropic drugs act via the biogenic amines. I knew nothing of opiates and could barely distinguish morphine from marijuana.

My entry into the opiate field stemmed from the pressures of the day related to the epidemics of heroin addiction in American cities and among our soldiers in Vietnam. My friend Jerry Jaffe, President Nixon’s “czar” of drug abuse, pressured me to “do something.” He put his money where his mouth was by creating a series of drug abuse research centers. Johns Hopkins was one of the initial grantees. I could have merely continued with our work on amphetamines and catecholamines, but chose instead to heed Jerry’s admonition and address the challenges of opiate research.

In those days – 1971–1972 – we knew much about the biosynthesis of neurotransmitters, their degradation, and their inactivation by reuptake or other mechanisms. However, how they acted at receptors had never been defined in molecular terms. Drug development typically utilized screens in intact rats. The paradigm derived from anti-convulsant development – hooking a rat to the house current and screening drugs for their ability to prevent convulsions. This was the means whereby Houston Merritt discovered the classic anti-convulsant phenytoin (Dilantin).

For such screens one needed veritable chemical engineering to come up with 25 g of material for screening. Structure-activity analysis was feeble, because one drug might be more active than another because of lesser metabolism or more efficient penetration to the target organ rather than greater potency at receptors. In terms of mechanism of action, pharmacologists administered opiates to rats and measured diverse biochemical markers with no way of knowing whether they were dealing with an initial receptor-related event, something secondary, or something unrelated to pharmacologic actions. Considering this morass, it was evident that identifying molecular receptors for opiates, other drugs and neurotransmitters could have a major impact.

1.1 Initial Findings

In 1970, several groups had identified the nicotinic cholinergic receptor in the electric organ of electric fish by the binding of the extremely potent, pseudo-reversible toxin α -bungarotoxin. Despite the fact that cholinergic receptors constitute up to

20% of weight by the electric organ, success depended on this unique toxin. The great pharmacologist Vincent Dole had already done armchair calculations indicating that presumed opiate receptors would amount to only about one-millionth by weight of mammalian brain. Hence, the very success of the nicotinic receptor work implied that conventional neurotransmitter receptors would remain inaccessible for the foreseeable future.

Accordingly, identification of opiate receptor binding in early 1973 utilizing reversible ligands was unexpected. The use of drugs labeled with high specific radioactivity, coupled with rapid but exhaustive washing to eliminate non-specific binding, made the system work. Radioligands based on diverse drugs soon led to the identification of receptors for most of the principal neurotransmitters, such as those for dopamine, norepinephrine, serotonin, glycine, and GABA. Differences in the binding properties of various ligands implied the existence of receptor subtypes. Receptor binding was rapidly adapted to high throughput analysis in the drug industry so that one could easily screen thousands of drugs a day with the chemists needing to synthesize only microgram quantities of drug candidates.

Major insights into how opiates mediate their pharmacologic actions came from the rather simple dissection of small regions of monkey brain in which opiate receptor binding was measured biochemically. Such studies were soon followed by autoradiographic microscopic localization of opiate receptors. Receptors were selectively enriched in areas of the brain mediating the “subjective” sense of pain which that is classically affected by opiates, e.g., the lateral thalamus. Multiple targets for euphoric actions of opiates emerged such as very high densities of receptors in the locus coeruleus, the source of most norepinephrine cell bodies, as well as areas of the limbic system.

Even the miotic actions of opiates can be explained by dense concentrations of receptors in the pretectal nuclei, which regulate pupillary diameter. Opiate analgesia at the spinal level could be explained by a very high density of opiate receptors in the substantia gelatinosa of the spinal cord on terminals of afferent pain fibers. Opiates act by inhibiting the release of pain-reducing peptide neurotransmitters from these neurons.

Work on opiate receptors evolved so rapidly that by May 1974, at a meeting of a Neurosciences Research Program, reports from diverse investigators provided insights into some of the biggest questions facing the field. Let me summarize some of the issues.

Vincent Dole opened the meeting by questioning the term “the opiate receptor.” He was concerned about *each* word. He noted that “the” suggested there was only a single opiate receptor, while differential effects of various opiates in animals and humans implied the possibility of multiple receptors. The word “opiate” referred to an agent of plant origin that presumably did not exist in mammals, raising the question of an endogenous ligand. The term “receptor” needed elucidation in terms of intracellular messengers that transform opiate recognition by a binding site into altered cellular function. The field moved so rapidly that when proceedings of the meeting were published a year later, a number of Dole’s prescient concerns had been addressed.

At the meeting in 1974, John Hughes and Hans Kosterlitz had presented their preliminary observations that brain extracts elicited influences on contractions of the guinea pig ileum and mouse vas deferens that mimicked morphine and were blocked by the opiate antagonist naloxone. In December 1975, they published the structures of the two enkephalin pentapeptides, the first endogenous opioid ligands.

As for the concept of multiple receptors, when the first batches of [³H]enkephalins were available, Kosterlitz noted that their binding properties to opiate receptors showed a different peptide/drug specificity than [³H]opiates. The enkephalin-prefering receptors were dubbed delta, because they were enriched in the vas deferens, while the morphine-prefering receptors were designated μ , for morphine. A year later Kosterlitz identified a third class of receptors, called κ , because they showed selective high affinity for ketocyclazocine. Remarkably, though the ligand techniques employed were relatively crude, molecular cloning has revealed that Kosterlitz was right on the mark, with the principal opiate receptor genes being indeed μ , δ , and κ .

What about connections to second messengers? Early insights emerged from efforts to differentiate agonists and antagonists. In initial receptor binding studies matched pairs of agonists and antagonists, e.g., morphine/nalorphine or oxymorphone/naloxone, displayed identical binding curves. A routine examination of ionic effects in our laboratory revealed that sodium ions selectively decreased the binding affinity of agonists with negligible effects upon pure antagonists and intermediate influences on mixed agonist–antagonist drugs.

When G proteins were subsequently characterized with GTP influencing ligand binding, it became evident that GTP and sodium synergistically differentiated agonists from antagonists, pointing to the notion that opiate receptors are G protein-coupled receptors. Workers in Marshall Nirenberg's laboratory first characterized effects of opiates upon adenyl cyclase.

Addiction is the *bête noire* of all psychoactive agents. The formal properties of the addictive process – tolerance, physical dependence, and compulsive drug-seeking behavior – are held in common by agents as diverse as alcohol, cocaine, and opiates. As opiates have been the paradigm for addiction research, all of us had hoped that conquest of the opiate receptor would resolve the riddles of addiction. No matter how hard we and other groups tried, however, we never found any meaningful alteration in opiate receptors as a product of the addictive process. Once we could measure enkephalins by radioimmunoassay, we hoped that alterations in their levels would explain it all, but nothing meaningful emerged.

1.2 Recent Advances

That is where matters stood in the mid- to late 1970s. What have we learned in the ensuing 30 years? The principal advances, largely at the molecular level, illustrate the complexity of opioid systems.

Cloning of μ , δ , and κ receptors was an important step forward. Mice with genetic deletion of each of these receptors as well as mice lacking two or three of the receptors have been generated, which we hoped would answer some fundamental questions. Which receptor subtypes mediate which forms of analgesia? Which receptor subtypes underlie addiction as well as lethal side effects such as respiratory depression?

Some answers have emerged, but conflicting and ambiguous findings abound. Difficulties of interpretation seem to rest in part on the surprisingly large variations among strains in opiate responsiveness. Many of the published studies have used mice with mixed genetic backgrounds with findings sometimes contaminated by differential strain influences. Hopefully, the uniform use of congenics will clarify such discrepancies.

There are too many conflicting findings to warrant an attempt to summarize the overall picture. The most extensive investigations have involved μ knockouts and have established that these receptors are primarily responsible for analgesia elicited by morphine and a variety of other opiates. Knockout of the μ receptors also abolishes tolerance and physical dependence to morphine. Hence, we are comforted that the binding sites all of us have been addressing for nearly 40 years are pharmacologically relevant.

However, the pharmacologic alterations observed with the three different receptor subtypes have failed to indicate that an agonist at only one of these subtypes will provide the long-hoped for less less-addicting, side-effect-free opiate analgesic. This conclusion is buttressed by the extraordinary effort of the pharmaceutical industry to develop drugs selective for receptor subtypes with negligible success.

Since the first identification of the enkephalins, substantial advances have been made in the biochemistry of opioid peptides, which are best differentiated by their precursors, pro-enkephalin, pro-opiomelanocortin (POMC), pro-dynorphin, and pro-nociceptin. Mice with targeted deletion of genes for the precursors of these peptides have been generated. In several cases the precursors give rise to multiple peptides. For instance, POMC is cleaved to yield ACTH as well as β endorphin. Knock-in mice have been designed permitting selective deletion of one or another peptide. These mutant mice have permitted investigations of the role of the various peptides in mediating analgesia and diverse behaviors.

Studies of mutants for peptide precursors as well for opiate receptor subtypes have asked which receptors are the “physiologic” targets for which peptides. As with opiate receptor knockouts, early studies utilizing mixed strains have produced ambiguous results, while few studies have employed congenic strains. Enkephalin deletion does lead to enhanced pain responses, while β endorphin loss seems to do the opposite, and very few effects are observed with dynorphin disruption. Studies comparing peptide and receptor mutants have so far failed to establish definitively the physiologic receptor for each of the peptides.

What about our understanding of addiction? Most pharmacologists have long assumed that tolerance and physical dependence reflect the following general model. Opiates exert some sort of effect on target cells, e.g., inhibition of adenyl cyclase.

To compensate, the target cell synthesizes more adenyl cyclase. When opiates are withdrawn one sees augmented cyclase responses. This model was demonstrated experimentally in early studies by Marshall Nirenberg by utilizing neuroblastoma cell lines expressing δ receptors. In recent years Eric Nestler has extended this approach to nuclear events involving acetylation of CREB and histones which that are influenced by addiction to cocaine and other drugs as well as opiates.

Some patterns have emerged. For instance, chronic cocaine administration represses the histone-methylating enzyme G9a in the nucleus accumbens, a dopamine-enriched cocaine target, leading to reduced levels of histone H3K9 dimethylation. Moreover, G9a downregulation increases preference for cocaine. Though the sequence of signaling steps that impacts histone alterations is not yet clear, elucidation of this pathway may lead to ways of manipulating the addictive process. The similarity in actions of cocaine and opiates fits with the view that addiction to most drugs is regulated by common mechanisms.

What is the bottom line? We know far more now than we did 35 years ago. However, we have not yet solved fundamental questions such as the nature of addiction, the functional roles of the different peptides or their relationship to receptor subtypes. In terms of Benjamin Franklin's challenge that we should come up with "useful knowledge," we must plead failure. There have been no major breakthroughs in the development of opiate drugs that can be attributed to our enhanced molecular understanding.

However, we need not feel discouraged. The same criticisms can be made of the cancer field. Our understanding of molecular features of cancer today vastly exceeds what was known 35 years ago. Therapeutic breakthroughs based on fundamental science, such as inhibitors of specific tyrosine kinases, are heartening. Nonetheless, the death rate from cancer has not changed notably. My suspicion is that the large body of seemingly conflicting opiate data that has accumulated in recent years will begin to form a pattern – a portrait which that will unexpectedly, and perhaps suddenly, afford the therapeutic advances we all seek.

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Chapter 2

The Evolution of Concepts

W.R. Martin

Abstract κ Opioid receptors were first proposed by the author based on the actions of benzomorphans, such as ketocyclazocine. κ receptors are involved with a wide range of actions, providing novel targets for drug development. This chapter will explore the pharmacology of κ receptors in a range of behavioral effects and their functions at the biological level.

Keywords κ Receptor • KOR • Electrophysiology • Dynorphin • Epilepsy • Stress • Depression • Learning • LTP • DRG • Ion channel • Kinase

2.1 Introduction

The early evolution of concepts of endogenous opioids and multiple receptors had its inception in a concerted program to develop safe, nonaddicting substitutes for opiates [1]. This endeavor was initiated but the Bureau of Social Hygiene and subsequently supported by the US Public Health Service under the auspices of the National Research Council. An empiric approach was taken in which a large number of chemicals, synthesized by University-based chemists and the pharmaceutical industry, were examined for their pharmacologic effects, particularly their analgesic activity and their abuse potential.

Although heroin and morphine addition were the initial driving force of this endeavor, the economic gains associated with the marketing of a less-addicting analgesic became the most important factor of the pharmaceutical industry's synthetic effort. From a societal perspective, however, the economics of drug abuse is by far the most important economic factor, since drug abuse costs the United States well over \$100 billion dollars a year. The search for safer and less-abusable

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analgesics has not been entirely successful. The evolution of ideas concerning multiple opioid receptors and endogenous opioid transmitters is still active.

The critical opioids in the pharmacologic dissection of multiple opioid receptors were *N*-allylnorcodeine, *N*-allylnormorphine, naloxone, cyclazocine, ethylketazocine, *N*-allylnormetazocine, and buprenorphine. Proceeding on the concept that allyl substitutions functioned as respiratory stimulants, Von Braun [2] synthesized *N*-allylnorcodeine and Pohl [3] studied the interactions between *N*-allylnorcodeine and morphine and first demonstrated that *N*-allylnorcodeine was capable of antagonizing the respiratory depressant effects of morphine.

Although Pohl's important observations were published in 1915 and were confirmed by Meissner [4], these findings lay dormant until they were resurrected again by Chauncy Leake. Dr. Leake, then of the University of California in San Francisco, stimulated efforts to synthesize *N*-allylnorcodeine and allylnormorphine. These endeavors have been briefly recounted [5], and, like Pohl's concept, were based on the hypothesis that allyl groups are respiratory stimulants.

The initial synthesis of *N*-allylnormorphine was controversial. As a consequence of resolving the synthetic issues, *N*-allylnormorphine (nalorphine) was independently synthesized by Weijlard and Erickson at Merck Laboratories [6] and by Hart and McCauley at the University of California [7]. Both Unna [8] and Hart and McCauley [7] also studied the pharmacology of this interesting compound and extended the observations of Pohl by showing that nalorphine antagonized other actions of morphine.

The issue of respiratory stimulant action can be clearly differentiated from its morphine antagonistic effect and from the respiratory stimulant actions of dinitrophenol. The observations of nalorphine's antagonistic effects were reluctantly accepted, as were speculations concerning its mechanism of action (Unna, personal communication). The clinical use of nalorphine for the treatment of acute morphinism was not pursued despite Unna's urging and was not demonstrated until Eckenhoff et al. [9] conducted the critical experiment in man.

To further elaborate on the importance of opiate addiction in stimulating research on opioid drugs, a Committee on Drug Addiction was formed by the Committee on Social Hygiene in 1920 to increase the understanding of addiction processes. Following the passage of the Harrison Narcotic Act and on the recommendation of the American Medical Association, clinics that provided narcotics to addicts were closed, leaving most addicts without a legitimate source for their narcotics. As part of the Committee's activities, they proposed a strategy for identifying new analgesics that would be devoid of the toxic and dependence-producing actions of the opium analgesics. Subsequently, the Federal government assumed the responsibility of continuing the activities of this committee.

Among the important activities were the initiation and continuation of a synthetic program, the development of an animal screening program, and finally assessment of new analgesics in humans for their ability to produce or sustain physical dependence. Many compounds were synthesized and evaluated by Dr. Eddy's laboratory at the National Institutes of Health, by Dr. Severs' laboratory at the University of Michigan, and by investigators of human subjects at the Addiction Research Center.

Most of the drugs studied were sufficiently like morphine that they were judged not to have any marked advantage. In retrospect, there may have been significant differences between the drugs; these were either not detected using the methods at hand, or differences were not pursued. Two examples of compounds that had unique pharmacological properties in human subjects were meperidine and normorphine. It was much more than difficult to produce physical dependence on these drugs than it was to produce physical dependence on morphine.

Doctor Harris Isbell has developed an interest in the use of *N*-allylnormorphine as an analgesic. He obtained the drug, however, at a time when he was very much involved in conducting his studies on alcohol and barbiturate dependence. Lasagna and Beecher [10] did study the analgesic actions of nalorphine and found it to be nearly as potent as morphine. Dr. Abraham Wikler attempted to substitute nalorphine for morphine in a dependent subject and observed that it precipitated a violent abstinence syndrome that could not be antidoted by morphine. He subsequently characterized precipitated abstinence in humans and in the spinal dog [11, 12]. These observations provided an important clue in the development of nonaddicting, safer analgesics and were pursued by several pharmaceutical firms that synthesized a number of compounds with antagonistic effects.

Thus, the driving force for the enormous commitment for development of opioid antagonists as analgesics was that they had analgesic activity and did not appear to substitute for morphine in morphine-dependent subjects. It is important to recognize the importance of the substitution technique for identifying morphine-like drugs devised by Himmelsbach [13]. Although Himmelsbach did not couch his concepts in receptor theory, his work was one of the first critical pieces of evidence that strongly indicated that opioids were exerting their effects by acting through a common mechanism. Himmelsbach [14] attributes the development of this technique to the observations of Eddy [15], who demonstrated cross-tolerance between morphine, codeine, and heroin in the dog.

Himmelsbach reasoned that cross-dependence could also exist, and that dependence was a major determinant of the addictiveness of analgesics. He demonstrated that a number of morphine congeners substituted for morphine in morphine-dependent subjects. These studies had several major implications. One of the drugs studied by Dr. Himmelsbach was desomorphine. Desomorphine did not produce dependence in the monkey; it substituted for morphine in morphine-dependent subjects, however. This was to be only the first of several drugs with the ability to sustain dependence that was much greater in humans than in the monkey. These observations, and others, led to the suggestion that opioid receptors differed in the intimate details of their configuration from one species to another [16, 17].

Another important innovation was the application of bioassay statistical techniques to not only suppression and precipitation data but also to subjective effects data as assessed by questionnaires. Harris Isbell introduced this technique to help strengthen the conclusions that had been reached concerning the abuse potentiality of phenazocine, the first of a series of benzomorphans that had a critical role in the formulation of concepts concerning multiple opioid receptors. Phenazocine was much less potent than morphine as an analgesic in humans. In humans, however,

phenazocine was three to four times more potent than morphine in constricting pupils and producing subjective effects and was eight times more potent in suppressing abstinence [18].

In addition to emphasizing the large differences in response to opioids among species, several other important lessons were learned through this quantitative comparison between drug measure and species. (1) Different experimental variables (e.g., pupillary diameter vs. subjective effects) that were measured using different scales (e.g., ordinal, nominal, or ratio) yield potency estimates that were not only equivalent, but had similar confidence limits. (2) The use of dose-response relationships became an important criterion for identifying changes in subjective states that were relevant to the drug effects. (3) The concomitant use of both a physiologic and behavioral measure provided an internal validation of the behavioral measures [19]. The effects of opioids on subjective states became an important criterion for differentiating the receptor subtypes.

Isbell's use of bioassay statistics and techniques to compare the relative potencies of opioids to suppress abstinence, to alter subjective states and to induce physiologic changes provided a powerful tool for quantitatively characterizing the pharmacologic profiles of drugs [18]. The use of crossover designs allowed for simultaneous and efficient assessment of relative potencies on several experimental parameters. Thus, valid assays could be obtained on studies employing four to six subjects using a four-point assay [20, 21]. This design allowed for the partitioning out of the between-subjects variance, and the error term for calculating the confidence limits of potency estimate was the residual part of the between-doses variance.

The seminal approach, however, had a major statistical problem in that different pharmacological effects were measured with different types of scales. For example, pupils were photographed and measured with a ruler (ratio scale). Some subjective states were measured using a nominal scale; others using an ordinal scale. Isbell's first effort (Table 2.1) revealed that the confidence limits of the potency estimated for the different types of measurement scales were similar despite differences in the inherent properties of the scales.

From a practical and empirical perspective, potency estimates obtained from dose-response relationships employing data bearing on the frequency of occurrence of signs and symptoms using nominal scales, data bearing on the subjectively estimated intensity of feeling states using ordinal scales, and the measurement of pupillary diameter from Polaroid photographs were in close agreement and had similar confidence limits [19].

Table 2.1 The relative potency of phenazocine and levophenacylmorphan in comparison to morphine in constricting pupils (interval scale), altering signs and symptoms (nominal scale), and suppressing abstinence (mixed scale)^a

	Pupils (miosis)	Questions	Suppression
Phenazocine	3.8 (1.3–5.6)	3.2 (2.3–5.0)	8.2 (4.2–17.2)
Levophenacylmorphan (NIH-7525)	5.2 (2.7–8.0)	6.11 (5.0–7.5)	9.1 (4.8–20.0)

^aFrom Fraser and Isbell [18]

These potency estimates further agreed with estimates of analgesic potency obtained in patients suffering from both acute and chronic pain. These observations were taken to mean that (1) the miotic phenomenon and changes in subjective states were probably the consequence of the drugs acting through a similar mechanism and that measures of subjective states were valid measures of drug effect; and (2) any lack of additivity among signs and symptoms, and deviations from linearity for nominal and ordinal scales, was probably small compared to between-subjects and across-time variance.

These latter issues were pursued experimentally. Thus, the frequency of occurrence of various signs or the intensity of symptoms and the degree of miosis produced by both morphine and heroin were found to be linearly related to the logarithm of dose. Hence, we knew that the principle of additivity was applicable to data obtained using nominal and ordinal ratio scales. We began to apply the criterion of dose responsiveness for the selection of questionnaire items [19, 22], yet another approach that enhances the rigor of additivity for our behavioral scales. Different signs were weighted such that the signs that exhibited lesser sensitivity were given greater weight. Thus, by weighting, different responses could be equated (e.g., pupils and liking).

The Himmelsbach method for scoring the intensity of opioid abstinence is composed of data derived from nominal, interval, and ratio scales that have different weighing values that are related to the severity of abstinence. A similar system for assessing abstinence was developed for precipitation and suppression studies in the dog [23, 24] that was composed of changes that were suppressed or precipitated in a dose-related way by agonists and antagonists and that were measured using nominal, ordinal, interval, and ratio scales. Those signs of abstinence, the frequency or intensity of which were related to the dose of the agonists in suppression studies and the dose of antagonists in the precipitation studies, were selected for measuring the intensity of abstinence, and each sign was weighted such that all signs made an approximately equal contribution to the abstinence syndrome score. Thus, the criterion of additivity and linearity were fulfilled.

By establishing linearity and additivity for items of subjective effects questionnaires, through a weighting and dosing relationship, a report of the two effects can be added. In a similar manner, the abstinence signs – yawning, piloerection, and body temperature – can be added. Through the technique of mapping, we have shown that there is a linear relationship between dose-related changes in score on the nominal, ordinal, interval, and ratio scales. This relationship is implicit when valid parallel line assays are obtained for different measures and effects. This is illustrated in Table 2.1.

These issues of measures and statistics have been discussed by Stevens [25]. Two important principles emerged. (1) Deviations from additivity and linearity for nominal and ordinal data are small compared to the unaccounted-for variance and (2) the frequency of occurrence of intensity of report are linearly related to the dose (logarithm) of the drug.

The use of pharmacologic syndromes has played a critical role in identifying receptor subtypes and in identifying specific drugs. In detailed studies of cyclazocine

in humans, it was apparent that cyclazocine produced effects that were not produced by morphine [26]. Although cyclazocine was a potent miotic (10–15 times or more potent than morphine and nalorphine), valid potency assays of this activity were not obtained. Further, cyclazocine in higher doses produced overt ataxia and subjects reported that they were sleepy and felt drunk. These signs and symptoms were not commonly observed in, or reported by, post addicts who had been administered morphine or heroin.

Cyclazocine and nalorphine produced feelings of well-being in some subjects, but not in others. They also produced feelings of dysphoric in more subjects when the dose was sufficient. The dysphoric effects of cyclazocine and nalorphine are complex. The most commonly reported symptom, with minimally dysphoric doses, is recall of disturbing memories. The patient can be distracted but has difficulty suppressing these thoughts. With larger doses, delusions, hallucinations, sleep with disturbing dreams, and anxiety states may be reported.

Cyclazocine was found to be 10–20 times more potent than morphine in equivalent measures. An attempt was made to make patients dependent on an equivalent dose of cyclazocine based on single-dose relative potency. A daily dose of 13.2 mg/70 kg was attained in six subjects. Some subjects found the dysphoric effects of cyclazocine especially disturbing and the dose of cyclazocine was incremented slowly. At the time these studies were initiated, we did not realize that cyclazocine had much longer duration of action than morphine, and hence our estimates of the equipotent dose of cyclazocine may have been high.

Regardless, when the administration of cyclazocine was terminated, we were presented with several surprises. The first was a long latency to onset of signs of abstinence. In fact, signs were not perceptible until the third day of withdrawal. Second, the abstinence syndrome was not associated with drug need. Most subjects were glad the study was over and none sought medication for relief of their symptoms. The third issue was the nature of abstinence syndrome.

Doctors Eddy and Isbell took the position that the cyclazocine abstinence syndrome was just mild abstinence. To help resolve this issue, Dr. Isbell provided me with unpublished data of E.G. Williams [27] who had studied the abstinence syndrome of subjects dependant on different stabilization doses of morphine in an attempt to determine the smallest dose of morphine that produced a clinically significant degree of physical dependence. A sign analysis of Williams' data and the cyclazocine abstinence data was done. The analysis indicated that the relative magnitude of the signs of cyclazocine abstinence was different from that of morphine abstinence, regardless of the level of dependence [26, 27].

The effects of cyclazocine shared certain characteristics with those of nalorphine [26], except that nalorphine was less potent and the maximum degree of ataxia was less. Whereas cyclazocine could produce overt drunkenness, nalorphine produced liminal ataxia that was only demonstrable with tandem gate walking. The latter difference was subsequently explained when studies were conducted in the chronic spinal dog, in which it was shown that nalorphine showed partial agonistic activity [24, 28]. When nalorphine was administered chronically in doses of 240 mg/kg/

day and then withdrawn, an abstinence syndrome emerged within 24 h and was qualitatively different from the morphine abstinence syndrome and similar to the cyclazocine abstinence syndrome.

Several investigators studied mixtures of morphine and nalorphine in human subjects and in animals, administered acutely and chronically [29]. Of particular importance were the observations of Houde and Wallenstein [30], who found that low doses of nalorphine antagonized the effects of 10 mg of morphine, whereas higher doses produced a lesser antagonism. The nalorphine biphasic dose response antagonism of morphine's analgesic action could not be explained by assuming that nalorphine was a competitive antagonist or a partial agonist of morphine. Houde and Wallenstein's observation [30] stimulated a mathematical formulation of receptor dualism [29, 31].

Naloxone antagonized the actions of cyclazocine in the chronic spinal dog [28] and in human subjects [32]. Naloxone in a high dose (15 mg/70 kg) antagonized miotic, respiratory depressant, and subjective effects produced by 1 mg/70 kg of cyclazocine in human subjects. Naloxone (0.2 mg/kg) partially antagonized the depressant effects of cyclazocine (0.063 mg/kg) on the flexor reflex of the chronic spinal dog. The same dose of naloxone completely antagonized the effects of 1.0 mg/kg of morphine. Blumberg showed that naloxone antagonized the analgesic effects of cyclazocine, nalorphine and pentazocine in mice [33].

Thus, four lines of evidence suggested that nalorphine and cyclazocine differed from morphine in their actions. (1) The nature of the subjective effects that they produced were different; (2) they produced different types of dependence; (3) interaction studies between morphine and nalorphine yielded biphasic dose response curves; and (4) the effects of several agonist–antagonists could be antagonized by large doses of naloxone.

These observations lead to the suggestions that there were two opioid receptors an M (morphine) and N (nalorphine). Further, the M and the N receptors operated in concert in some, but not all, physiologic systems. The process of a concerted action was "pharmacologic dualism." It was suggested that morphine acted as an agonist and nalorphine as a competitive antagonists at the M receptor. Further nalorphine acted as a partial agonist at the N receptor [29]. This concept (pharmacologic dualism) was an elaboration on my concept of pharmacologic redundancy, which postulated parallel neuronal pathways employing different transmitter, as well as co-transmitters and co-receptors as alternative mechanisms for the conduct of function [34].

The hypothesis that there are two opioid receptors that exhibit the principle of receptor dualism reconciled many observations. It was soon apparent, however, that it left other observations unexplained in terms of receptor theory.

The first analgesic with predominantly N agonistic activity to be marketed was pentazocine. It was not scheduled as a narcotic because studies at the Addiction Research Center indicated that it did not produce as much euphoria as morphine, did not substitute for morphine in morphine-dependent subjects, did not appear to produce physical dependence, and was not liked by post-addict subjects when administered chronically [35].

There were sporadic case reports of abuse of pentazocine. For this reason, and because we had developed new concepts, we decided to reinvestigate the abuse potentiality of pentazocine. One of the important developments in opioid pharmacology was the synthesis of naloxone and the elucidation of its pharmacology. Blumberg had encouraged the synthesis of naloxone with the end of obtaining a more potent antagonist with fewer side effects (e.g., respiratory depression and psychotomimetic effects) [36]. Foldes [37] conducted extensive studies with naloxone showing that it antagonized the respiratory actions of opiate analgesics. Lasagna found that naloxone produced a modest degree of both analgesia and hyperalgesia in patients with pain.

Our task was to assess the abuse potentiality of naloxone [38]. We found that it did not induce subjective changes, did not produce miosis when administered chronically, did not produce physical dependence, and, when administered to morphine-dependent subjects, was seven times more potent than nalorphine in precipitating abstinence [39]. We concluded that naloxone was an opioid antagonist that was devoid of agonistic activity. Naloxone was of great importance in further clarifying the mechanism of action of pentazocine and provided critical proof that morphine was acting as an agonist.

In our reinvestigations of pentazocine, we confirmed several of the observations of Fraser and Rosenberg [35]. Low doses of pentazocine produced a subjective state similar to that produced by low doses of morphine characterized by elevations of MBG scale scores, which measure feelings of well-being. In this regard, pentazocine was about one-fourth as potent as morphine [40]. Further, doses above 40 mg produced dose-related elevation on the LSD and PCAG scale scores, which measure, respectively hallucinations, delusions, and anxiety (LSD) and apathetic sedation (PCAG), and a decrease in the MBG scale scores.

The fact that lower doses of pentazocine produced elevations of MBG scale scores raised the question of whether pentazocine could be a weak partial agonist at the M receptor and a less potent but strong agonist, at the N receptor. To test this hypothesis, subjects were made dependant on decreasingly lower doses of morphine and the ability of pentazocine to suppress the morphine and abstinence syndrome was assessed. In short, pentazocine did not clearly suppress abstinence in subjects dependent on morphine in doses as low as 30 mg/day and as high as 240 mg/day.

When subjects who were dependent and stabilized on 240 mg/day of morphine were administered pentazocine, it precipitated an abstinence syndrome and in this regard was 1/50 as potent as nalorphine. Thus the doses of pentazocine that were necessary to precipitate abstinence were greater than those necessary to cause miosis, analgesia, and subjective effects. When subjects were administered pentazocine in doses of 522–684 mg/day and then abruptly withdrawn, a mild abstinence syndrome emerged that was quantitatively similar to that seen in cyclazocine- and nalorphine-dependent subjects.

Further, an abstinence syndrome could be precipitated in pentazocine-dependent subjects with naloxone in doses approximately ten times larger than necessary to precipitate an abstinence syndrome in morphine-dependent subjects. At this

juncture our operating hypothesis was that pentazocine, like nalorphine and cyclazocine, was a competitive antagonist at the M receptor and either a partial or a strong agonist at the N receptor.

We had been aware that cyclazocine and nalorphine produced a subjective syndrome consisting of dysphoria and an apathetic sedation. The fact that pentazocine produced more feeling of well-being than did nalorphine and cyclazocine, and yet resembled them in many other ways, was a problem – “the pentazocine problem.”

To determine if this problem had a receptor-based explanation, an extensive group of studies was initiated in the chronic spinal dog [23, 41–43]. These studies developed methods that yielded data in the dog that provided potency estimates on a variety of physiologic parameters (pupillary diameter, pulse rate, respiratory rate, body temperature, amplitude of the flexor reflex, and the latent of the skin twitch reflex). In addition, procedures were developed for conducting valid assays of the potency of drugs in suppressing signs of abstinence in the maximally abstinent chronic spinal dog.

A large group of dogs was made dependent on morphine; a prototypic “M” agonist. Another group was made dependant on cyclazocine; a prototypic “N” agonist. Over 20 prototypic drugs were studied. Morphine-like drugs (see Table 2.2) by and large produced a similar pattern of effects suppressing the flexor and skin twitch reflexes, constricting pupils, lowering body temperature, and slowing pulse rate. Further these agents as well as other that resembled morphine suppressed the morphine abstinence syndrome in a dose-related way. Of some importance were the observations that neither meperidine nor normorphine produced morphine-like effects or suppressed the morphine abstinence syndrome. Hence, although they are morphine-like drugs in other species, they do not appear to be morphine-like in the dog.

Buprenorphine in single doses also produced a morphine-like pattern of effects, but differed from morphine in that it produced a lesser maximal effect. Further it suppressed abstinence signs; the slope of its suppression dose-response line was less than that of morphine, however. Buprenorphine also precipitated abstinence in stabilized morphine-*dependent* dogs; the slope of the precipitation dose-response line, however, was less than that of naloxone and naltrexone. These data were consistent with the hypothesis that the buprenorphine was a partial agonist of the morphine-type.

In contrast to morphine-like drugs, cyclazocine, nalorphine, and pentazocine were relatively ineffective in suppressing the thermally evoked skin twitch reflex, but produced a profound depression of the pressure-evoked flexor reflex. They also, especially in longer doses, dilated pupils and increased heart and respiratory rate, but did not depress body temperature to the degree morphine did.

Keats and Telford [44] in their study of the analgesic properties of a series of *N*-substituted benzomorphans, had observed that *N*-allynormetazocine (NANM; SKF 10,047) produced severe dysphoria and little analgesia. NANM was selected as a prototypic and relatively selective dysphoriant and was studied in the chronic dog. It produced less depression of the flexor reflex than morphine or ethylketazocine, did not depress the skin twitch reflex, increased pupillary diameter, pulse rate, and respiratory rate, and produced a canine delirium.

Table 2.2 Relative potency of μ and κ agonists in suppressing and precipitating abstinence in morphine (A)- and cyclazocine (B)- dependent dogs and in producing changes in the nondependent dog (C)^a

Drug	Morphine-dependent dogs		Cyclazocine-dependent dogs		Single dose	
	(A)		(B)			
	Suppression potency	Precipitation potency	Suppression potency	Precipitation potency		
D-Propoxyphene	0.2				0.12	
Propiram					0.14	
Codeine					0.06–0.1	
Morphine	1.0		1		1	
Oxycodone	1.2					
Methadone	4.9					
Ketobemidone	5.1					
Phenazocine	8.1					
Levorphanol	9.0					
Dilauidid	15.4					
Fentanyl	70.5					
Etorphine	200.3					
Buprenorphine					257	
Pentazocine		0.002	0.19		0.3	
Nalorphine		0.08	0.24 (PA)	0.009	0.5	
<i>N</i> -allyl normetazocine (NANM)		0.13				
Cyclazocine		0.47	1.0		3.3	
Naloxone		1				
Naltrexone		3.4		1	0	
Ethylketazocine			4.41		9	
Ketazocine			0.2		1	

PA nalorphine is a partial agonist

^aPotency estimates for suppression studies are expressed as milligrams of morphine or cyclazocine that are necessary to produce the same degree of suppression as the experimental drug. Naloxone is used as a standard drug in precipitation studies in morphine-dependent dogs and naltrexone in cyclazocine-dependent dogs

NANM's respiratory stimulant action probably has a different mechanism of action than morphine's in the dog. Morphine causes panting by resetting hypothalamic thermoregulatory center that downregulated the set point and thus body temperature. In contrast, NANM stimulated respiration, while producing a modest hyperthermic reaction. In all probability the respiratory stimulant actions of nalorphine, which are seen in relatively high doses, are a consequence of nalorphine's σ activity [41].

Other prototypic drugs studied were ketazocine and ethylketazocine, which depressed the flexor reflex, had little effect on the latency of the skin twitch reflex, produced sedation, and were potent miotics.

Studies in the morphine- and cyclazocine-dependent dog are summarized in Table 2.2. Several points are of importance. Nalorphine precipitated abstinence in both

the morphine- and cyclazocine-dependent dog. In the cyclazocine-dependent spinal dog, however, it exhibited a ceiling effect. These observations were in keeping with the observations in the nondependent dog, namely that nalorphine's agonistic effects exhibited a ceiling and that it was probably a partial agonist of the κ type (see below).

Of great importance were the observations that three groups of drugs suppressed the cyclazocine abstinence: (1) morphine; (2) cyclazocine, nalorphine, and pentazocine, which exhibited excitation effects such as mydriasis and tachycardia; and (3) ethylketazocine and ketazocine, which constricted pupils, but did not suppress the morphine abstinence syndrome. The excitatory effects of cyclazocine, nalorphine, and (to some extent) pentazocine resemble the effects of NANM.

To further compare the pharmacologic properties of NANM with those of the prototypic drugs, morphine and cyclazocine dogs were made dependent on 10 mg/kg/day of NANM administered in equally divided i.v. doses six times a day [43]. This proved to be a difficult experiment to execute. As the dose levels were increased, dogs exhibited canine delirium and loss of appetite and weight. By slowly escalation the dose, a stabilization dose of 10 mg/kg was eventually obtained, and precipitation and withdrawal studies were conducted.

This study showed that chronic administration of NANM induced tolerance to its ability to produce canine delirium, tachypnea, and anorexia. The withdrawal abstinence was mild, consisting of a decrease in body temperature, miosis, bradycardia, tachypnea, and an increase in the amplitude of the flexor reflex. This syndrome was unlike that seen in either morphine- or cyclazocine-dependent animals. The naltrexone-precipitated abstinence syndrome was yet different, consisting of hyperthermia, tachycardia, tachypnea, and an increase in the amplitude of the flexor reflex. These data further showed that some of the effects of chronically administered NANM could be antagonized by naltrexone, whereas others could not. These observations led to the suggestion that NANM might have multiple modes of action.

These and other observations could be reconciled by the hypothesis that (1) there were three opioid-related receptors, μ , κ , and σ [41]; (2) these receptors could exert their effects on several physiologic systems through different but converging pathways (receptors dualism and pharmacologic redundancy) [29]; and (3) drugs that interact with opioid receptors could act as competitive antagonists partial agonists and strong agonist.

2.2 Reflections

In the relatively brief time – two decades – since these hypotheses were proposed, an enormous body of data has been generated that supports them. Further, they have been extended in two major directions: (1) additional types of opioid-related receptors have been identified and (2) endogenous opioid transmitter substances have been discovered. These observations have had, and will continue to have, an enormous impact on neurochemistry, physiology, neuropsychopharmacology, and psychology, as well as on mental health.

2.2.1 Pharmacologic Implications

The first clues concerning the existence of multiple opioids came from studies in humans that were subsequently elaborated on using the chronic spinal dog. The conclusions were drawn from analyses of the patterns of pharmacologic effects using agonists and antagonists of different specificities and differed from other classic analyses of receptor subtypes only in that these comparisons used signs derived from changes in central nervous function for the comparisons. For these pattern comparisons to become meaningful, valid bioassay techniques had to be developed for the various central nervous system functions under study such as subjective effects, pupillary diameter, function of homeostats, and reflex activity. The second element was the use of receptor theory in the design of experiments and conceptualization of hypotheses. The third major ingredient in this endeavor was the very large synthetic effort that yielded a rich diversity of structural modification of important drugs. In this regard the synthetic efforts of Sidney Archer, William Michne, Jack Fishman, John Lewis, and Everett May were particularly important.

In a relatively short time, it was demonstrated that relatively minor structural modifications of opioid drugs could change the specificities for μ , κ , σ , and δ receptors and could alter their activity, yielding agonists, partial agonists, and competitive antagonists. It was also apparent that opioid ligands had a number of reactive sites that could interact with a variety of moieties on opioid receptors. These general observations lead to the formulation of the steric theory of multiple opioid receptors, which offers a theoretical basis for explaining not only the multiplicity of opioid receptors but also differences in their efficacy and activity [17].

The steric theory has several components:

1. It assumes that the opioid receptor has nuclear sites that are responsible for initiating the pharmacologic action of the drug or transmitter, as well as satellite sites that play two roles: (a) determination of the affinity of the drug for the receptor and (b) the orientation of the drug on the receptors.
2. Changes in the configuration of these two components of the receptors may have several effects on drug receptor interactions. The following terms are coined to designate the possible types of changes. *Allomorphism* is a change in the position of the active moieties of the nuclear part of the receptor. Such changes will result in a change in the specificity of drugs for the receptor. *Allosterism* is a change in the positions of moieties of the satellite sites. These result in changes of affinity of the drug for the receptor and in the orienting properties of the receptor toward the drug. *Allotaxia* is the property whereby the drug can occupy the receptor in several positions.

These types of changes in the receptors can hypothetically be interactive. Clearly, changes in the relative positions of satellite moieties could alter both the affinity and the allotaxic properties of a family of drugs and hence alter both the K_d values and the activity of the drug. On the other hand, allomorphic changes will result in a change in the number of receptors of different specificities. These types of changes may result in complicated dose-response curves.

Depending on one's perspective, the concept of opioid antagonists had a slow acceptance by the medical and pharmacologic community. The concept of multiple opioid receptors and the application of receptor theory to opioids had a somewhat more rapid acceptance. The delineation of each receptor subtype has extended our basic understanding of general receptor theory, as well as the complexity of body function and evolution. In turn, the complexities of microstructure and function have provided those who have a bent for using pharmacologic approaches to function and evolution a wonderful opportunity for identifying drugs with unique specificities. I believe that a strong case can be made for the proposition that the discovery of most multiple receptors types has been a consequence of pattern identification using tissues of diverse origins and response drugs.

I have tried in this account to present most of the critical events that either directly or indirectly have influenced my thinking and conclusions about multiple opioid receptors and receptor dualism. It was my very good fortune to have had Drs. Klaus Unna, Harris Isbell, and Abraham Wikler as my teachers, collaborators, and friends. They played critical roles and had seminal influences in the development of opioid antagonists and agonist-antagonists as therapeutic agents and pharmacologic tools. They also recognized the importance of systematic, quantitative, and reliable observations in drug comparison, both in humans and animals, a perspective that was essential to the analysis of the mechanisms of action of opioid receptors.

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Chapter 3

Pharmacology of Opioid Drugs

Brian M. Cox*

Abstract Extracts of the opium poppy have been used for the relief of pain since antiquity. The active principle, morphine, continues to this day to be one of the most effective ways of alleviating moderate and severe pain. It is also one of the most addicting drugs known to man. This chapter describes the history of research on opium and the pharmacologic properties of morphine and related drugs. It briefly summarizes critical studies in the development of current knowledge of their antinociceptive actions; their effects on arousal, respiration, locomotor activity, and behavioral reinforcement; endocrine effects; and actions on peripheral tissues including the gastrointestinal tract, the genital tract, and the heart and circulatory system. The last section provides an overview of the absorption, metabolism, and distribution of morphine and other major opiate drugs.

Keywords Absorption • Analgesia • Antinociception • Arousal • Distribution • Endocrine • Endogenous opioids • Endorphins • History • Gastrointestinal tract • Opium • Metabolism • Morphine • Receptor • Respiratory depression

3.1 A Brief History of Studies on Opiate Drug Pharmacology

The opium poppy has been used to provide symptomatic relief of pain since early antiquity. The active principal, morphine, was isolated in 1805 by Serturner, [1] but its structure was not determined until 1925 [2] and a complete synthesis was not achieved until 1956 [3]. Opium products were used quite extensively and without

*The opinions and assertions contained herein are the private opinions of the author. They are not to be construed as official or reflecting the views of the Uniformed Services University of the Health Sciences, or the U.S. Department of Defense, or the Government of the United States.

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any governmental control during the nineteenth century, as pain relievers, sedatives, and for their euphoriant properties. Samuel Taylor Coleridge in 1797 claimed that his celebrated poem “Kubla Khan” was inspired by an opium-induced “reverie.” Thomas de Quincy described his experiences with opium in his semi-autobiographical book *Confessions of an English Opium-Eater* (1822). The early history of opiate drug studies is described by Eddy and May [4] and Brownstein [5].

As the science of experimental pharmacology developed in the first third of the last century, the properties of opium and its major active principal, morphine, were studied experimentally. The analgesic, respiratory depressant, constipating, and addictive properties of morphine were characterized in experimental animals and in man [6].

During this period several new synthetic drugs with potent analgesic activity were developed, the best known being methadone and meperidine (also known as pethidine). At the same time, chemists were manipulating the structure of morphine itself in an attempt to develop derivatives that would retain analgesic activity with reduced respiratory depressant and/or addictive properties. In this way heroin (diacetylmorphine), hydromorphone, levorphanol and a number of other semi-synthetic drugs were developed.

Many, including methadone and meperidine, were incorrectly claimed to be non-addicting at the time of their introduction, but these claims were very quickly demonstrated to be false. Further studies of structures related to meperidine led to the synthesis of the potent short-acting opiate, fentanyl, and its congeners alfentanil and sufentanil, by Janssen and colleagues. Later a series of interesting morphine-like drugs was developed from thebaine [7], another alkaloid isolated from opium. These drugs included etorphine, one of the most potent of the morphine-like drugs, now used as a veterinary analgesic and immobilizing agent, and buprenorphine, a close analog of etorphine that is used both as an analgesic and in the treatment of heroin addiction.

The major opiate drugs in current clinical use are listed in Table 3.1.

The social menace presented by the escalation of opiate drug use during the first two decades of the twentieth century played an important role in the search for nonaddicting potent analgesic drugs [8]. A Committee on Drug Addictions was formed, and the Harrison Narcotics Act (1914) was passed. Research on opiate drugs was encouraged and a United States Narcotic Farm was established in 1935 at the federal prison in Lexington, Kentucky, where many convicted opiate drug traffickers were held. With the creation of the National Institutes of Health in 1948 the research component at Lexington became a U.S. Public Health Service Hospital under the name of the Addiction Research Center.

Over a period of several decades, Himmelsbach and his colleagues at the Addiction Research Center developed effective procedures for the quantitative evaluation of behavioral, neurological, and subjective symptoms of addictive drugs and the application of statistical techniques to their quantitative analyses. These studies were among the earliest quantitative psychopharmacology studies conducted in human subjects; the techniques developed in the course of this work and the resulting publications provided the basis for many future studies on the actions

Table 3.1 Opiate drugs in clinical or veterinary use

Category	Drug	Primary use
Strong agonists	Morphine	Pain relief
	Hydromorphone	Pain relief
	Levorphanol	Pain relief
	Oxymorphone	Pain relief
	Oxycodone	Pain relief
	Meperidine	Pain relief
	Methadone	Pain relief; treatment of opiate drug dependence
	Fentanyl	Pain relief
	Alfentanil	Anesthesia
	Sufentanil	Anesthesia
	Etorphine	Anesthesia, immobilization (large animals only ^a)
Weak agonists	Loperamide	Relief of diarrhea (does not enter CNS)
	Diphenoxylate	Relief of diarrhea (does not enter CNS; may also have nonopiod actions)
Weak agonist/monoamine reuptake inhibitor	Codeine	Pain relief; cough suppressant
	Hydrocodone	Pain relief
	Propoxyphene	Pain relief
Partial agonist	Tramadol	Pain relief
Mixed agonists/antagonists	Buprenorphine	Pain relief; treatment of drug dependence
	Butorphanol	Pain relief (no abuse potential)
	Nalbuphine	Pain relief (no abuse potential)
Antagonists	Pentazocine	Pain relief (little abuse potential)
	Naloxone	Opiate overdose
	Naltrexone	Opiate overdose; treatment of drug dependence

^aEtorphine is only approved for veterinary use

of psychoactive agents in man. At the time these studies were performed, testing of drugs in prisoners was a generally accepted procedure provided that the health of the participants was carefully monitored. Studies to be conducted in prisoner populations today are subject to much more stringent regulation. Nevertheless, these studies were carefully conducted and have provided invaluable insight into the effects of high doses of opiate drugs in a population with substantial prior experience of heavy opiate drugs use.

Among other achievements, Himmelsbach and his colleagues demonstrated the ability of synthetic opiates to substitute for morphine, thus attenuating the withdrawal syndrome [9]. Dole et al. [10] later exploited this observation in their introduction of the use of methadone as a maintenance therapy discouraging the continued use of illicit opiates and providing the basis for the major current treatment approaches for heroin and other drug addictions.

Others at Lexington, including Harris Isbell, H.F. Fraser, and William Martin added quantitative physiologic measures to the behavioral profiles and detailed descriptions of subjective effects of opiate drugs following acute and chronic administration. Collectively, these studies have resulted in a very complete understanding of the pharmacology of these drugs in humans and in primates. Martin and colleagues later conducted additional studies that extended into the 1970s on physiologic measures of opiate drug effect in dogs.

3.1.1 Receptors for Opiate Drugs

Himmelsbach did not describe his results and those of his colleagues in the context of a receptor theory for opiate drug action [8], but the careful quantitative studies of many chemically different agents with opiate drug properties that emerged from the Addiction Research Center, along with related studies from other research groups in the United States and elsewhere, clearly pointed to a common mechanism of action for drugs as chemically dissimilar as morphine, meperidine, and methadone. However, elucidation of the mechanisms of action of drugs producing effects via the central nervous system (CNS) was difficult since the function of the CNS itself was understood at that time only in a most rudimentary way.

During the middle of the last century three major themes in the studies of opiate drug pharmacology strongly supported the possibility that morphine acted in the brains of vertebrates by activating a specific form of receptor. This concept was first explicitly proposed by Beckett and Casy [11], based on their studies of the antinociceptive properties of a series of synthetic agents related to meperidine, a molecule that lent itself more readily than morphine to stepwise chemical manipulation and in particular to the preparation of stereoisomers.

In the course of these studies they developed a number of enantiomeric pairs of opiate analgesic drugs. Antinociceptive activity in this chemical series was largely restricted to one enantiomer, and all the active enantiomers had the same conformation (relative to D-alanine). The concept of an endogenous receptor for a plant alkaloid that was not found in animals was, of course, controversial. Nevertheless, the receptor concept gained acceptance, and was further expanded by additional studies of the structure-activity relationships for other novel synthetic antinociceptive agents by Portoghese [12, 13] and Lewis et al. [7].

The relevance of the stereo-selectivity of opiate-like actions was based on the understanding that the physical properties of each of the enantiomers (molecular weight, charge, hydrophobicity, etc.) of each member of a pair of enantiomers were very similar, but their spatial characteristics were not superimposable; it was therefore probable that they interacted with a tissue component in a way that required a specific spatial orientation of critical functional groups – a feature that would be anticipated for action through a specific target receptor but not for actions depending simply on charge or hydrophobicity.

Natural morphine itself is the levorotatory enantiomer of a molecule with five optically active carbon atoms. Not all actions of opiate drugs show the same degree of stereospecificity; for example, the ability of opiates to release histamine or to activate cholinesterase were reported not to be stereo-specific [14] as was the lethality induced by high dose levels of levorphanol or dextrorphan (the D-isomer of levorphanol) in Swiss Webster mice [15, 16]. (Note that levorphanol still exhibits a lower LD₅₀ than dextrorphan in mice because lower dose effects are mediated via μ-opioid receptors regulating respiration).

The second theme that emerged during these early years was the existence of drugs chemically similar to morphine that appeared to antagonize the antinociceptive and other properties of morphine and its congeners. In the early years of the last century an *N*-allyl-substituted analog of codeine, and later *N*-allylnormorphine (subsequently called nalorphine), were synthesized, based on the belief that *N*-allyl substitutions conferred respiratory stimulant properties that might mask or prevent the respiratory depressant actions of morphine or codeine.

By the 1940s it was appreciated that *N*-allylnorcodeine and nalorphine were capable of antagonizing the analgesic actions of morphine as well as the respiratory depression [17]. However, while antagonism of morphine by nalorphine could be demonstrated, nalorphine alone was shown to induce an antinociceptive effect that was almost comparable to that of morphine [18, 19].

The introduction of naloxone, another semi-synthetic drug related to morphine, clarified the issue. Naloxone was completely devoid of analgesic, respiratory depressant, and addictive properties, but it effectively and completely antagonized the analgesic and respiratory depressant actions of morphine [20, 21]. Further studies on the role of the nitrogen substituent in morphine and related drugs revealed a striking requirement for a relatively small substituent in this position for optimum antinociceptive activity; methyl and ethyl groups conferred greater activity than hydrogen, while side chains much longer than pentyl resulted in reduced activity, with allyl or cyclopropylmethyl substituents giving an antagonistic effect [22]. The importance of the size of the nitrogen substituent in determining if the effect was predominantly agonist or antagonist, taken together with the stereospecificity for opiate actions, provided further indirect support for the receptor model of opiate drug action, at a time when it was not possible to isolate or characterize receptors directly.

A third line of evidence for specific receptors for opiate drugs emerged from studies of the actions of opiate on neurotransmitter release in the gastrointestinal tract and other peripheral tissues. The inhibition of peristalsis by morphine was first demonstrated by Trendelenburg [23, 24]. Later, Schaumann et al. [25] and Trendelenburg [26] in Germany, Kosterlitz and Robinson [27] in Scotland, and Paton [28] in England confirmed that the relative potencies of a wide range of opiate drugs in producing antinociception *in vivo* were very similar to their relative potencies in inhibiting the contractions of isolated guinea pig ileum segments induced by activating the cholinergic neurons of the myenteric plexus.

Paton [28] showed that this action was mediated by a reduction of acetylcholine release; the smooth muscle response to acetylcholine was unaffected by morphine. About the same time, it was found that nalorphine [29] and naloxone [30] could

antagonize the morphine-induced reduction of acetylcholine release from guinea-pig myenteric plexus neurons, and that the ability to inhibit acetylcholine release resided predominantly in the analgesically active isomer of enantiomeric opiate drug pairs [29]. These results supported the earlier proposal by Paton [28] that the myenteric plexus offered an experimentally accessible “paradigm” of drug action at selected synapses related to pain perception in the CNS.

The guinea pig ileum and later another isolated tissue neuroeffector junction model, the mouse vas deferens preparation, became widely used models for quantitative experimental studies of opiate drug action. Kosterlitz thereafter strongly supported the importance of demonstration of antagonism of an effect by naloxone as the critical requirement by which this effect might be classified as “opiate-like.”

The concept of specific receptors for opiate drugs became generally accepted by most groups working in the field by the late 1960s, about the time that techniques offering a realistic chance of directly measuring the drug-receptor interaction were introduced. The availability of radioactively labeled drugs molecules opened the way for measurement of binding of the radiolabeled drug to binding sites in tissues where drug effects were exerted. Early studies on the tissue binding of radiolabeled opiates suffered from the problem of very high binding to tissue components unrelated to drug action. Goldstein et al. [31] noted that binding to the receptors should be high affinity, saturable and stereospecific. It should be possible to identify the receptor component of total binding despite the presence of an excess of other nonspecific binding sites by computing the fraction of total binding that was displaced by an excess of an active opiate drug enantiomer such as levorphanol but not by its inactive enantiomer dextrophan.

Using this approach to dissect the components of the binding by brain tissues of [³H] levorphanol, Goldstein and colleagues found only about 2% of total labeled opiate binding was saturable and stereospecific and thus might be expected to reflect receptor binding. The radiolabeled levorphanol that was available for these studies was of low specific activity by today’s standards, making it necessary to use relatively high drug concentrations to measure any binding.

Within a couple of years, three groups obtained [³H]-labeled opiates with significantly higher specific activity, making it possible to study binding with lower free drug concentrations (significantly reducing nonspecific binding). At the same time ligand binding assays using glass fiber filters were developed, permitting more extensive “washing-out” of nonspecifically bound drug. With these technical enhancements Pert and Snyder [32], Terenius [33, 34], and Simon et al. [35] all demonstrated quantitatively reliable specific drug binding that was now usually greater than 70% of the total observed binding under their experimental conditions. Competition studies quickly confirmed that the labeled sites in these studies exhibited binding properties that were strikingly similar to the expected binding properties for opiate receptors as predicted from structure-activity studies of analgesic action *in vivo*. The future development of these studies in analyses of the structure and functions of receptors for opiate drugs will be discussed elsewhere in this volume.

3.1.2 *Endogenous Ligands for Opioid Receptors*

The publication of direct experimental evidence for receptors for opiate drugs was soon followed by the proposal that these endogenous receptors must be subject to regulation by an endogenous ligand. No known endogenous agent was capable of producing the spectrum of effects induced by morphine, leading Collier [36] to predict in print (and others more cautiously during discussions at meetings) that an endogenous ligand for these receptors must exist.

In vitro assays that might be used to facilitate a search for an endogenous ligand for opiate drug receptors were now available. The radioligand binding assays used by the Terenius, Snyder, and Simon groups allowed many samples to be compared easily, but unfortunately were highly sensitive to interfering substances in tissue extracts such as salts and endogenous nucleotides. The in vitro neuro-effector assays such as the isolated guinea pig ileum and mouse vas deferens assays, although less sensitive to irrelevant tissue components, were less readily adapted for the analysis of large sample numbers. However they offered the major advantage that antagonism of any apparent opiate effect by selective antagonists such as naloxone could be tested in these preparations, thus eliminating false positive effects induced by salts and other tissue components that do not directly interact with the ligand binding site of opioid receptors.

After these two types of assays were employed, evidence for the presence of endogenous opioids in mammalian brain quickly emerged. These results were first reported in a meeting of the Neurosciences Research Program held at MIT in late spring of 1974 [37]. Terenius and Wahlstrom [38] then reported inhibition of radio-labeled opiate drug binding to brain membranes by a brain extract, as did Pasternak et al. [39]. Shortly thereafter, Hughes [40] reported opiate-like activity of pig brain extracts on the mouse vas deferens preparation.

Chemical characterization quickly followed. Hughes et al. [41] reported the structure of two peptides isolated from pig brain with potent naloxone-reversible opiate-like activity on both the mouse vas deferens and guinea pig ileum preparations. These closely related pentapeptides were named [Met^5]enkephalin and [Leu^5]enkephalin by Kosterlitz and colleagues. This study was remarkable in that it was also the first use of the newly developed technique of mass spectrometry to identify the chemical structures of novel highly potent low-molecular weight endogenous neurotransmitters.

After publication of the enkephalin structure, it was immediately recognized that the [Met^5]enkephalin sequence was contained within the previously published sequence of the pituitary peptide β -lipotropin (β -LPH), part of the precursor peptide from which adrenocorticotropin (ACTH) was produced in pituitary.

Earlier in 1975, Goldstein and colleagues had reported that extracts of pituitary from either pig [42] or cattle [43] also contained products that exerted opiate-like action on the guinea-pig ileum preparation, although these extracts showed some differences in properties. A rapid analysis of previously prepared semi-purified pituitary extracts showed that a C-terminal fragment of the pituitary peptide β -LPH

did indeed exert potent opiate activity [44, 45] closely resembling the activity reported by Teschemacher et al. [43]. This peptide soon became known as β -endorphin. Subsequently, Goldstein et al. [46] isolated and characterized yet another very potent highly basic opioid heptadecapeptide from porcine pituitary glands, which they named dynorphin. This peptide showed similar properties in the guinea pig ileum assays to the porcine pituitary extract first reported by [42]. Dynorphin was later shown not to be unique to the pig; it has since been found in all tested mammalian brains and other tissues.

The three families of endogenous peptides that play physiological and pathophysiological roles in the brain and periphery soon became known as “opiod” peptides (meaning “opiate-like”), reflecting the similarities in their actions to those of the opiate drugs. The generic term “endorphins” (meaning endogenous peptides inducing actions similar to those of opiate drugs) began to be used around this time.

The discovery of the endorphins required that the actions of morphine and related drugs be viewed in a new light. It was now obvious that opiate drugs were active on many physiologic systems because they activated the receptors normally activated by endogenous opioid peptides. The endogenous opioids presumably serve a modulatory physiologic role in the regulation of these systems. The structures of the mRNAs coding for the three families of endogenous opioids – preproopiomelanocotin (POMC) [47] preproenkephalin, [48] and preprodynorphin (also called pro-enkephalin B) [49] were soon determined.

3.1.3 *Heterogeneity Among Receptors for Opioids*

The surprising identification of more than one endogenous agent capable of activating the receptors for opiate drugs immediately raised the question of whether these agents all activate the same endogenous receptor. Martin and colleagues had previously reached the conclusion that at least two receptor types, which they called M for morphine and N for nalorphine, [50] must be involved in the action of morphine and its congeners, based on the interactions of these two drugs in patients [8, 18, 19].

With further studies completed, Martin now proposed the existence of three receptors for opiate drugs with the new names μ , κ , and σ , derived from the names of their preferred ligands, morphine, ketocyclazocine, and SKF 10,047, respectively [51]. Since publication of these studies, others have concluded that the receptors designated σ , responding preferentially to the synthetic opiate-like drug, SKF 10,047 (*N*-allyl-normetazocine), are not in fact members of the family of opioid receptors since actions at this site are not antagonized by naloxone.

Soon after Martin’s work was published, Lord et al. [52] reported the results of quantitative analyses of the actions of the newly discovered opioid peptides in comparisons with morphine-like drugs, first as competitors for radioligand binding sites in brain membranes and second as naloxone-reversible agonists in two isolated neuro-effector bioassays. They also interpreted their results as indicative of the presence of three distinct types of receptor, two of which appeared similar to the

two opioid receptor types defined by Martin et al. [51] – the μ and the κ type receptors – but Lord et al. [52] proposed a different third receptor type which they labeled δ (preferentially responsive to [D-Ala²]-substituted enkephalins and also endogenous enkephalins).

Others soon provided additional pharmacological data that supported this classification of opioid receptors [53, 54]. It took another 20 years for these receptors to be cloned (see Chaps. 6, 7, and 8), but these cloning studies confirmed the early insights of the Martin and Kosterlitz groups.

The eventual cloning of the δ and then of the μ and κ receptors yielded one more surprise. Using homology cloning techniques, receptor clones closely resembling those of the three “classic” opioid receptors but with some differences in coding sequence were quickly identified. Expression and characterization of these previously unknown (and identical) receptor clones – named provisionally ORL-1 [55], KOR-3 [56, 57], or LC132 [58] – made it clear that the novel receptor type had negligible affinity for ligands binding to μ , δ , or κ opioid receptors.

Two groups used activation of the expressed “orphan” clones, (measuring inhibition of adenylyl cyclase activity in cell lines expressing ORL1 or LC132) to screen brain extracts for endogenous ligands. Both groups identified the endogenous ligand as the same novel heptadecapeptide, naming this nociceptin [59] or orphanin FQ [60]. The precursor peptide was identified and the nociceptin/orphanin FQ gene was soon cloned [61, 62]. There is still lack of agreement on a common name for this peptide; many use a combined name, nociceptin/orphanin FQ or orphanin FQ/nociceptin, or some abbreviation of these terms (e.g., N/OFQ). The receptor has become known as the nociceptin orphanin peptide receptor (or NOP receptor).

The close structural homologies between the NOP sequence and the sequences for μ , δ or κ opioid receptors, and the sequence homology of pre-proN/OFQ and preprodynorphin, make it clear that the genes for N/OFQ and its NOP receptor are evolutionarily related to the other peptide/receptor pairs in the opioid gene family (and despite the lack of antagonism by naloxone at NOP receptors, the receptors are still in the opioid receptor family in terms of structural similarity, pace Dr. Kosterlitz). The currently accepted nomenclature for opioid receptor types and their major endogenous ligands, are shown in Table 3.2.

3.1.4 Locating Opioid Receptors: Autoradiography and In Vivo Imaging

Opioid actions within the brain were mapped to specific regions [63], raising the question of whether or not these same regions contained the opioid receptors. Receptor binding assays showed a high correlation between regions important in opioid action and receptor expression [64]. However, receptor binding assays are limited in their resolution, leading to the development of autoradiographic approaches capable of showing receptor expression at the cellular level [64–68]. Initially performed for μ receptors, these approaches have been extended to the

Table 3.2 Classification of opioid receptor types

IUPHAR-NC recommended nomenclature ^a	Other names	Presumed endogenous ligands
μ , mu, or MOP ^b	MOR	β -endorphin (not selective)
	OP ₃	Enkephalins (not selective) endomorphins ^c
δ , delta, or DOP ^b	DOR	Enkephalins (not selective)
	OP ₁	β -endorphin (not selective)
κ , kappa, or KOP ^b	KOR	Dynorphin A
	OP ₂	Dynorphin B α -neoendorphin
NOP	ORL1, LC132	Nociceptin/orphanin FQ
	OP ₄	

^aThis table is based on the recommendations of the International Union of Pharmacology Receptor Nomenclature Committee (IUPHAR-NC). Their full recommendations can be viewed at:), <http://www.iuphar-db.org/DATABASE/FamilyMenuForward?familyId=50>.

^bThe well-established Greek terminology for opioid receptor types using the descriptors, μ (mu), δ (delta) or κ (kappa) is recommended, but the receptor type should additionally be defined as MOP, DOP, KOP, or NOP when first mentioned in a publication

^cNo mechanism for the endogenous synthesis of endomorphins has been identified; their status as endogenous ligands for the μ opioid receptor is tentative

other classes as well [69–71] While many sites were labeled with selective radioligands, sites lacking highly selective ligands were visualized using blocking approaches with selective competitors or even digital subtraction techniques [72]. The cloning of the receptors also opened new avenues for their localization, including *in situ* hybridization of the mRNA's encoding the receptors [73–77] and immunohistochemistry of the receptor itself [78–82].

3.1.4.1 Imaging OP Receptors in Human Brain

With the recent development of *in vivo* imaging technologies, it has now become possible to locate important target sites for opiate drugs in human brain. Positron emission tomography (PET) permits the localization of positron emitting ligands in reasonably discrete regions of human brain, together with an estimate of their local concentration. The radionuclides used are ¹¹C and ¹⁸F; analogs of potent opiate drugs containing one of these radionuclides have been synthesized for PET studies.

A comprehensive review of the use of PET ligands for the analysis of opiate distribution and function in human brain has recently appeared [83]. Limiting factors are the difficulty of discriminating specific from nonspecific ligand binding, and the limited spatial resolution of the detection equipment currently available, which precludes anything more than very general views of receptor distribution in the brains of laboratory animals. In humans, the resolution is adequate to permit quantitative estimates of drug concentrations in defined brain regions but not in discrete regions within the larger structures.

Radiolabelled PET ligands developed from clinically used opiate drugs have been administered to volunteer subjects and the sites within the brain at which the PET ligand is concentrated have been identified. In some studies comparison has been made between ligand binding in control conditions relative to binding in altered physiological or pathological states, but their interpretation is difficult. A function-related reduction in PET ligand binding may be caused by receptor down-regulation or by reduced receptor availability because of occupancy of the receptors by endogenous opioids (reducing their availability to the PET ligand). In some cases the two potential mechanisms can be differentiated by the time course over which the change in binding occurs; down-regulation is presumed to take some time, while endogenous peptide release can occur rapidly, but in chronic pathological conditions this discrimination cannot be easily be made.

At present the value of these studies is also constrained by the limited availability of positron emitting ligands with ideal properties as high and selective affinity for specific OP receptor types. The most selective opioid receptor PET ligand is [¹¹C]carfentanil ([¹¹C]CAF), with approximately 130-fold selectivity for μ receptors vs. δ , and 1,800-fold selectivity for μ vs. κ receptors. [¹⁸F]Cyclofoxy has only modest (three to fourfold) selectivity for μ receptors while ¹¹C and ¹⁸F analogs of the opioid antagonist diprenorphine (DPN) are nonselective among μ , δ , and κ receptors [83].

The PET technique was first used to analyze opioid receptor distribution in human brain by Frost et al. [84] using [¹¹C]CAF as the ligand. High receptor concentrations were observed in the basal ganglia and thalamus, with lower concentrations in various cortical regions. Specific [¹¹C]DPN binding was found to be relatively high in brain regions thought to be implicated in both the sensory discriminative (lateral) and affective/motivational (medial) components of pain perception pathways [85]. Chronic pain conditions such as rheumatoid arthritis have been associated with reduced binding of opiate PET ligands; this has been attributed to the release of endogenous opioids as a result of the chronic pain [86]. An extensive literature on other applications of opiate PET ligands for *in vivo* opioid receptor studies in human brain has been summarized recently [83].

The following sections provide an overview of the major pharmacological actions and clinical uses of opiate drugs. Cellular mechanisms of opiate drug action and issues relating to the mechanisms underlying opiate drug tolerance, dependence and addiction are beyond the scope of this review.

3.2 Antinociception

Morphine is particularly effective against dull sustained pain rather than against the short sharp pains induced by electric shocks, but the perception of all types of pain can be reduced by adequate doses. The pain relief induced by low doses of morphine is specific for pain sensation rather than other sensory modalities such as touch, hearing, sight, smell, taste, or proprioception, although high doses of opiate drugs

will cloud consciousness, at which point responses to these other types of sensory stimuli are also affected. Consciousness is usually only affected by doses of morphine that significantly depress respiration.

With moderate to high morphine doses the degree of pain relief can be profound. In recent years, opiate drugs and particularly fentanyl and its congeners have been used as anesthetic agents, usually in combination with a weak anesthetic such as nitrous oxide or high doses of benzodiazepines to induce clouding of consciousness and reduce recall of the surgical experience. At anesthetic doses of opiates, respiration must be maintained artificially but with respiratory support and the addition of a muscle relaxant to facilitate surgery, opiate drugs can provide complete pain relief for surgery with less stress to the heart than with conventional gaseous or volatile anesthetics.

3.2.1 Experimental Assessment of Pain Relief

Reduced pain perception induced by opiates is commonly called “analgesia” (absence of pain) although the patient may well not have complete relief of pain. In experimental animals, the effects of opiate drugs are assessed by measuring the response of an animal to some kind of noxious painful stimulus (usually transient). Such stimuli include heat (mice or rats are placed on a hot surface that is maintained at about 55°C and the time to a withdrawal response such as lifting of foot from plate or jumping is measured, or to heat from a lamp that is focused onto a small region of skin on the tail or nose; again latency to an avoidance behavior is measured); pressure (applied through a probe placed on the tail, limb, or toe (again latency to withdrawal is measured); brief electrical shocks (applied to the feet via an electrified cage floor grid or through electrodes applied to the tail or other area of skin-threshold current to induce a defensive response is measured); or painful chemical stimuli (e.g., injection of small amounts of an acid such as formic acid or a local inflammatory agent into the plantar region of a foot or acetic acid or other irritant into the peritoneal cavity-protective or avoidance behaviors induced by the chemical irritant are scored).

More recently, studies in which nocifensive protective or avoidance behaviors induced by the implantation of proliferating tumor cells into limb bones have been used to evaluate the potential effects of opiate drugs in the treatment of cancer pain. In all these cases the ability of opiate drugs to extend the response latency, increase the shock threshold for motor response, or to reduce protective or avoidance behaviors, is called an “antinociceptive” action. For opiate drugs, demonstration of antinociception in laboratory animals is usually indicative of analgesic actions of an agent in man.

In humans, the perceived intensity of pathological or experimental pain can be assessed by patient questionnaires, or by asking subjects to mark the severity of their pain on a visual-analog scale from 0 (no pain) to 10 (most severe pain imaginable). Despite the apparent simplicity of such scoring systems and their

potential sensitivity to numerous confounding variables, many carefully controlled studies have shown remarkably consistent responses for particular drugs among different laboratories and over significant time periods.

3.2.2 Target Sites in Brain Mediating Opiate Antinociception

Until recently studies of the sites of action of opiate drugs have been conducted in laboratory animals. It has long been known that the undifferentiated fine nerve terminals distributed widely through skin and pain-sensitive organs can be activated by local injury, by changes in local pH, and by locally released mediators, including the peptide, bradykinin [87]. An early study by Lim et al. [88] demonstrated that opiate drugs suppressed the response to pain largely by actions in the CNS, in contrast to the antinociceptive actions of aspirin, which appeared to be mediated locally in close proximity to the sites of action of local pain mediators. Lim et al. [88] recorded the vocalization, tachycardia, and hyperpnea induced by bradykinin when injected directly into arterioles supplying the spleen in conscious dogs in which the vascular supply to the spleen was isolated by perfusion of this organ with blood from a donor dog. They showed that while aspirin induced antinociceptive effects when injected into the vascular supply to the spleen (i.e., to the donor dog) but not when administered centrally to the recipient dog, morphine and meperidine failed to induce significant antinociception when applied exclusively to the recipient dog spleen (via the donor dog circulation). In contrast, morphine or meperidine induced complete suppression of the vocalization and other nocifensive behaviors induced by splenic injection of bradykinin when injected into the systemic circulation in dogs, despite the lack of access of the morphine or meperidine to the splenic site at which bradykinin induced pain. These studies led to a search for the primary sites of action of morphine in the CNS.

A common approach in CNS site-of-action studies was for small amounts of an opiate drug to be injected into a discrete region of brain or into the cerebral ventricles under conditions where drug diffusion was significantly limited. Three CNS regions emerged from these studies as major target sites for opiate analgesia. A region in the vicinity of the aqueduct between the third and fourth cerebral ventricles was identified by Tsou and Jang [89]. Subsequent studies by Herz, Teschemacher and their colleagues (summarized in Hertz et al [90] and by Pert & Yaksh [63] confirmed the critical importance of the mid-brain periaqueductal gray region (PAG) as a primary target site for the antinociceptive actions of morphine. The presence of opiate-sensitive neurons in PAG that project to the rostroventral medulla (RVM; see below) has subsequently been confirmed by Osborne et al. [91].

The role of the PAG in the regulation of the response to noxious stimuli was initially uncertain. That the PAG (and RVM) were not relay sites in afferent spinothalamic or cortical pathways became apparent following the demonstration by Satoh and Takagi [92] that morphine activates a descending pain regulatory pathway that attenuates the response to noxious stimuli at the level of the spinal cord. Satoh and

Takagi recorded activity high in the ascending pain pathways in the spinal cord of cats during electrical stimulation of the splanchnic nerve.

Systemic administration (i.v.) of morphine reduced the activity in the ascending nociceptor pathway. However, transection of the brain stem above the level of the recording electrode largely abolished the antinociceptive action of systemic morphine, but decerebration by transection of the brain at the interfollicular level (above the medulla, pons and most of the mid-brain) was without effect on the action of morphine, suggesting that pontine and/or brainstem sites of action were particularly critical for the antinociceptive action of morphine.

The potential role for sites in the RVM was suggested by experiments by Dey and Feldberg [93]. They applied morphine directly into the subarachnoid space under the ventral surface of the brain stem caudal to the pons in cats and observed a very marked antinociceptive response. The role of neurons located in the nucleus reticularis paragigantocellularis (NpGC) and nucleus raphe magnus (NRM) in the actions of morphine in RVM was later demonstrated [94].

Understanding of the role of the RVM in analgesia was further advanced by the work of Howard Fields and colleagues who identified two classes of neurons in NRM projecting down the spinal cord to serve a modulatory role at the dorsal horn synapse of primary afferent nociceptor neurons and dorsal horn transmission neurons (projecting from the spinal cord to the thalamus and on to the cortex). Agonists acting at μ receptors disinhibit primary neurons in NRM, increasing their firing rate [91, 95]. Activity in these neurons inhibits transmission at nociceptor synapses in the dorsal horn, thus producing analgesia.

Receptors of the μ -type are also expressed by a second class of NRM neurons (secondary cells) projecting to the dorsal horn of spinal cord; μ -agonists directly inhibit these cells whose normal activity facilitates pain transmission in dorsal horn [96]. Thus, there are two sites of μ -mediated antinociception in NRM. The NRM primary cells are also subject to direct inhibition by κ -agonists; these drugs tend to facilitate pain transmission in dorsal horn and exacerbate nociceptive responding.

Primary NRM neurons are also subject to direct inhibition by N/OFQ acting through NOP receptors, and N/OFQ is known to increase pain perception after spinal cord administration. Thus both κ - and NOP agonists can reverse μ -mediated antinociception at this site [97]. (NOP receptors are also present on secondary cells in NRM where they may contribute to N/OFQ-mediated antagonism of hyperalgesia associated with increased activity in the secondary cells of NRM). The dual action of μ -selective opiates in the RVM contributes significantly to the antinociceptive actions of systemically administered opiate drugs.

Thirdly, opiates have also been shown to reduce nociceptive responses when applied to the dorsal horn of spinal cord in rats or man [98, 99]. Intrathecally applied morphine inhibited the release of the major transmitter in primary afferent neurons, substance P, into the sub-arachnoid space is reduced [100]. Opioid receptors of the μ -type are found in close proximity to substance P-containing terminals at both pre and postsynaptic locations in the dorsal horn [101]. The organization of nociceptive transmission in the dorsal horn of spinal cord is complex and remains incompletely understood [102]. Intrathecal administration of morphine

is now used clinically to provide localized relief of pain with less respiratory depression than would be seen with equivalent doses given systemically.

Dynorphin is also implicated in hyperalgesic effects mediated at the spinal cord level, particularly after various forms of neural injury and in animal tolerant to the actions of μ -agonists. Spinal cord injury causing hyperalgesia is known to increase the local expression of dynorphin [103]. In this case, however, κ -receptors may not be involved. Des [Tyr¹]-dynorphin, a prominent dynorphin metabolite, can activate NMDA receptors to induce pain [104]. In a mouse strain that does not upregulate dynorphin expression in dorsal horn after injury, neuropathic pain is not observed [105].

Section of the dorsal roots of primary afferent neurons close to the spinal cord is followed by degeneration of the nerve terminals in the dorsal horn, and results in the loss of a significant fraction of the μ -opioid receptors that are expressed in the dorsal horn [106]. These results suggested that some of the dorsal horn OP receptors are expressed in primary afferent neurons. The conclusion has subsequently been confirmed and extended, particularly in studies by Stein et al. [107] The receptors are located on sensory nerve terminals in joints and other tissues, where they appear to be regulated by endogenous opioids – principally β -endorphin and [Met⁵] enkephalin – that are released from immune cells including macrophages, lymphocytes, and plasma cells, and from cells of the synovial lining [108].

The extent of opioid peptide release is related to the severity of inflammation. Furthermore, local application of opiate drugs has been shown to reduce joint pain [107]. Thus the peripheral terminals of primary afferent neurons offer a fourth potential target site for an antinociceptive action of systemically administered opiate drugs. However, while a local action of opiates has been demonstrated to relieve joint pain and to be of clinical value in some cases, it is not certain that this is a significant site for the overall antinociceptive actions of low systemic doses of morphine.

These studies demonstrate that there are at least three primary sites of action mediating the antinociceptive effects of opiate drugs acting at μ -receptors: the PAG, the NRM, and NpGC in RVM and the dorsal horn of spinal cord (Fig. 3.1).

When opiate drugs are administered systemically, they probably act in part at all three sites (and potentially at peripheral nerve endings and other central sites). At the lowest doses, morphine may act preferentially in the RVM and PAG, but at slightly higher doses spinal cord actions are also important. There is evidence that combined action at more than one site leads to a significant potentiation of the observed analgesic effect. This was first demonstrated as a profound synergy between morphine doses administered at separate supraspinal and spinal sites [109]; subsequent studies also revealed synergistic interactions between the PAG, RVM and the locus coeruleus within the brainstem, [110] and between peripheral and central sites [111]. The “multiplicative” effect of actions by opiates at different points on the pain-regulating pathway, to use a term coined by Yeung and Rudy [109], may explain the effectiveness of opiates in pain relief, and may provide a partial explanation of the apparent ability of opiates to induce significant analgesia at low levels of μ receptor occupancy.

to thalamus and cortex

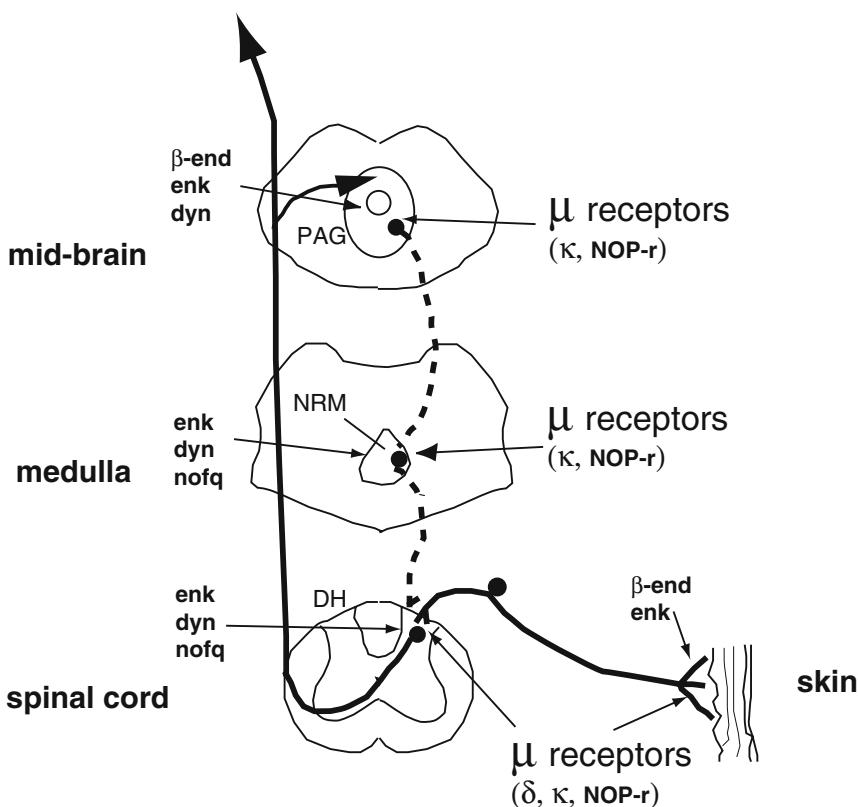


Fig. 3.1 Location of expression of endogenous opioids and opioid receptors in relation to sites of action of opiate drugs in neural pathways regulating nociception. The illustration depicts regions where nociceptor pathways connect at the level of peripheral nociceptor nerve terminals, spinal cord, medulla, and mid-brain. (PAG periaqueductal gray region; NRM nuc raphe magnus; DH dorsal horn of spinal cord; β -end beta-endorphin; enk enkephalins; nofq nociceptin/orphanin FQ.)

3.3 Other Actions of Opiate Drugs

Enkephalins and dynorphins are expressed widely in many brain regions; β -endorphin expression is more restricted, but the proopiomelanocortin neurons of the hypothalamic arcuate nucleus can deliver β -endorphin to many widely dispersed forebrain and mid-brain structures and there is local release of β -endorphin in several brain stem structures. Similarly, opioid receptors of all types are also widely distributed throughout the brain in many different functional systems. It is therefore not surprising that morphine and related drugs induce effects on many physiological systems that are not directly related to the relief of pain. Some of these other actions are exploited therapeutically, but they are mainly regarded as side effects in man.

that ultimately limit the doses of morphine and related drugs that can be given safely to relieve pain. The following section summarizes the major “side effects” of opiate drugs, again with an emphasis on the prototypic drug morphine.

3.3.1 Regulation of Central Arousal Mechanisms; Sedation and Anesthesia

It has long been known that opiate drugs reduce arousal, exert sedative effects and at high doses (relative to those required to reduce the response to noxious stimuli) can induce an anesthetic state. The induction of somnolence is implied in the name linking morphine with Morpheus, the god of sleep.

The ability of the drugs to reduce arousal contribute to the spectrum of potentially beneficial actions that opiates can induce in addition to the reduction of pain sensation when opiates are used in palliative care. During the middle years of the last century there was interest in exploiting the sedative actions of opiates in new approaches to anesthesia. The demonstration by Janssen [112] that high doses of haloperidol or related butyrophenones (drugs then known as neuroleptics) exhibited a unique pharmacology including induction of a pseudo-anesthetic state lead to the use of these drugs as co-anesthetic agents, in combination with another potent Janssen drug, fentanyl (or one of its congeners), to obtund pain.

Later, the combination of neuroleptic drug with an opiate was dropped. High doses of a short-acting opiate drug (for pain relief) in combination with a muscle relaxant (to facilitate surgery) and a benzodiazepine (to avoid recall of the surgery) became an important anesthetic drug combination, valued because of the reduced cardiovascular stress relative to the use of gaseous or volatile anesthetics. Short-acting drugs such as fentanyl, sufentanil, and remifentanil are the most commonly used opiates for anesthesia.

The mechanisms underlying arousal are complex and multifaceted. Early studies emphasized the role of the central reticular activating system; later, a histaminergic hypothalamic arousal system was described, [113] and a noradrenergic arousal/alerting pathway from the locus coeruleus has been proposed [114]. Recently, the hypocretin/orexin pathways from the lateral hypothalamus to many brain stem and forebrain structures [115] have been reported to play an important role in the maintenance of alertness.

Noradrenergic neurons in the locus ceruleus (LC) that innervate the cortex and other forebrain structures were proposed to play a significant role in vigilance and alertness; for a review, see Aston-Jones et al. [114] Almost all neurons of the LC express μ -opioid receptors in relatively high concentration; these neurons thus stand out prominently in coronal brain sections at the level of the LC when processed to display tyrosine hydroxylase (as a marker of noradrenergic neurons) by immunohistochemistry, or to display the mRNA for μ -opioid receptors by *in situ* hybridization.

Activation of μ -receptors in LC neurons by morphine or [Met^5]enkephalin produces a marked hyperpolarization that can be readily reversed by naloxone or other μ -selective antagonists [116, 117]. Aghajanian had earlier reported marked elevation of firing rates in LC neurons of morphine-dependent animals when withdrawal symptoms were elicited by morphine withdrawal or administration of an opiate antagonist [118]. The studies of Aston-Jones emphasized the broader role of LC neurons in eliciting vigilance and arousal by a general facilitation of activation of cortical neurons when LC neurons were active [114]. These observations indicate that inhibition by morphine of norepinephrine release in forebrain structures may play an important role in morphine-induced sedation.

The role of the hypocretin system has been reported much more recently [115]. Hypocretin/orexin is expressed in a discrete cluster of neurons in the lateral hypothalamus. These neurons innervate numerous structures throughout the brain including the LC and most forebrain structures. Loss of hypocretin neurons is associated with narcolepsy [119]. Hypocretin neurons express μ -receptors [120], and activation of these receptors by morphine or [Met^5]enkephalin results in hyperpolarization and a marked reduction in firing rate [121]. Since administration of a selective μ -antagonist increases the rate of firing in the absence of any exogenous opiate it is likely that the hypocretin neurons are under tonic regulation by hypothalamic endogenous opioids. In addition to direct action of opiates on hypocretin-containing neurons, opiates or endogenous opioids may also exert presynaptic inhibition of excitatory inputs to the hypocretin neurons [122]. The lateral hypothalamus and hypocretin system is also implicated in the expression of opiate withdrawal. Firing rates are increased, and there is increased hypocretin mRNA expression [123]. The mechanism of arousal by the hypocretin-orexin system appears to require the participation of histamine [122]; the hypothalamic histaminergic system [113] is activated by the local release of hypocretin.

Collectively these results suggest that a major component of opiate drug-induced somnolence and sedation results from inhibition of hypocretin-mediated activation of histaminergic arousal pathways [119, 122].

3.3.2 Respiratory Depression

Morphine is a potent respiratory depressant. The effects of opiate drugs on the control of respiration have recently been reviewed by Pattinson [124]. Depression of respiration is a limiting side effect in some patients receiving opiates for pain relief. In humans and many other species the primary cause of death after a lethal dose of morphine is usually failure of respiration. The rate of respiration is slowed and minute volume decreased by doses of morphine that are not much larger than those required for analgesia, leading to hypercapnia and hypoxia.

Peripheral and central chemoreception is depressed by opiates making the respiratory centers less responsive to the respiratory stimulus exerted by an elevation in blood pCO_2 and reduced pO_2 [125], thus explaining the need for extreme care in the

use of opioids with patients with underlying pulmonary disease. Enkephalins, μ -, and other opioid receptors are present in several peripheral sensory sites including the carotid bodies [126] and the vagi, as well as in afferent neuronal pathways from the lungs and airways.

μ -Receptors are also present in brain stem structures concerned with the regulation of respiration, including the nucleus tractus solitarius (NTS) and the pre-Bötzinger nucleus complex. In the NTS, μ -receptors are notably located on the endings of afferent neurons from the respiratory tract and peripheral sensory receptors regulating respiration, where they inhibit activity particularly of the dorso-lateral and medial regions of the nucleus [127]. Respiratory rhythm generation is coordinated by interactions between the brainstem respiratory centers in the retro-trapezoid and parafacial neuron complexes and the nearby pre-Bötzinger complex (a neuronal complex identified in rodents, but not yet characterized in man). It is further modulated by activity from pontine structures, including the Kolliker–Fuse nucleus, the parabrachial complex, and the LC [124]. Opioid peptides and opioid receptors (particularly μ -receptors) are significantly expressed in each of these brain areas. The relative importance to the overall inhibition of respiration by opiate drugs of actions at the various levels of the respiratory reflex (carotid body, NTS, respiratory nuclei complex) and/or the pontine and cortical respiratory modulatory centers remains unknown.

Dissociating pain control from respiratory depression has long been a goal in opioid drug development, but no opiate drugs showing good analgesic actions in the absence of respiratory depression have yet been identified. However, while μ -receptors can clearly mediate respiratory depression, there is a suggestion that their mechanisms may differ in some way from the μ receptors involved in pain control [128, 129].

Opiate drugs are also effective inhibitors of the cough reflex; codeine is frequently prescribed to suppress cough. This action may be mediated through μ -receptors in NTS (probably after systemic or local metabolism of codeine to morphine). A recent study reports that local administration of a μ -selective agonist DAMGO into NTS in the rabbit suppresses cough induced by tracheobronchial stimulation [130]. The actions of other cough suppressants may involve other receptor types. The “over-the-counter” cough suppressant, dextromethorphan, is the (+)-isomer of the opiate receptor agonist morphinan analog, levomethorphan. However, the activity of dextromethorphan at opiate receptors is very weak; it is likely that its cough suppressant actions are mediated through NMDA receptors or as yet uncharacterized receptors.

3.3.3 Locomotor Stimulation and Behavioral Reinforcement

While sedation and respiratory depression are common side effects of moderately high doses of morphine and related drugs in humans and many other species, in other species moderate doses of morphine and other μ -selective agonists may induce stimulant effects. It has long been known that morphine (or heroin and other

opiates) may increase motor activity in horses, with potential benefit to those gambling on their relative speeds over short distances [131]. This facilitatory action is strong enough for racehorses to be tested regularly for doping with opiate drugs. Other species, including mice and some strains of rat, may also show predominantly stimulant actions after morphine.

The quantitative reliability of the running response (i.e., locomotor stimulation) in some mice strains led to the use of measures of locomotor activity by Goldstein and Sheehan [15] in early studies of the mechanisms underlying opiate drug tolerance. This response to opiate drugs was shown to be genetically controlled. By selectively breeding the mice based on the locomotor response to an opiate, it was possible to select two different sub-strains with markedly different motor responses to the drug [132]. The strain and species differences in the primary locomotor actions of morphine (sedation and lethargy or motor stimulation) presumably result from species/strain-dependent differences in the role of μ -receptors and their relative levels and locations of expression in critical brain structures.

The role(s) of other opioid receptor types in motor regulation is less clear. It has been suggested that μ -receptors preferentially increase horizontal activity while decreasing vertical activity (rearing); in contrast δ -receptor agonists increased both horizontal and vertical activity in mice [133]. Subsequent studies suggest that the stimulant response to opiates in mice is related to modulation of dopaminergic and possibly noradrenergic pathways in the striatum and forebrain. In cats, a marked psychomotor excitation is induced by opiate peptides such as β -endorphin [134].

These motor stimulant actions of opiates are closely related to opiate-induced behavioral reinforcement (i.e., drug-induced reward); actions of opiate that underlie the addictive liability of this class of drugs. Again, interactions of opiates with endogenous dopaminergic pathways arising in the ventral tegmental area, with pathways projecting to the nucleus accumbens, the prefrontal cortex and other forebrain structures are thought to play a major role in these effects. A more extensive review of this topic is beyond the scope of this chapter.

3.3.4 Nausea and Emesis

In animals with a functional vomiting reflex (including humans), morphine and related drugs can induce nausea and emesis. This action is observed with therapeutic doses of morphine presenting an additional constraint on the achievement of adequate pain relief by opiate drugs in patients particularly sensitive to this action. Nausea and emesis induced by opiate drugs appears to be mediated by μ -receptors in the area postrema (also known as the chemoreceptive emetic trigger zone) [135], an area of the brain that is functionally outside the blood–brain barrier and therefore sensitive to circulating levels of agents that may not cross the blood–brain barrier. Activation of neurons in the emetic trigger zone by opioid peptides [136], drugs or by physiological stimuli such as repetitive motion activates the vomiting center to induce emesis. In very high doses, μ -receptors in the vomiting center itself may

actually directly inhibit the vomiting reflex, but this inhibitory action is not likely to be observed with therapeutic doses of opiates.

Opiate-induced emesis cannot be studied in rodents since these animals do not exhibit the vomiting reflex. However, administration of morphine or other opiates to ferrets induces episodes of vomiting, and this species has been used for studies of agents that might be useful in reducing morphine-induces emesis in humans and ferrets [137, 138]. Other studies on opiate emesis have been conducted in dogs or cats.

3.3.5 Endocrine Effects

Opioid receptors of all types are present in relatively high concentrations in the hypothalamus, where they are located on a number of neuronal systems critical to endocrine function. Endogenous opioids play an important inhibitory role in the regulation of luteinizing hormone releasing hormone (LHRH) secretion. Thus, morphine-like drugs can suppress LHRH release indirectly inhibiting LH release.

Opiate effects on endocrine function have been reviewed by Howlett and Rees [139]. Opiates can suppress ovulation in females and reduce testicular function in males. TRH release is also reduced by morphine in rat, but morphine may increase TRH release in humans. Naloxone alone does not significantly affect TSH release, suggesting that endorphins do not play a major role in the regulation of TRH and TSH. In the rat, morphine is a potent stimulant of CRH secretion, leading to elevated levels of ACTH and corticosterone in plasma. This maybe in part a response to stress induced by other actions of opiates such as respiratory depression. In man, morphine suppresses ACTH release and lowers cortisol levels, while naloxone tends to increase ACTH and cortisol, suggesting that naloxone may remove an endogenous inhibitory tone on CRH release that might be mediated by endogenous opioids. In contrast, the hypothalamic release of growth hormone releasing hormone (GHRH) is facilitated in rat, with elevated circulating levels of growth hormone after morphine treatment, although morphine effects on GH levels in man are modest. Prolactin release is also elevated by morphine, methadone and related drugs in man and laboratory animals; low doses of naloxone antagonize this effect. Although it has been known for decades that opiates also can lower testosterone levels in men [140], it has become recognized as an increasing clinical problem only recently.

3.3.6 Peripheral Actions

3.3.6.1 Gastro-Intestinal Tract

Opiate drugs reduce transit of gut contents through the gastro-intestinal tract (GIT). These actions are exerted at multiple levels. The ability of opiates to inhibit peristalsis [25] through inhibition of acetylcholine release from myenteric plexus

neurons [28], has already been described. In addition, morphine induces a stimulation of the circular muscle of many regions of the GIT; this appears to be mediated through release of 5-hydroxytryptamine in the circular muscle of the GIT [141, 142]. Thus the pyloric sphincter controlling release of stomach contents into the duodenum is contracted, with delayed emptying of the stomach. Tone is also increased in the circular muscle of the small and large intestine; at the same time the peristaltic reflex is inhibited. The result is a significant retardation of oral to anal movement of gut contents, prolonging the time in transit and increasing the reabsorption of water, particularly in the colon.

Gut contents become dry and compacted leading to constipation that can sometimes be severe. These effects appear to be predominantly caused by μ -receptor activation, but other opioid receptor types are present although their functions in GIT are not fully understood. Interestingly, there is some evidence suggesting that the μ -receptor mechanisms involved in regulation of GIT function may be slightly different from those associated with pain control [143, 144].

The delayed transit of gut contents induced by opiate drugs results in significant dehydration of the stool (facilitated by an antisecretory effect of opiates) and constipation that can be severe and distressing to patients. Along with respiratory depression, nausea, and emesis, this is a significant factor limiting the use of opiate drugs for patients in severe pain. For the most part, clinicians have recommended to their patients that they always take a mild GIT stimulant such as senna, bisacodyl, and/or a stool softener to antagonize the constipating actions of morphine. This is inconvenient and not always effective.

The feasibility of utilizing μ -receptor antagonists that cannot enter the CNS to block the effects of systemically administered morphine on the GIT, while leaving intact the centrally mediated analgesic actions of morphine, has long been a goal of therapy. Dragonetti et al. [145] tested the use of several quaternary analogs of opiate drugs for this purpose; the charged quaternary analogs cannot enter the CNS because of their low lipid solubility. Novel peripherally restricted opiate antagonists have also been developed although their place in therapy remains uncertain [146].

More recently, a controlled clinical trial has shown that quaternary naltrexone promotes laxation within 4 h in about 50% of patients with advanced illness receiving moderate to high doses of morphine [147]. The lack of effect in roughly 50% of patients is interesting; it could be that an inadequate dose of quaternary naltrexone was used but it seems more likely that the limited efficacy is related to the fact that part of the reduction of propulsive activity in GIT by opiates is mediated by a central action that cannot be blocked by an antagonist that is excluded from the CNS because of its very low lipid solubility.

3.3.6.2 Genital Tract

Endogenous opiates are expressed in organs of the genital tract [148], and opiate drugs and opioid peptides inhibit contractions of the vasa deferentia in some species. This action was exploited in the use of mouse vasa deferentia bioassays in the original discovery of the enkephalins [41]. There is great species variability in

the level of expression and the types of OP receptor expressed in vasa deferentia. Thus, mouse expresses μ , δ , and κ receptors, hamster expresses exclusively δ , while rat vasa seem to express only μ (although an early study had suggested that a postulated novel type of receptor, named ε , with proposed high and specific sensitivity for β -endorphin was expressed in this tissue, it appears that in fact the receptor is a μ receptor expressed at such low levels that partial agonists such as morphine are essentially without effect) [149]. The physiological significance of the expression of opioid receptors in the vasa deferentia is unknown.

3.3.6.3 Heart and Cardiovascular System

Fentanyl and related opiate drugs are used clinically as anesthetics in part because they can give complete pain relief without inducing cardiac depression (unlike most gaseous and volatile anesthetic agents). Endogenous opioids are expressed and stored in cells in the atria, along with many other bioactive peptides [150]. Opioid receptors are also expressed in the atrium and in conducting tissue in the heart, but their functions are not well understood. In recent years careful evaluation of the effects of morphine and related agents after hypoxic damage to the heart suggests that opiate drugs are cardioprotective in the face of ischemic insult, even when administered during reperfusion after the ischemic insult [151, 152].

Morphine is commonly used to relieve the pain associated with myocardial infarct; the work of Gross and colleagues [151] suggests that in addition to pain relief, there may also be a cardioprotective effect conveyed by the opiate drug. The mechanisms underlying opioid cardioprotective actions are complex, multifaceted, and may vary for opioids with selective preference for different opioid receptor types.

In addition to effects on cardiac function, there has long been evidence that morphine and related drugs have selective inhibitory effects on transmitter release from sympathetic nerve endings [26], including the sympathetic innervation of the peripheral circulation. These actions appear to be species-specific; generally opiates do not significantly depress vascular tone in man.

3.4 Opiate Drug Absorption, Metabolism, and Distribution

3.4.1 *Absorption*

Morphine is commonly administered orally, sublingually or by intramuscular or subcutaneous injection. Oral administration is convenient but variable because of extensive metabolism in the liver; much of the drug is metabolized at first pass through the liver and bioavailability is estimated at less than 40%. However, despite this morphine and other opiates (some of which may be less extensively metabolized in the liver) are often given by the oral route (sometimes in a sustained-release formulation) because of the convenience and avoidance of the need for injection. In severe pain cases, morphine may be given sublingually, with the advantage that

the patient may be able to titrate the amount of drug taken to a dose that is adequate for pain relief while reducing troublesome side effects to a minimum. Opiates may also be given rectally in patients unable to take drugs by mouth. For other patients, parenteral injection (subcutaneous, intramuscular or even intravenous) may be necessary to obtain adequate blood levels to control severe pain.

Absorption from the gastrointestinal tract is dependent on environmental pH since morphine is a weak base. At the acid pH of the stomach most of the drug is ionized, with limited ability to cross the plasma membrane of mucosal cells and enter the circulation. In the less acid environment of the small intestine a larger fraction of the drug is in the nonionized base form with a higher lipid solubility, permitting it to cross into the blood stream more readily. Absorption across the intestinal wall may also be influenced by the expression of transporter proteins such as MDR-1 (P glycoprotein) and related transporters that use ATP to provide energy to pump the drug from the cytoplasm of the mucosal cells back into the intestinal lumen.

The variability between individuals with respect to the extent of oral absorption of morphine may be partly related to genetic or environmentally determined differences in the level of expression of drug transporters in intestine. However, MDR-1 and other drug transporter are probably more important in controlling the amount of morphine crossing the blood–brain barrier into the CNS (see below) than in their effect on absorption from the gastrointestinal tract.

Synthetic opiate drugs such as fentanyl may also be administered as a skin patch for absorption via the transdermal route. The drug is highly lipid soluble; it diffuses across the skin layers into the blood stream to yield therapeutic blood concentrations. The estimated elimination rate for fentanyl delivered by the transdermal route is about 17 h [153] – long enough to ensure that blood concentrations of the drug achieved by this route of administration are relatively stable over periods of hours to days – contrasts sharply with the very short duration of fentanyl action when given intravenously.

The route is useful in the treatment of long-term moderate-to-severe pain as seen often in cancer patients. The advantages are that stable blood levels are obtained in most patients. The transdermal route also avoids the need for daily or more frequent injections or oral administration (which some patients find difficult to tolerate). However, it may be necessary to use supplementary dosing with an opiate by other routes (e.g., sub-lingual) to treat “break-through” pain.

3.4.2 Metabolism

Once absorbed from the small intestine, morphine passes by the hepatic portal system to the liver where it is subject to extensive metabolism by both oxidative and conjugating drug metabolizing enzymes. Cytochrome P450 enzymes demethylate morphine to yield normorphine [154], a product retaining ability to activate opiate receptors but with reduced ability to enter the CNS.

In rodents, demethylation by cytochrome P450 enzymes is a major metabolic pathway for morphine; a substantially smaller fraction of administered morphine is subject to *N*-demethylation in humans [155]. Several P450 isoforms can demethylate morphine, but CYP3A4 may be the major isoform involved. There are no important polymorphisms affecting morphine metabolism by CYP3A4, but the presence of other CYP3A4 substrates may slow its metabolism by competition. The P450 enzymes are also inducible by chronic exposure to hepatic enzyme inducers, with the result that the extent of metabolism to normorphine may be increased after exposure to hepatic enzyme inducers.

Other opiate drugs are also subject to metabolism by cytochrome P450 enzymes. *N*-dealkylation is a significant metabolic route for meperidine and methadone [156, 157]. Codeine is a pro-drug, requiring O-demethylation (in the three-position) to generate morphine, which is responsible for its analgesic activity. CYP2D6 is the primary P450 isoform involved in the O-demethylation of codeine. Clinically significant polymorphisms of CYP2D6 are known; poor 2D6 metabolizers are insensitive to the analgesic actions of codeine [158].

The major metabolic conversion of morphine in man is glucuronidation by UDP-glucuronyl transferase [155]. This enzyme attaches O-linked glucuronide groups to replace hydroxyl groups at the three- and six-positions of morphine (Fig. 3.2). Glucuronidation in the three-position abolishes the ability to activate opioid receptors, while glucuronidation at the six-position creates a product (morphine 6-glucuronide) that is reported to have high activity at opioid receptors; [159] the di-glucuronide is without receptor activity.

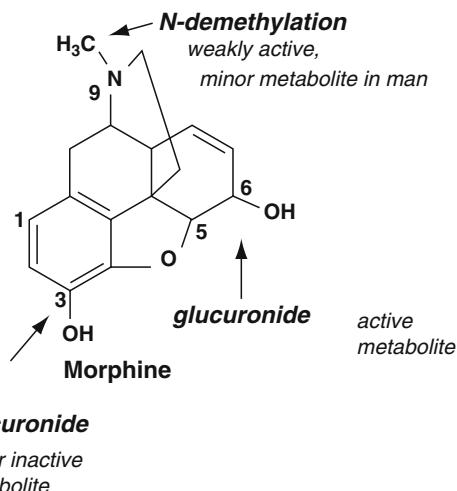


Fig. 3.2 Structure of morphine, showing regions of the morphine molecule where the major metabolic transformations occur (see text for details)

There are polymorphisms in UDP-glucuronyl transferases that can affect their ability to metabolize morphine. These enzymes can also be induced by some hepatic enzyme inducers; both factors are additional sources of variability between individuals in their susceptibility to morphine and related drugs.

Almost all significant metabolism of morphine and related drugs occurs in the liver. However, cytochrome P450 and glucuronidating enzymes are also present in the endoplasmic reticulum of other cells and some local metabolism may occur. In the wall of the gastrointestinal tract this metabolism may have some effect on the extent of absorption of some opiates. Other tissues with measurable ability to metabolize opiates include the lung and skin. Morphine and its metabolites (along with many other related drugs and their metabolites) are excreted in the urine, and also via the bile into the feces.

3.4.3 Distribution

At blood pH, unmetabolized morphine is partially ionized; the nonionized form can enter cells and cross the blood–brain barrier into the CNS, the primary target for its therapeutic actions. However, opiate drug penetration into brain is significantly attenuated by the expression of MDR-1 and other drug transporters in the choroid plexus and the cells surrounding blood vessels in the CNS. Morphine is a good substrate for MDR-1, although measurable brain uptake can still occur with normal MDR-1 expression.

Morphine entering the cytoplasm of cells in the cerebral blood vessel wall and astrocytes surrounding cerebral blood vessels is returned to the vessel lumen by MDR-1. As a result, morphine concentrations in the CNS are usually several-fold lower than in serum. Genetic deletion of the MDR-1 in mice allows more of the morphine to enter the CNS; significantly lower doses of morphine are needed to induce comparable analgesic effects [160]. There is a negative relationship between morphine analgesic response and the level of expression of MDR-1 in individual mice [161]. Other therapeutically useful opiates drugs are also MDR-1 substrates, making the level of expression of MDR-1 and related transporters a critical determinant of the central activity of most opiate drugs.

For example, a recent study has shown that a threefold lower concentration of the synthetic opiate alfentanil is required for comparable levels of analgesia in *mdr1a* $-/-$ mice relative to *mdr1a* $+/+$ animals, although brain concentrations of the drug are not significantly different at equi-analgesic dose levels [162]. Since the levels of MDR-1 influence the analgesic potency of morphine, it was particularly interesting to note that chronic administration of morphine increases MDR-1 levels, perhaps contributing to the development of tolerance [163, 164].

Loperamide, a synthetic opiate, is an even better drug transporter substrate than morphine. It is completely excluded from the CNS by the action of MDR-1 [165]. This makes it a clinically useful as a peripherally restricted opiate drug for the control of gastrointestinal hypermotility (see Sect. 3.3), which does not exhibit the centrally mediated actions of opiate drugs. The lack of any abuse liability for

loperamide demonstrates clearly the significance of drug transporters in controlling access of this class of drugs to the CNS.

The expression and/or function of drug transporters vary by genotype. Several common polymorphisms in MDR-1 and other transporters have been identified in human subjects, some of which appear to reduce the transporter efficiency and others which appear to affect the level of transporter expression. Thus some of the variability in sensitivity to opiates may be related to polymorphisms in drug transporter expression, together with other genetic factors influencing morphine analgesic response [166]. However, the functional relevance of many transporter polymorphisms are not yet fully understood and at this time it is not possible to correlate opiate hyper or hyposensitivity with commonly expressed specific MDR-1 polymorphisms in man.

3.5 Epilogue

Morphine is unique among currently used therapeutic agents in the longevity of its use to alleviate pain and suffering in humans. Its known use as a therapy for pain dates from the dawn of recorded history, yet it remains today a drug-of-choice for the treatment of moderate to severe pain of various causes in a large number of patients. Studies on the pharmacology of opiate drugs have had a major impact on the development of novel experimental approaches in the fields of neuroscience and pharmacology.

The early observation that opiate drugs induce physical and psychological dependence led to a very extensive study in animals and humans of the addictive properties of opiate drugs. These studies in human subjects at the Lexington Narcotics Farm were among the first uses of quantitative psycho-pharmacological outcome measures to assess the effects of a drug on mental and psychiatric functions. The chemical structure of morphine was determined relatively early in the history of pharmaceutical chemistry, and since that time an enormous number of chemical analogs have been synthesized. The goal of identifying very potent analgesic agents without any abuse liability may not have been realized, but the attempts to achieve that goal have yielded one of the richest areas of structure-activity studies in pharmacology.

The sharply defined structural requirements for analgesic activity in turn resulted in understanding that normal brain must express a receptor target through which morphine and related agents act, and an early use of radiolabeled drugs to identify binding to receptors targets in brain and other tissues. Although binding to opioid receptors was demonstrated relatively early in the era of receptor pharmacology, it took a surprisingly long time for opioid receptors to be cloned. Studies of opiate drug effect on isolated peripheral tissues in the mid-years of the last century led to the use of such “simple” models of neural functions as a way of gaining insight into the actions of drugs, and also provided bioassay preparations that were of great importance in the discovery of endogenous opioid peptides.

Many of the techniques now used to study the biosynthesis, processing, storage, release, function, and degradation of bioactive peptides were first developed in studies seeking to understand the role and functions of endogenous opioids. Once opioid receptors were cloned, a search for homologous receptors revealed the existence of a hitherto unknown opioid-like receptor type, and the discovery of its endogenous ligand. Studies of the properties of opioid receptors have played an important role in the recognition that many GPCR may function as homo- or heterodimers (or higher trimers) with properties subtly modified by the polymerization. We can confidently expect future studies of the actions of opioids to yield yet more surprises and yet more complexity in the receptor-mediated regulation of the functions of cells and the behavior of animals and man.

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Chapter 4

Opioid Receptors: The Early Years

Gavril W. Pasternak

Abstract Opioid receptors were first demonstrated long after they were proposed. Pharmacological studies of opiates in patients and animal models have typically predicted concepts subsequently confirmed at the molecular level. The initial binding of opiates, the identification of the opioid peptides and the concept of opioid receptor multiplicity are excellent examples. This review will discuss many of these early studies in the context of our current understanding of opioid action.

Keywords Opiate receptor • Receptor dualism • Sodium effect • Opioid binding sites • Receptor binding

4.1 Introduction

The opioid receptors were first demonstrated in 1973 [1–3], long before the cloning of any of the G-protein coupled receptors and the elucidation of their general structure. Indeed, it was nearly 20 years before the first member of the family was cloned [4, 5]. Despite the lack of many of the biological tools available today, many of the concepts of opioid pharmacology were determined during this early period. This includes the general classification of mu, delta and kappa receptors [6, 7], the proposal of kappa receptor subdivisions [8–10] and even multiple classes of mu receptors [11]. Although some of the interpretations of this early work need to be reexamined, much of it is still relevant. This chapter will review some of the early studies in light of our current knowledge. With thousands of articles, a comprehensive overview is not possible. Some of the more prominent areas of study are presented below.

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4.2 Early Attempts to Demonstrate Opiate Binding Sites

The pharmacological concept of receptors, based upon the observation of rigid structure–activity relationships, stereospecificity, and the observation of maximal pharmacological responses, goes back to the turn of the century [12–15]. Opioid structure–activity relationships established for literally thousands of semisynthetic and synthetic compounds [16–19] revealed very rigid requirements for activity including strict specificity [20, 21]. Furthermore, both *in vivo* testing and bioassays clearly fulfilled the other criteria expected of a receptor-mediated action, including cross tolerance and dependence.

Based upon these observations, Beckett and Casey attempted to map an opioid receptor binding site [22–26]. With such strong pharmacological evidence in favor of a receptor mechanism of action, it is not surprising that many groups attempted, unsuccessfully, to label it biochemically prior to 1973 [27–35]. Most groups used the concept of stereospecificity; i.e., looking at differences in the ability of the active and inactive stereoisomers to compete binding [29]. However, the low specific activity of the radioligands available at that time made it very difficult to detect the low levels of binding sites. The specific activity of the radioligands required that studies be performed using radioligands concentrations orders of magnitude higher than their K_D values, leading to high levels of nonspecific binding that impeded the ability to detect the low levels of specific binding. These problems were not appreciated. Most biochemists at the time considered micromolar affinities to be high affinity and did not anticipate that neurotransmitters could bind drugs with low nanomolar affinities. Thus, investigators anticipated opioid affinity for the receptors in the micromolar, a belief that was further supported by estimates that morphine concentrations within the brain sufficient to produce analgesia were approximately 0.1 μM . With the benefit of hindsight, it is now clear that these technical issues led to the failure of these early attempts.

4.3 Identification and Characterization of Opiate Binding Sites

4.3.1 *Identification of Opioid Binding Sites in Nervous Tissue*

In 1973 three laboratories reported the biochemical demonstration of opioid binding sites using ^3H -labeled naloxone [36], ^3H -dihydromorphine [2] or ^3H -etorphine [3]. The success of these three groups rested primarily upon their use of high specific activity radioligands (>1 Ci/mmol), enabling them to examine low ligand concentrations and have sufficient specific binding to see the signal. These reports fulfilled the major criteria required to document a relevant binding site. These binding sites demonstrated the same stereospecificity observed pharmacologically, and unlabeled compounds inhibited binding with the same relative potency observed in bioassays and *in vivo* (Table 4.1). Furthermore, compounds without opioid-like activity in standard pharmacological assays did not inhibit binding at concentrations under

Table 4.1 Inhibition of [³H]naloxone binding by a series of opioids

Compound	IC ₅₀ value (nM)
(-)Etorphine	0.3
(-)Levallorphan	1
(-)Levorphanol	2
(-)Nalorphine	3
(-)Morphine	7
(-)Cyclazocine	10
(-)Naloxone	10
(-)Hydromorphone	20
(-)Methadone	30
(±)Pentazocine	50
(±)Methadone	300
(±)Meperidine	1,000
(±)Propoxyphene	1,000
(+)3-Hydroxy- <i>N</i> -allylmorphinan	7,000
(+)Dextrorphan	8,000
(-)Codeine	20,000
(-)Oxycodone	30,000

IC₅₀ values were determined by log probit analysis and are taken from the literature [265]. Note that dextrorphan is the (+) isomer of levorphanol and (+)-3-hydroxy-*N*-allylmorphinan is the (+) isomer of levallorphan. Values are IC₅₀, which are higher than calculated K_D values

10 μM (Table 4.2). Codeine and oxycodone initially seemed out of place, but many now believe that these agents are not active and must be metabolized to morphine and oxymorphone, respectively, to be active. All the ligands displayed very high affinities, with K_D values well below 10 nM.

Additional studies characterizing these sites soon followed. However, it is important to remember that the concept of distinct classes of opioid receptor had not yet been fully developed at this time. For the most part, these initial reports examined mu binding sites, particularly the studies employing the antagonist ³H-naloxone and the agonist ³H-dihydromorphine. However, the agonist ³H-endorphine is less selective and labels a variety of classes of opioid receptor. The lack of any structural information on the receptor led to a series of studies establishing its composition, starting with its protein structure based upon its sensitivity to enzymatic treatments [3, 37, 38]. The proteases trypsin and chymotrypsin both effectively lowered binding, whereas DNAase, RNAase, neuraminidase, and phospholipase C were all without effect [37, 38]. Furthermore, reagents known to react with proteins, particularly those targeting sulphydryl groups, also impacted binding [39, 40]. One interesting difference among laboratories arose regarding the importance of phospholipids.

Several groups reported that opioid binding was quite sensitive to phospholipase A [41, 42], but one did not [3]. This probably reflected technical differences in the enzyme treatments, but subsequent studies by this same group demonstrated that the fatty acids generated by phospholipase A inhibited binding [43]. These effects of phospholipase A appeared to be related to its actions on phosphatidylserine.

Table 4.2 Drugs ineffective in altering [³H] dihydromorphine binding

Acetyl-β-methycholine	Epinephrine	Ornithine
Acetylsalicylic	Ethosoximide	Orphenadrine
γ-Aminobutyric acid	Fenfluramine	Oxytropane
Arecoline	Glutamic acid	Pargyline
Atropine	Glycine	Peganone
Bicuculline	Hemicholinium-3	Pentylenetetrazole
Bretylium	Histamine	Phenelzine
Brocresine	Hydroxyamphetamine	Phenobarbital
Bromodiphenhydramine	Hydroxychloroquine	Phenylpropanolamine
3α-(5H-Dibenzo[a,d]-cyclohepten-S-yl)	Hydroxyzine	Pilocarpine
Bulbocapnine	Hyoscine	Promethazine
Butylcholine	Imipramine	Serotonin
Caffeine	3-Iodo-L-tyrosine	Taurine
Carbamylcholine	Iprindole	Tetrabenazine
Carboxinamine	Isoproteronol	Δ-Tetrahydrocannabinol
Chlordiazepoxide	Leucine	
Chlorpheniramine	Mecamylamine	B-(2-Thienyl)-isopropylamine
Cobefrin	Methergine	
Colchicine	Methdilazine	Theronine
Cycramine	3-Methoxytyramine	Tranylcypromine
	3-Methoxy-4-hydroxy-mandelic acid	Trifluoperazine
Decamethonium		Tripolidine
Desipramine	A-Methyldopa	Tryptamine
Diethylpropion	Neostigmine	Tryptophan
Doxepin	Nialamide	Urecholamine
Dopamine	Nicotine	Valine
Ecolid	Nortriptyline	
Ephedrine	Octopamine	

Drugs at a final concentration of 3 μM were preincubated with rat brain homogenate in a standard binding assay using ³H-dihydromorphine. None of the listed compounds produces significant inhibition of stereospecific binding [265]

Whereas phospholipase A lowered binding, exposing brain membranes to phosphatidylserine alone enhanced opioid binding and reversed the effects of phospholipase A [42]. Furthermore, phosphatidylserine decarboxylase, which converts phosphatidylserine to phosphatidylethanolamine, also inhibited binding. Another lipid implicated in these early studies of opioid binding was cerebroside sulfate [44, 45]. There has been little work on the role of lipids in recent years.

4.3.2 Localization of Binding Sites

A number of groups looked at the localization of the binding sites in an effort to understand the circuitry and mechanisms of receptor action. These included both regional studies within the nervous system as well subcellular approaches.

It is interesting to note that the opioid receptors were among the very first to be examined autoradiographically in brain tissue, a general approach that was then extended to a wide range of receptors.

4.3.2.1 Cellular Localization of Opioid Receptors

Subcellular studies revealed an association of the binding sites with synaptosomes, which are “pinched off nerve endings” generated by gentle glass/Teflon homogenization [1, 46], results consistent with its proposed function as a transmitter receptor. Following lysis of the synaptosomes, binding was associated with the synaptic membranes. Injection ^3H -etorphine *in vivo* revealed an association of radiolabel with synaptic membrane fractions [47]. Early studies identified binding sites both pre-and postsynaptically. Lamotte and co-workers [48] addressed the issue by lesioning dorsal roots and then measuring binding within the cord. In brief, they found a decrease of approximately 50%, implying the presence of sites both pre- and postsynaptically. However, this did not take into consideration the possibility of presynaptic receptors within the neurons intrinsic to the spinal cord. Autoradiographic approaches also documented the peripheral localization of opioid binding sites on the vagus nerve, as well as documenting the axoplasmic flow of the binding sites along the nerve [49]. Other investigators using electrophysiological approaches, also observed actions consistent with postsynaptic localizations [50].

4.3.2.2 Regional Localization of Opioid Receptors

Regional studies using homogenate binding documented dramatic differences in the levels of binding among brain structures (Table 4.3) [51–54]. In these homogenate studies, high levels of binding were noted in areas associated with the limbic system, brain regions known to be important in opioid action [55, 56]. No significant specific opioid binding was detected in the white matter, again implying an association with neuronal elements.

Although a valuable approach, regional homogenate binding studies suffer from the limited detail possible due to the need for sufficient tissue to do the binding. On the other hand, autoradiographic studies labeling sites *in vivo* or *in vitro* provided a detailed distribution of the sites within the brain [57–63]. Opioid receptor autoradiographic approaches were among the first developed for neurotransmitters. Again, the issue of the selectivity of the early drugs was not appreciated. The most detailed early approach was that of Atweh and Kuhar. Although their distribution seems to correspond most closely to mu receptors, their radioligand, ^3H -diprenorphine, is not selective and will label a range of opioid receptor subtypes. It did not take long, however, to utilize selective agents to document differences in the regional distributions of mu, kappa and delta sites [64–71]. Many of these studies were facilitated by the availability of tritium-sensitive film, obviating the need for emulsion approaches [72], and enabling the development of a digital subtraction approach to map binding lost with a competitor [73]. Clearly, more recent studies have mapped the subtypes

Table 4.3 Opioid-receptor binding in regions of monkey brain

³ H-Dihydromorphine binding (fmol/mg protein)			
<i>Cerebral hemispheres</i>		<i>Extrapyramidal areas</i>	
Frontal pole	11.9	Caudate nucleus (head)	19.4
Superior temporal gyrus	10.1	Caudate nucleus (body)	9.0
Middle temporal gyrus	7.1	Caudate nucleus (tail)	8.9
Inferior temporal gyrus	6.0	Putamen	11.7
Precentral gyrus	3.4	Globus	7.7
Postcentral gyrus	2.8	Internal capsule	5.4
Occipital pole	2.3	<i>Midbrain</i>	
<i>White matter areas</i>		Superior colliculi	10.6
Corpus callosum	<2	Inferior colliculi	6.7
Corona radiata	<2	Interpeduncular nucleus	13.7
Anterior commissure	5.4	Raphe area	8.2
Fornix	<2	Periaqueductal gray	31.1
Optic chiasm	<2	<i>Cerebellum-lower brain stem</i>	
<i>Limbic cortex</i>		Pons (ventral)	1.4
Anterior amygdala	65.1	Cerebellar cortex	<2
Posterior amygdala	34.1	Dentate nucleus	1.9
Hippocampus	12.5	Floor of fourth ventricle	6.3
<i>Hypothalamus</i>		Pyramidal tract	3.0
Medial hypothalamus	24.2	Lower medulla	5.8
Anterior hypothalamus	24.3	<i>Spinal cord (thoracic)</i>	
Posterior hypothalamus	24.7	Dorsal column (white)	3.1
Hypothalamus	32.2	Lateral cord (white)	3.3
Mammillary bodies	5.0	Gray matter	8.8
<i>Thalamus</i>			
Medial thalamus	24.6		
Lateral thalamus	7.8		

Adapted from Kuhar et al. [51]

far more comprehensively using *in situ* hybridization to map mRNA and immunohistochemical approaches [74–78]. However, each has its own limitations. mRNA suffers from the potential mismatch between the cell bodies and the receptors, which may be on axons far removed. Immunohistochemical approaches offer an opportunity to directly visualize the receptor proteins, but recent studies have questioned the issues of selectivity and cross labeling other, unrelated, proteins. Thus, the results of autoradiographic approaches which assess “functional” (i.e., based upon ligand binding) sites still provide a valuable map of the receptors.

4.3.2.3 Development

Developmentally, these sites show their greatest increase during the first 3 weeks after birth in the rat with a caudal to rostral development of regions [79–81]. Zhang and Pasternak [82] found similar results for the traditional mu-selective sites, with

levels at birth approximately 40% of those in adults which slowly increased over time. However, the appearance of a very high affinity site, the μ_1 site, had extremely low levels at birth and increased dramatically over the next 2 weeks. This increase in density of μ_1 sites corresponded with the increased analgesic sensitivity of the animals to morphine, with the analgesic ED_{50} dropping 40-fold, from 56 mg/kg, s.c. in 2-day-old rats to 1.4 mg/kg, s.c. in 14-day-old rats. In contrast, the ED_{50} for morphine-induced respiratory depression actually increased, from 2.5 mg/kg, s.c. in the 2-day-old rats to 9.3 mg/kg, s.c. in the 14-day-old rats. Thus, the sensitivity of the rats to morphine correlated well to the high affinity μ_1 site and as the rats became more sensitive to morphine analgesia, they became less sensitive to its respiratory depressant effects. These respiratory results were similar to those seen earlier showing that neonatal rats were highly sensitive to morphine, as assessed by LD_{50} [83]. These developmental studies supported the concept that morphine analgesia and respiratory depression are mediated through distinct mechanisms and correlated quite well with the naloxazone/naloxonazine studies implicating the μ_1 binding sites with morphine analgesia and not respiratory depression [11, 84–87].

4.3.3 Phylogeny

Phylogenetically, opioid binding sites have been reported in a wide range of species. In general, ontogeny recapitulates phylogeny. Initial studies by Snyder's group suggested very low levels in invertebrates [88]. Among the vertebrates, binding generally increased progressively in higher animals. Other studies utilizing radiolabeled enkephalin analogs have reported opioid binding sites in insects and in mollusks [89, 90]. However, it is important to keep in mind that these studies typically did not attempt to address the concept of receptor classes. Finally, binding levels can also vary significantly among different strains of single species, and these levels correlate quite well with the sensitivity of the strains of opioids [91–93].

4.3.4 Correlating Opiate Binding Sites with Opiate Actions

Establishing the relevancy of these binding sites was a major objective in the early years. The demonstration of stereospecificity and the correlation of binding and pharmacological potency provided the first evidence. This was then supported by the localization of the binding sites to brain regions known to be important for opioid action and the association of binding sites with neural tissue, more specifically synaptic membranes.

Perhaps the strongest initial evidence correlating binding activity with pharmacological actions comes from studies by Creese and Snyder [1, 94] in which they examined binding sites on the nerve plexus of the guinea pig ileum. They demonstrated

a strong relationship between binding in the brain and in the guinea pig ileum binding assay. More important, they correlated the binding with the activity of the opioids on the ileum contraction assay, the classical approach for studying opiates for many years [95–97].

4.4 Discrimination of Agonist and Antagonist Binding

The opioid receptors were among the first of the neurotransmitters receptors to be identified through binding approaches. Thus, it is not surprising that it provided many other “firsts.” It was the first to be used to identify an unknown endogenous ligand, the opioid peptides, as described elsewhere in this volume. It also was the first to document the conformational changes between agonist and antagonist binding.

One of the advantages of the opioid system in those early studies was the availability of a number of useful radiolabeled agonists and antagonists, along with the very small structural differences between agonists and antagonists that enabled comparisons between their binding. Using binding approaches, a number of treatments were uncovered that discriminated the binding of agonists compared to antagonists, including monovalent divalent cations, enzymes, reagents and even assay incubation temperatures [36, 38–40, 98–103]. These effects appear to be important in stabilization of different, interconverting states of the receptor, with most stabilizing the antagonist conformation.

4.4.1 Sodium Effect

The ability of sodium ions to enhance antagonist binding and lower agonist binding was first reported in 1973 [36], the same year of the original description of the binding. It had not been anticipated and was the result an interesting series of events. After noting in December of 1992 that the sodium salt of ethylenediaminetetraacetic acid (Na_2EDTA) markedly enhanced ^3H -naloxone binding, I suggested to Adele Snowman that she try it. However, when she examined ^3H -dihydromorphine binding she saw a profound decrease the binding, the opposite effect. Initial skepticism of the findings of the other quickly gave way to amazement when the agonist and antagonist were tested in the same assay with the same results, with the chelater increasing the binding of the antagonist ^3H -naloxone and decreasing the binding of the agonist ^3H -dihydromorphine. We then determined that the major cause of the binding changes was the sodium ions and extended the study on the effects of sodium ions to a series of paired ^3H -labeled agonists and antagonists (Table 4.4).

The discovery of the sodium shift also proved valuable in evaluating unlabeled compounds, enabling an initial assessment of the agonist/antagonist character of the compound from simple binding studies. The IC_{50} values of the compound were determined against ^3H -naloxone binding in the presence and absence of sodium chloride (100 mM) and their ratio determined ($\text{IC}_{50+\text{NaCl}}/\text{IC}_{50-\text{NaCl}}$). Since sodium ions decrease agonist affinity, its inclusion in the assay shifted the IC_{50} values, yielding a

Table 4.4 Effect of sodium ions on radiolabeled opioid agonists and antagonists

Drug	Change in binding by NaCl (100 mM)
<i>Agonist</i>	
³ H-Dihydromorphine	-70%
³ H-Oxymorphone	-44%
³ H-Levorphanol	-28%
<i>Antagonists</i>	
³ H-Nalorphine	+45%
³ H-Naloxone	+141%
³ H-Levallorphan	+29%

Binding each of the radioligand was assessed in the presence and the absence of sodium chloride (100 mM) [36]

Table 4.5 Sodium shifts of the opioids on ³H-naloxone binding

	IC ₅₀ values (nM)		
	Control	NaCl (100 mM)	Sodium shift
Naloxone	1.5	1.5	1.0
Natrexone	0.5	0.5	1.0
Diprenorphine	0.5	0.5	1.0
Cyclazocine	0.9	1.5	1.7
Levallorphan	1.0	2.0	2.0
Nalorphine	1.5	4.0	2.7
Pentazocine	15.0	50.0	3.3
Etorphine	0.5	6.0	12
Meperidine	3,000	50,000	17
Levorphanol	1.0	15	15
Oxymorphone	1.0	30	30
Dihydromorphine	3.0	140	47
Propoxyphene	200	12,000	60
Phenazocine	0.6	80	133

IC₅₀ values for each compound were determined against ³H-naloxone in the presence and the absence of sodium chloride (100 mM) and the ratio determined [36]

ratio greater than 1 (Table 4.5). Antagonists retain their potency in the presence of sodium and have ratios of approximately one, with intermediate values for partial agonists. This technique has proven extremely valuable in the classification of new opioids and has been employed with a wide range of G-protein coupled receptors.

When radiolabeled opioids of even higher specificity activity became available, our laboratory identified a novel binding site with a tenfold higher affinity than the sites originally observed for both the agonist ³H-dihydromorphine and the antagonist ³H-naloxone [104]. This high affinity site was markedly influenced by the inclusion of sodium ions. Sodium ions appeared to stabilize the antagonist conformation, since inclusion enhanced the number of high affinity binding sites seen for ³H-naloxone while virtually eliminating the high affinity ³H-dihydromorphine site.

4.4.2 Divalent Cations

Not all ions preferentially lower agonist binding. Certain divalent cations, for example, selectively enhance agonist binding (Table 4.6) [98]. Mn⁺² seemed to be the most effective, although Mg⁺² also was effective while calcium had little effect. It is interesting to note that the divalent cations appear to counteract the effects of sodium ions. Their ability to enhance agonist binding was far more prominent in assays containing sodium ions while treatment with divalent cation chelators, such as EDTA, enhanced the effects of sodium ions. Much like sodium shifts, manganese shifts can also be employed to define the agonist/antagonist character of a ligand, although they are not frequently used.

These ionic effects are selective and not merely the result of increasing ionic strength. Whereas sodium ions have dramatic effects, potassium ions and ammonium ions have little. A variety of anions, including chloride, sulfate, fluoride, bromide, iodide, thiocyanate, perchlorate and formate also were inactive.

4.4.3 GTP

In the early days of opioid receptor binding studies, the concept of G-protein coupled receptors was still evolving. The role of G-proteins (termed N proteins at the time, for nucleotide) in receptor binding was first appreciated with β-adrenergic receptors in classic studies by Lefkowitz [105–107]. Soon afterwards, the role of nucleotides, particularly GTP and its stabilized analogs GppNHP and GTPγS, was established in opioid binding [103]. Again, the inclusion of stabilized GTP analogs markedly impaired the binding of agonists and the effect was potentiated by the inclusion of sodium ions.

4.4.4 Protein Modifying Reagents, Enzymes and Temperature

A number of other treatments targeting proteins also discriminated between agonist and antagonist binding (Table 4.6) [38–40, 98, 99]. Treating tissue with a wide variety of reagents, particularly those targeting sulphydryl (SH) groups lowered the binding of agonists far more effectively than antagonists. Equally interesting was the relationship between the actions of these reagents and sodium, with the reagent treatment making the agonist binding even more sensitive to sodium ions.

Similarly, proteolytic enzymatic treatments lowered agonist binding far more than that of antagonists (Table 4.5). As with the reagents, sodium dramatically enhanced the differential effect. Even lowering the temperature of the binding assay to 0°C enhanced antagonist binding while lowering agonists binding, presumably by altering membrane fluidity [99].

Table 4.6 Effects of reagents, enzymes, and cations on the binding of opioid agonists and antagonists^a

Reagent	Change in binding (%)			
	Assayed with NaCl		Assayed without NaCl	
	³ H-Nal	³ H-DHM	³ H-Nal	³ H-DHM
<i>N</i> -ethylmaleimide	-15%	-91%	-25%	-47%
Iodoacetamide	-10%	-90%	-15%	-55%
Mersalyl acid	-24%	-77%	-41%	-65%
1-Ethyl-3-(3dimethylamino propyl) carbodiimide	-19%	-70%	-11%	-48%
5,5-Dithiobis-(2-nitrobenzoic acid)	0	-79%	0	-16%
p-Chloromercuribenzoate (10 μM)	-10%	-75%	-40%	-45%
<i>Enzyme</i>				
Trypsin	-21%	-95%	-35%	-63%
Chymotrypsin	-29%	-55%	-38%	-46%
Phospholipase A	-50%	-85%	-81%	-77%
<i>Divalent</i>				
MnCl ₂	+8%	+105%	-1%	+17%
MgCl ₂	+17%	+58%	-1%	+24%
CaCl ₂	-15%	+28%	-23%	-9%

Tissue was assayed with either ³H-dihydromorphine (³H-DHM) or ³H-naloxone (³H-Nal) either after treatments with the stated reagent or enzyme of in the presence and the absence of indicated divalent cation with or without sodium. All enzymes and reagents were washed from the tissue prior to the assay [38–40, 98]

4.5 Endogenous Ligands: Endogenous Morphine

The biochemical identification of opioid receptor binding sites within the brain quickly raised the question of endogenous ligands. Efforts were made by a number of groups. The first public disclosure of the endogenous opioids came at a meeting of the Neuroscience Research Program in Boston in 1974 (Fig. 4.1) [108]. A small conference of less than 50 scientists, it included most of the major investigators in the opioid field. It was a dramatic event when John Hughes, who was there with Hans Kosterlitz, announced that they had isolated, although not fully purified, an opioid material from the brain using their tissue contraction assays to follow the isolation. At the same session, Lars Terenius told the audience that he, too, had uncovered a morphine-like substance using receptor binding techniques. Our own efforts which also were reported came from an observation made in the course of the various treatments looking at agonist and antagonist binding [39]. When incubating the tissue for the treatments, we observed a 50% increase in control binding. Subsequent studies then established that this resulted from an increase in the number of sites and not affinity. We then demonstrated that the supernatant from the



Fig. 4.1

incubated membranes competed binding, confirming that the increased binding was due to the dissociation of an opioid-like material from the receptor. Indeed, many groups now routinely incubate their brain tissues as a part of their standard tissue preparation to facilitate this dissociation and “open” the receptors. It also is of note that following the incubations, sodium ions no longer significantly increased ^3H -antagonist binding, although they still lowered ^3H -agonist binding. Thus, the increased binding seen with the ^3H -antagonists likely represents the accelerated dissociation of the endogenous ligands, which are agonists, from the receptor.

4.5.1 Endogenous Opioid Peptides

The first description of the enkephalins and their structure was reported by Kosterlitz and Hughes [109], although a number of articles had been published using partially purified extracts [110–112] and discussed at Committee on Problems

of Drug Dependence and the International Narcotics Research Conference in the spring of 1975. The work of Kosterlitz and Hughes opened a major field of research both for the opioids as well as in the more general role of neuropeptides. Soon after the isolation and characterization of the enkephalins, other members of the opioid family were uncovered, including dynorphin and β -endorphin [113–117] as discussed elsewhere in this volume. The enkephalins were named by Kosterlitz, as “within the head,” while Goldstein coined the term dynorphin based upon its very high potency. Simon proposed calling the whole family of opioid peptides the endorphins, a contraction of “enodogenous morphine” – without the “e.” These discoveries were followed by the isolation of three distinct precursor peptides and genes for each family. The enkephalins have been associated with the delta receptors, dynorphin A with the kappa₁ receptors and β -endorphin with mu, although it also retains a similar high affinity for delta.

All these endogenous opioid peptides share the enkephalin sequence (Tyr-Gly-Gly-Phe-Leu or Tyr-Gly-Gly-Phe-Met) at the N-terminus, with differing extensions at the C-terminus. However, inspection of the precursor peptides revealed a number of additional potential ligands that also contained the enkephalin sequence with extended sequences at the C-terminus. After a single malt or two, it was not uncommon for Kosterlitz to refer to these compounds as extended enkephalins while it was said that Goldstein might counter by referring to the enkephalins as truncated dynorphins. The role of dynorphin A has been established. However, the full pharmacological significance of the additional opioid peptides are not clear. It seems likely that they each may have their own receptors and functions, but this has not been documented, with the exception of the recent identification of the sensory neuron specific receptor (SNSR) which binds BAM-22, an extended enkephalin [118–121].

Soon afterwards another set of endogenous peptides were reported, the endorphins [122]. These were quite distinct in that they were highly mu-selective and they did not contain the enkephalin sequence. Much work has been done on these agents, but many questions remain.

4.5.2 *Endogenous Morphine*

A major question has long been whether the brain makes its own morphine. Evidence now suggests that it does [123–128]. The first suggestion that morphine was present within the brain came from immunohistochemical studies by Gintzler and Spector using antibodies developed against morphine. Since then a number of groups have demonstrated the presence of morphine within the brain, as well as the enzymatic machinery needed to synthesize it. Many of these studies have been criticized, suggesting that the morphine within the CNS was from dietary sources. However, more recent work reports that morphine can be synthesized in neuroblastoma cell lines using defined media, which overcomes this concern [128].

4.6 Multiple Opioid Receptors

The concept of multiple opioid receptors came from clinical trials and classical pharmacological approaches. Martin first proposed the concept of opioid receptor multiplicity with his proposal of “Receptor Dualism” in 1967 [129]. This suggestion of morphine (M) and nalorphine (N) receptors was based upon clinical studies looking at the combination of these drugs in patients [130, 131], studies that were then replicated in animal models. In these studies, nalorphine, the N-allyl analog of morphine, at low doses reversed morphine analgesia. However, higher nalorphine doses elicited its own analgesia, implying that the drug was an antagonist at the morphine sites and an analgesic acting through a different mechanism, the N receptors. Martin’s classification was expanded a number of years later with the suggestion of mu, kappa and sigma receptors [6]. Although the sigma receptors initially proposed by Martin were opioid, the term sigma receptors now refers to a cloned protein with no homology to any of the G-protein coupled receptors and to which opioids do not bind, but which can influence opioid actions [132–136].

Clinicians also have observed responses in patients that implied multiple opioid receptors. The hallmark of the clinical management of pain is the need to individualize therapy. This comes from observations that patients can vary dramatically in their responses to a range of mu opioids. Some patients respond well to one opioid, while others do better on a different one. The doses of drug needed for a good response also can vary significantly. These observations have been recapitulated in animal models. Different mouse strains, for example, display differing sensitivities to opioid analgesia [137]. However, studies with the CXBK mouse have provided the most dramatic insights into the diversity of mu opioid responses. The CXBK mouse is insensitive to morphine [91, 138]. Yet, these same mice respond normally to other opioids, including a number of mu drugs, such as heroin, methadone, fentanyl and morphine-6 β -glucuronide (M6G) [139, 140]. Together, these studies illustrate the importance of the genetic backgrounds in opioid responses and support the proposal of mu opioid receptor multiplicity [11].

Martin’s proposal of mu and kappa classes of opioid receptors, along with the discovery of the enkephalins and their proposed receptor, termed delta [7], led many groups to try to identify them biochemically. Delta sites were easily detected using stabilized enkephalin derivatives [141–145]. However, demonstrating kappa sites proved difficult, primarily due to the lack of selective ligands and their relatively low levels in rat and mouse brain. The drugs used to define kappa sites were typically benzomorphans, which labeled both mu and kappa sites. Many of these drugs were mu antagonist/kappa agonists and required complex blocking approaches in the binding studies to selectively compete away the mu and/or delta sites, leaving the kappa sites [146–150]. These were not simple assays and questions remained regarding the identity of the kappa receptor. This changed with the synthesis of U50,488H [151], a very potent and highly selective kappa drug. Thus, within only a few years after their proposal, mu, delta and kappa binding sites were demonstrated in brain.

4.6.1 Mu Receptors

Although the suggestion of multiple mu receptors initially came from clinical observations and the responses of patients to different mu opioids, it did not take long for this concept to be supported by binding studies with the suggestion of mu₁ and mu₂ sites [11]. However, cloning studies have now shown that the situation is far more complex with a vast array of MOR-1 splice variants [152–158]. Obviously, the situation with multiple mu receptors is far more complex than originally proposed. The evidence for the initial classification of multiple mu receptors is described below, but the relationship between the cloned variants with those defined pharmacologically has not yet been resolved.

4.6.1.1 Mu₁ and Mu₂ Receptors

Despite the evidence suggesting different receptor mechanisms for the mu opioids, the early reports of opioid binding did not reveal evidence of binding heterogeneity [1–3, 37], although this changed with the availability of higher specific activity radioligands [104]. Insights into the concept of multiple mu receptors came from several antagonists synthesized by our group, naloxazone and naloxonazine [84, 86, 159, 160]. Studies *in vivo* found that these antagonists selectively blocked morphine analgesia without influencing morphine-induced respiratory depression [84, 86, 161, 162], gastrointestinal transit [163, 164] or physical dependence [165]. More detailed studies found that supraspinal morphine analgesia was sensitive to naloxonazine while spinal morphine analgesia was not [166]. Morphine induces the release of a number of hormones. Again, naloxonazine was able to distinguish among them, preventing the morphine-induced stimulation of prolactin release but not growth hormone [167, 168]. Additional studies implicated the naloxonazine/naloxazone-sensitive receptors in feeding and other morphine actions [169–178]. This selective blockade of morphine actions by naloxonazine/naloxazone contrasted with the actions of the mu-selective antagonist β-funaltrexamine [179–182], which blocked all mu actions [183]. However, studies using these drugs must be interpreted cautiously. Although both antagonists are relatively selective for the mu₁ site, this selectivity is only seen with their irreversible blockade of binding sites. Thus, it is necessary to eliminate the free drug to obtain a selective action, either by washing membranes or permitting sufficient time *in vivo* for the free drug to be eliminated, which is why the drug is administered 24 h prior to testing.

The actions of naloxazone and naloxonazine result from the selective blockade of a very high affinity binding component in brain [11, 84, 86, 159, 160, 184]. This site differed pharmacologically from the classical mu and delta receptors. Whereas the classical mu and delta receptor were highly selective for their respective classes of drug, the high affinity site bound most mu and delta ligands. Binding studies confirmed that treatment with naloxonazine or naloxazone eliminated the high affinity binding component from both radiolabeled mu and delta agonists. Thus, this site appears responsible for selected morphine actions, as well as those of a

number of delta ligands. The naloxonazine-sensitive binding site was termed mu₁. The morphine-selective site was termed mu₂ and the delta receptor remained the enkephalin-selective site previously demonstrated [11]. Thus, morphine and the enkephalins interacted with three sites. The pharmacology of mu₁, mu₂ and delta sites has been extensively reviewed [158, 185–191].

Rothman independently proposed three receptors for the mu and delta receptors soon after the proposal of mu₁ and mu₂ receptors using sophisticated computer modeling approaches [192–194]. In his model, he referred to them as complexed and non-complexed sites. In this regard, he may have been prescient since there is some evidence suggesting that the mu₁ site, which he termed the μ_{cx}/δ_{cx} site, may involve a dimer of the mu and delta receptors. However, this remains to be demonstrated conclusively.

4.6.1.2 Morphine-6 β -Glucuronide Receptors

Morphine undergoes extensive metabolism in humans. One of its metabolites is M6G. Early studies indicated that it had analgesic activity [195] and was demonstrated to have high affinity for opioid binding sites [196]. However, its full analgesic activity was not appreciated until it was administered intracerebroventricularly where it was found to be as much as 100-fold more potent than morphine whereas it was twofold more potent than morphine given systemically [197, 198]. This raises interesting issues clinically since M6G levels in the blood following chronic morphine dosing can be higher than those of morphine [199]. Furthermore, its levels can increase even further in patients with renal insufficiency. Clearly, these observations raise the question of how much of the analgesia seen with chronic morphine results from morphine itself or from M6G. The importance of M6G in morphine actions, however, seems to be relatively restricted among species. Although humans glucuronidate both the 3' and the 6' positions of morphine, other species, including mice and rats, glucuronidate only the 3' position. This is important when interpreting morphine studies in rats and mice since they do not convert morphine to M6G [200].

M6G also is important in understanding the pharmacology of mu receptors. Although it has high affinity for mu sites and its actions are antagonized by mu-selective antagonists, its actions can be readily distinguished from those of morphine. One of the most dramatic examples was the comparison of the two drugs in CXBK mice. Morphine has long been known to have very limited activity in CXBK mice. Yet these same mice respond normally to M6G. Although both drugs act through mu receptors, their receptor mechanisms must differ. This conclusion is further supported by the antagonist 3-methylhaptrexone [201, 202]. This drug effectively blocks the analgesic and rewarding actions of heroin and M6G at doses that do not influence morphine. There is also additional information using knockout animals to demonstrate differences between the action of morphine and M6G at the molecular level [186, 203]. While the identification of multiple splice variants of the cloned mu receptor MOR-1 might be involved, these questions remain unanswered.

4.6.2 *Delta Receptor Subtypes*

Delta receptor subtypes were first proposed using classical pharmacological approaches with selective antagonists [204]. In this study, Porreca and colleagues compared two nonequilibrium antagonists, naltrindole 5'-isothiocyanate (5'-NTII) [205] and [D-Ala²,Leu⁵,Cys⁶]enkephalin (DALCE) [206] on the analgesic actions of [D-Pen²,D-Pen⁵]enkephalin (DPDPE) and [D-Ala²]Deltorphin II. In brief, they found that 5'-NTII selectively blocked the effects of [D-Ala²]deltorphin II while DALCE selectively blocked the actions of DPDPE. The [D-Ala²]deltorphin II/5'NTII site was termed delta₁, while the DPDPE/DALCE site was then termed delta₂. These antagonists have been extensively used to elucidate a wide range of actions of delta subtypes [204, 206–216].

With the cloning of the receptors, many investigators anticipated identifying these delta subtypes at the molecular level. However, unlike MOR-1 with its dozens of functionally active splice variants, functional DOR-1 splice variants have not been identified and to date there is no evidence for a second delta receptor gene. Thus, many questions remain regarding these subtypes. The pharmacological evidence is compelling, but we do not yet have a molecular understanding of it. There also is evidence at the genetic level supporting the concept. In a DOR-1 knockout mouse, Pintar and colleagues found that the loss of DOR-1 led to a loss of spinal delta analgesia, but supraspinal DPDPE retained analgesic activity [217]. Indeed, one of the non-peptide delta ligands, BW373U69, was actually more potent in the DOR-1 knockout mouse. More work is needed to explain these observations and to provide a molecular basis for the pharmacology of delta ligands.

4.6.3 *Kappa Receptors*

Martin proposed kappa receptors based upon the pharmacology of the benzomorphan ketocyclazocine in the chronic spinal dog [218]. However, the definitive identification of kappa receptors in binding assays proved difficult. Indeed, Simon initially published a report suggesting that kappa binding sites did not exist [219]. The major problem encountered by investigators was the lack of specificity of the radio-ligands available to label the sites. Most of the drugs available were mixed agonist/antagonists. Pharmacologically, their agonist actions at the kappa receptors were easily seen while their antagonist activity at mu sites was not discernable unless the drug was given in combination with a mu drug. Binding assays, on the other hand, showed labeling of both receptor classes. Attempts to compensate for this lack of selectivity initially utilized blockers. In this regard, investigators were lucky in that highly selective mu and delta ligands were available. Thus, blocking the mu and delta sites with these selective agents would leave only the kappa receptors. This approach was used effectively in homogenate binding assays [149, 150, 220–222] and autoradiographically [68], but the approach was not optimal. Characterization of the kappa receptors was greatly facilitated by the discovery that its abundance was

markedly higher in guinea pig brain, including the cerebellum, which has little mu or delta binding [221]. However, the greatest advance came with the development of highly selective kappa drugs, starting with U50,488H in 1983 [151]. The general availability of another highly selective kappa agent, ³H-U69,593 opened the way for detailed studies of kappa receptors in a variety of tissues.

4.6.3.1 Kappa₁ and Kappa₂ Receptors

There has long been controversy about whether or not there are subtypes of kappa receptors. However, studies by Zukin and coworkers [8] clearly demonstrated two kappa receptor subtypes. Using radiolabeled ³H-ethylketocyclazocine, they identified two classes of binding that were neither mu nor delta. They defined the U50,488H-sensitive site as kappa₁ receptor and the other as kappa₂, a classification that has become well accepted. The cloned kappa receptor KOR-1 corresponds to the kappa₁ site. Defining the kappa₂ receptor proved more difficult. However, there is now evidence that it corresponds to a heterodimer of KOR-1 and DOR-1 [223].

Binding studies also have suggested that the U50,488H-sensitive sites can be further subdivided into kappa_{1a} and kappa_{1b} based upon their sensitivity towards dynorphin B and α -neoendorphin [9]. Although both U50,488H and dynorphin A competed both the kappa_{1a} and the kappa_{1b} sites equally well, competing the binding in brain in a monophasic manner and with high affinity, dynorphin B and α -neoenorphin revealed high and low affinity components in competition studies differing in affinities by approximately 100-fold. The site sensitive to dynorphin B and α -neoendorphin was termed kappa_{1b} and the other site as kappa_{1a}. It is interesting that both the compounds that distinguish between them are endogenous opioids, raising the interesting question of relationship of these kappa sites to some of the other endogenous ligands other than dynorphin A.

4.6.3.2 Kappa₃ Receptors

Kappa₃ receptors were first described twenty years ago using naloxone benzoylhydrazone (NalBzOH) [224, 225]. Pharmacologically, the compound was a mixed agonist/antagonist. It was a mu antagonist, reversing morphine actions at low doses. However, higher doses elicited analgesia with a pharmacological profile quite distinct from most of the opioids [225, 226]. This mixed agonist/antagonist character was quite similar to the prior studies looking at nalorphine/morphine interactions in patients reported almost 40 years earlier [130, 131] and raised the possibility that NalBzOH might be acting through the same site.

The receptor selectivity of NalBzOH in binding assays proved complex. Competition studies with the unlabeled drug against mu binding revealed a high affinity, consistent with its reversal of morphine analgesia [9, 227]. However, when we examined the binding of radiolabeled ³H-NalBzOH we noticed a marked

increase in binding levels compared to values seen with ^3H -naloxone. More detailed characterization of the binding, revealed that approximately 1/3 of the binding corresponded to mu sites while the remainder had a selectivity profile different from any site known at the time (Table 4.7). Neither the kappa₁-selective ligand U50,488H nor the delta-selective compound DPDPE competed the binding at concentrations 100-fold greater than their affinities for their respective receptors. However, a number of benzomorphans long considered to be kappa drugs, including ketocyclazocine, ethylketocyclazocine, (-)SKF10,047 and cyclazocine, all showed high affinity for the site, leading us to consider the site as within the kappa receptor family. It was interesting that a number of additional ligands also showed high affinity for this site, including the morphinan levorphanol and nalbuphine (Table 4.7).

Many of these drugs elicited their actions in large part through this site, including nalorphine [228] and levorphanol [229] while nalbuphine acted through a combina-

Table 4.7 Selectivity of opioid receptors in brain

Class	Drug	K_i values (nM)					
		Mu ₁	Mu ₂	Kappa ₁	Kappa ₂	Kappa ₃	Delta
Mu	Morphine	0.5	2.5	49		32.8	278
	DAMGO	0.5	2.1	>350		8.2	>500
	PL-017	5.4	16.5	>350		88.8	>100
	(-)Naloxone	1.3	3.7	5.3		8.4	106
	(+)Naloxone	>500	>500			>350	>500
Kappa	(-)EKC	0.17	0.24	0.21	44	1.4	4.7
	(+)EKC			>350		>350	
	Ketocyclazocine			1.8		4.5	
	U50,488H	370	>500	6.1	484	>350	>500
	Tifluadom			0.87	39	6.5	
	(-)SKF10,046			2.9		6.9	
	(+)SKF10,046			>350		>350	
	WIN44,441			0.32		0.2	
	(-)Levallophan	0.25	1.0	0.95		2.2	5.4
	(+)Levallophan	>500	>500			>350	>500
	Levorphanol			8.1		5.6	
	NalBzoH	0.3	0.8	0.6		0.9	
	Cyclazocine			0.99	65	1.5	
Delta	Pentazocine			11.8		79.5	
	Nalbuphine			3.3		5.8	
	norBNI	27.5	115	3.5		103	23
	DPDPE	82	457	>350	>10,000	>350	2.9
	DADL	0.9	7.2	>350		85.9	1.9
Endogenous opioids	DSLET	1.4	14			259	2.3
	β -Endorphin	0.98	3.1	80		10.7	2.6
	α -Neoendorphin	5.6	19			67	4.2
	Dynorphin A	0.69	2.2	0.19	1.7	14.2	8.7
	Dynorphin B	6.0	17			63	6.8

These K_i values are from the literature [9]

tion of kappa₁ and kappa₃ mechanisms [230]. Both levorphanol and nalbuphine are approved for clinical use. However, many of these drugs were not particularly selective, acting through more than one receptor. For example, levorphanol analgesia shows unidirectional cross tolerance with morphine [231], consistent with both a mu and kappa₃ mechanism of action. Rats tolerant to morphine showed normal responses to levorphanol. However, animals tolerant to levorphanol showed cross tolerance to morphine. Levorphanol has been extensively used clinically, where it is potent analgesic with a longer duration of action. Even though levorphanol has a significant kappa₃ component to its analgesia, it is important to note that it does not produce an untoward number of psychotomimetic effects, suggesting that this kappa-related action may be mediated through the kappa₁ site. This is supported by the suggestion that highly selective kappa₁ drugs given clinically do elicit these side-effects. In addition to its traditional actions as an opioid analgesic for somatic pain, levorphanol also is active against neuropathic pain [232], a particularly difficult clinical pain to treat. This observation raises the question of whether the kappa₃ receptor might be a reasonable target for drug development.

The limited selectivity of NalBzoH for the kappa₃ site has hampered its study. However, recent advances in the molecular biology of the opioid receptors and the synthesis of a series of new, very potent ligands for this site have now provided greater insight (S. Majumdar and G.W. Pasternak, in preparation). Using an ¹²⁵I-version of one of the compounds, we found a very high affinity binding site ($K_D << 1 \text{ nM}$) in a triple opioid receptor knockout mouse with disruption of the MOR-1/DOR-1/KOR-1 genes. This observation is at odds with a prior autoradiography study which failed to observe any high affinity ³H-NalBzoH labeling in these same mice [233]. However, their failure to detect the sites probably reflects the limited sensitivity of tritiated, as opposed to ¹²⁵I-ligands. Thus, current evidence indicates that the sun has not set on kappa₃ receptors [234].

Characterization of this site revealed a very distinct selectivity profile. Again, many of the benzomorphans showed very high affinity for the sites, as did levorphanol, levallorphan and buprenorphine. In contrast, a wide range of selective mu, delta and kappa₁-selective agents did not compete binding at doses under 1 μM . These kappa₃ ligands elicit a potent analgesia that persists in the triple knockout mice and shows no cross tolerance to morphine and no evidence of dependence with chronic administration. This response was readily antagonized by levallorphan, confirming an opioid mechanism.

4.6.4 *Epsilon Receptors*

β -Endorphin has played an important role in opioid systems. One of the initial opioid peptides, it is larger than the others (31 amino acids) and derives from the processing of a prohormone pre-pro-opiomelanocortin, which also is responsible for generating ACTH and MSH. It has been suggested by some that β -endorphin is the endogenous ligand for mu receptors in view of its reasonably high affinity for

this site, even though it labels delta sites about as effectively. However, there is evidence that β -endorphin may have its own, distinct receptors [235, 236]. In brain, it is difficult to distinguish between β -endorphin-selective binding and cross-labeling of other sites for which the ligand has high affinity. Cuatrecasas and coworkers reported a high affinity β -endorphin binding site in lymphocytes (K_D 3 nM) that was insensitive to a wide range of opioids examined at 1 μ M, including naloxone, morphine, and a series of enkephalin analogs [235]. Little has been reported in recent years on this binding, perhaps due to technical difficulties in making 125 I- β -endorphin with selective iodination of [Tyr²⁷]. This is important since iodinating [Tyr¹] markedly diminishes the affinity of the ligand. 3 H- β -endorphin does not have the sensitivity needed to see the sites in the lymphocytes.

4.6.5 Others

Other opioid or opioid-like receptors have been proposed. The most closely studied is the ORL₁ receptor (opioid receptor like) [237] which was reported by a number of groups [238–247] soon after the initial reports of the cloning of the delta receptor. The cloning of the receptor left many questions since its endogenous ligand was unknown at the time. It was subsequently identified independently by two groups and termed orphanin FQ by one [248] and nociceptin by the other [249]. OFQ/N has similarities to dynorphin A, the replacement of the Tyr¹ with Phe¹. This position is quite important for opioid receptor binding, so it was not surprising that OFQ/N showed little affinity for any of the opioid receptors and the opioids showed virtually no affinity for ORL₁. Antisense mapping of ORL₁, initially termed KOR-3 by our group, suggested a close association with the kappa₃ receptor. A wide range of antisense probes targeting coding exons 2 and 3 all downregulated kappa₃ analgesia, but probes targeting exon 1 were ineffective, leading us to speculate that ORL₁ was related to, but different from, the kappa₃ receptor. These issues have not yet been resolved, but recent evidence has suggested that the kappa₃ receptor may involve heterodimerization, much like the kappa₂ site [223].

A very different receptor related to the opioid peptides has also been proposed [250]. Termed the zeta receptor, it has been implicated in growth and proliferation of a wide range of tumors [251–260]. It has been implicated in tumor growth and its presence is reported in skin and cornea as well as the brain. It has been suggested that the receptor is selective for [Met⁵]enkephalin, with other opioids and opioid peptides having poor affinity.

More recently, cloning studies identified a series of G-protein coupled receptors in sensory neurons that are activated by BAM-22, a peptide generated from proenkephalin [121]. These receptors have been termed sensory neuron-specific receptors (SNSR) based upon their localization to only sensory neurons. Their regional distribution has been determined as well as their role in pain perception [119, 120, 261–263]. What distinguishes these receptors is that they are pronociceptive and their activation by BAM-22 enhances pain perception.

4.7 Conclusion

The opioid field has evolved quite rapidly since the cloning of the opioid receptors in 1992. Three major families of receptors have been isolated and alternative splicing shown to be important in the actions of some. Other receptors, such as ORL₁ and the sensory neuron-specific receptors are related. However, the cloning does not provide an explanation for the far greater number of receptor suggested from pharmacological studies.

Traditionally, the pharmacological identification of opioid receptors has led to their identification at the molecular level. It will be interesting to see if this carries over to the ones that have not yet been isolated. One possibility is that additional genes remain to be cloned. Alternatively, the range of proposed opioid receptors may be due to heterodimerization. Already, heterodimerization has provided an explanation for the kappa₂ receptor and recent evidence suggest a similar importance in the kappa₃ receptors. In these situations, as well as an ORL₁/MOR-1 dimer [264], the pharmacology is quite different from that of each component expressed alone. These heterodimers may include only opioid receptors, such as MOR-1/DOR-1 and DOR-1/KOR-1 or involve dimerization of related receptors, such as MOR-1/ORL₁, which yields a heterodimer in which opioids can compete OFQ binding and vice versa [264], competitions that cannot be seen when expressing either receptor alone. In addition, opioid receptors can dimerize with a range of non-opioid receptors, including substance P and α -adrenergic receptors. With the wide range of G-protein coupled receptors, the potential of additional subtypes of opioid receptors may be quite large. It will be interesting to see how these issues evolve.

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Chapter 5

Endogenous Opioids

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Abstract Most of the endogenous opioids that have been identified are peptides that originate from four distinct precursor proteins, proenkephalin, prodynorphin, proopiomelanocortin (POMC), and pronociceptin/orphanin FQ. All four of these precursors are differentially processed by endopeptidases and carboxypeptidases to give rise to a large number of peptides that have biological activity. In many cases, the amount of processing affects the bioactivity of the resulting peptides; in some cases the longer forms of a particular peptide bind with higher affinity to one of the opioid peptide receptors, in other cases the shorter forms bind with higher affinity to the receptor. Also, in the case of β -endorphin, one form of the peptide is an agonist at opioid peptide receptors while a shorter form lacking four C-terminal residues is an antagonist at the same receptor. Thus, processing of the endogenous opioid peptides plays a critical role in generating the bioactive form(s) of the peptide, and regulation of this processing can greatly influence the physiological activity of the peptide.

Keywords Proenkephalin • Prodynorphin • Proopiomelanocortin • Enkephalin • Dynorphin • Endorphin • Nociceptin • Orphanin FQ • Prohormone convertase • Carboxypeptidase E

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5.1 Introduction

5.1.1 Discovery of Endogenous Opioids

The first endogenous opioid peptides were isolated and identified by Hughes, Kosterlitz, and colleagues in 1975, and named Met-enkephalin and Leu-enkephalin [1]. Soon after this important discovery, Li and Chung reported the sequence of a distinct peptide with opioid activity; this peptide was named β -endorphin [2]. Other forms, named α - and γ -endorphin were also detected, but these forms are not the major ones either in abundance or in biological effects. Because β -endorphin and the enkephalins show weak binding to the κ opioid peptide receptors, Goldstein et al. [3, 4] searched for the endogenous ligand for these receptors and discovered the dynorphins in 1979.

Independently, Kangawa and Matsuo discovered α -neoendorphin [5], and later, with other colleagues, identified additional κ -selective ligands named β -neoendorphin and dynorphin A 1-8 [6, 7]. Many additional peptides with opioid activity were discovered in bovine adrenal medulla and other tissues; these were subsequently found to be encoded by the same precursor that gave rise to the enkephalins and to result from incomplete processing [8–12]. A search for endogenous ligands for ORL-1 (opioid receptor-like 1) – the “orphan” G protein-coupled receptor with substantial sequence homology to the opioid peptide receptors – led to the simultaneous discovery in 1995 of a peptide named ophain FQ by Reinscheid et al., and nociceptin by Meunier et al. [13, 14].

The above molecules are well-studied peptides that are discussed further below. Other “endogenous” molecules have been reported and may also represent endogenous ligands for the μ opioid peptide receptor. Because the μ opioid peptide receptor binds to most of the peptides mentioned above with lower affinities than the other opioid peptide receptors, it has been proposed that additional endogenous μ ligands exist.

A search for non-peptide opioid ligands in bovine brain and adrenal resulted in the purification of a compound that was chemically indistinguishable from morphine [15]. Other related compounds were also detected in these tissues [15–17]. However, it is not clear if these compounds are synthesized in animals or if they are absorbed from dietary sources [18].

Another strategy to identify an endogenous μ opioid peptide receptor ligand was developed by Zadina et al. [19]; they prepared a number of synthetic peptides and tested them in receptor-binding assays. Surprisingly, the most potent of the synthetic μ opioid peptide receptor agonists were subsequently identified in extracts of brain and other tissues; these were named endomorphin [19]. However, the precursors of these peptides have not yet been identified even after many years of searching. Thus, it remains to be proven that the endomorphins are endogenous opioid ligands.

In addition to the above peptides, a fragment of β -hemoglobin, named hemorphin, has been shown to bind to opioid peptide receptors [20]. A variety of N-terminally extended forms of hemorphin have been identified in plasma and in brain [21, 22].

The cloning of the cDNA for the precursors of enkephalin, dynorphin, β -endorphin, and nociceptin/orphanin FQ (N/OFQ) led to the discovery of additional sequences within these precursors that encode bioactive peptides [13, 14, 23–33]. Some of these other peptides bind to opioid peptide receptors (discussed in other chapters in this book). The precursor of β -endorphin, named proopiomelanocortin (POMC), encodes peptides with non-opioid functions, such as α -melanocyte stimulating hormone (α MSH) and adrenocorticotropic hormone (ACTH).

From the analysis of the precursors and their bioactivities, it is clear that the extent of the processing of the precursor can alter the bioactive properties of the peptides. A classic example of this, albeit not relating directly to peptides with opioid activity, is the processing of POMC into ACTH in the anterior pituitary, and the further processing of ACTH into α MSH and a C-terminal fragment in the intermediate pituitary [34]. The biological activity of these two products are much different: α MSH stimulates melanocytes and also plays a role in feeding/body weight regulation, while ACTH stimulates steroid release from the adrenal cortex [34].

In the case of opioid peptides, the pentapeptides Met- and Leu-enkephalin have the highest affinity for δ opioid peptide receptors, lower affinity for μ receptors, and much lower affinity for κ receptors, while C-terminally extended enkephalin-containing peptides such as metorphamide and BAM 18 bind to all three opioid peptide receptors with high affinity [35, 36]. Thus, unlike classical neurotransmitters such as acetylcholine, which are either active (when acetylcholine) or inactive (when cleaved into acetate and choline), the bioactive peptides can be modulated by the extent of processing during their biosynthesis and also following secretion from the cell.

5.1.2 Biosynthesis of Opioid Peptides

Like most neuropeptides, the opioid peptides are produced from larger precursors by the selective action of a small number of peptidases and other post-translational processing enzymes (Table 5.1). First, the signal peptide that directs the protein to be imported into the endoplasmic reticulum is co-translationally removed by a signal peptidase [37]. This enzyme is not specific for prohormones, but acts on any signal peptide containing peptide which includes the majority of secretory proteins as well as many proteins destined for the cell surface or internal organelles such as lysosomes, endosomes, and Golgi [37]. If N-linked carbohydrates are added (proenkephalin and POMC each contains a site for this modification), this also occurs in the endoplasmic reticulum, and then subsequent processing of the sugar side chains occurs in the Golgi [38]. O-linked glycosylation, which is smaller than N-linked glycosylation, occurs in the Golgi [38]. Other events such as phosphorylation, which is found on some proenkephalin- and proopiomelanocortin-derived peptides, also occurs in the Golgi [38].

The proteolytic processing of the precursors into smaller fragments usually begins in the late Golgi compartment named the *trans*-Golgi network and continues

Table 5.1 Enzymes involved in the post-translational processing of neuropeptide precursors

Posttranslational processing step	Enzyme(s)	Subcellular location	Modification site/consensus sequence	References
Cleavage of signal peptide	Signal peptidase	ER	Enzyme cleaves after Ala, Ser, Gly, Thr or Cys, usually 20–25 amino acids from N-terminus of precursor	[169]
N-glycosylation	Oligosaccharyltransferase adds sugar	ER	Asn is modified. Asn-Xaa-Ser/Thr (Xaa=any amino acid except Pro)	[38]
O-glycosylation	Many enzymes process sugar	Golgi	Ser or Thr are modified. No consensus site is known	[38]
Phosphorylation	Many	Golgi	Ser or Thr are modified. Ser/Thr-Xaa-Asp/Glu	[38]
Sulfation	Casein kinase I	Golgi	Tyr is modified. Surrounding region is acidic, with few basic or hydrophobic residues, or disulfide bonds nearby	[38]
Proteolytic cleavage at sites containing basic amino acids	Tyrosylprotein sulfotransferase	Golgi	Lys-Arg and Arg-Arg most common. Sequences of Arg-Xaa(N)-Lys/Arg where n=0, 2, 4, or 6 and Xaa is any amino acid except Cys. Some sites contain a single Arg and no additional upstream basic amino acid	[41–43]
Proteolytic cleavage at sites without basic amino acids	Prohormone convertases 1/3 and 2 in secretory vesicles; several protein convertases and furin in trans-Golgi	Golgi and secretory vesicles	Less common than cleavage at basic residues, but for some peptides this is an important step. No clear consensus site. Cleavage may occur in secretory pathway, or in other parts of the cell (lysosomes, or extracellular environment)	[98, 170]
Removal of C-terminal basic residues	Carboxypeptidases E in secretory vesicles and carboxypeptidase D (CPD) in trans-Golgi	Golgi and secretory vesicles	Requires C-terminal basic residue. Preference Arg≥Lys>>His	[50, 51, 55]
C-terminal amidation	Peptidyl-glycine- α -amidating-monoxygenase (which possesses monooxygenase and lyase activities)	Secretory vesicles	Requires C-terminal Gly (NH group of the Gly becomes the amide). Enzyme reaction is two-step process	[55]
Acetylation	Unknown	Secretory vesicles	Modification of N-terminal amine. On occasion, the Ser side chain is acetylated (as in di-acetyl α -melanocyte-stimulating hormone)	

as the prohormone is routed into immature and then mature secretory vesicles [39]. Several peptidases are involved with this processing. Initially an endopeptidase cleaves the precursor at sites usually containing the basic amino acids Lys and/or Arg [40]. Secretory pathway enzymes that cleave prohormones to the C-terminal side of Lys and Arg residues have been identified and alternatively named prohormone convertases or proprotein convertases; both of these are abbreviated as PCs. The major PCs for the production of opioid peptides are PC1 (also known as PC3, and sometimes referred to as PC1/3) and PC2 [40–43], these are discussed further below. Other PCs that may also function in the processing of opioid peptides include PACE4, PC5, PC7, and furin, a PC-like enzyme that is located in the *trans*-Golgi network [44].

Following the action of the endopeptidase, a carboxypeptidase (CP) is required to remove the C-terminal basic residues [45]. The major peptide-producing CP is carboxypeptidase E (CPE), also known as carboxypeptidase H and enkephalin convertase [45–47]. Another enzyme that contributes to peptide processing is carboxypeptidase D (CPD), which is located along with furin in the *trans*-Golgi network [48, 49]. Both CPE and CPD are discussed further below.

Additional post-translational processing steps may occur following the proteolytic cleavages. For example, if a C-terminal Gly is present after proteolytic processing, this will be converted to an amide group by the enzyme peptidylglycine- α -amidating monooxygenase (PAM) [50, 51]. Whereas C-terminal amidation is relatively common for peptide hormones that circulate in plasma, only one of the major opioid peptides (metorphamide) has this modification [52, 53].

In cow, but not human, rat, mouse, or most other species, a second amidated opioid peptide, named amidorphin, has been detected in adrenal medulla; in bovine brain, a shorter fragment of this peptide was detected [53, 54]. This amidated brain peptide corresponds to residues 114–133 of mouse proenkephalin (Fig. 5.1), and the cow sequence contains a Gly on the C-terminus (in human, rat, mouse, and frog this residue is an Ala, and therefore not a substrate for amidation). In addition to amidation, another rare modification is N-terminal acetylation, which is found in β -endorphin produced in some tissues such as the intermediate pituitary and several brain regions [55–57]. Other peptides derived from POMC (such as joining peptide and α MSH) also are acetylated on the N-terminus and α MSH can undergo an O-acetylation of the N-terminal Ser to form diacetyl MSH [55]. The amidation enzyme has been identified [50, 51, 55] and is discussed further below; the acetylation enzyme has not yet been identified.

5.2 Overview of Opioid Precursors and Peptide Products

The isolation and sequence analysis of the cDNAs encoding the opioid peptide precursors has provided a better understanding of the relationship of the various peptides that had been described, and also allowed for the prediction of novel

peptides. In some cases (described below) these novel peptides turned out to have biological activity. Until recently, peptides were measured using radioimmunoassay or radioreceptor assays, and because these methods are rarely specific for a single form of the peptide, [58] it was difficult to be sure what precise form was being measured in the tissue extract. Recently, techniques using mass spectrometry have been developed that provide the precise mass and sequence of many peptides in a single experiment [59–65].

It is also possible to quantify the relative level of a peptide between two samples, or even obtain absolute quantification of a peptide's levels using mass spectrometry if the sample is spiked with the appropriate isotopic standards [61, 66, 67]. In the following discussion, the focus is primarily on peptides that have been conclusively identified by sequence analysis (Edman sequencing in past years, tandem mass spectrometry in recent years).

5.2.1 *Proenkephalin*

Proenkephalin contains six copies of Met-enkephalin; four of these are released from proenkephalin by cleavage by the PCs at sites containing pairs of basic residues (Fig. 5.1). The other two Met-enkephalin-containing peptides, named octapeptide and heptapeptide contain a single basic residue on the C-terminus of the enkephalin sequence, and these are not completely converted into Met-enkephalin (Table 5.2). Likewise, one of the Met-enkephalin-containing sequences (position 211–215 of proenkephalin) is not completely converted into Met-enkephalin because C-terminally extended forms of this peptide have been detected by radioimmunoassay and mass spectrometry. For example, the peptide metorphamide corresponds to proenkephalin 211–218 with a C-terminal amide group, and BAM18 (originally named for “bovine adrenal medulla” peptide of 18 residues) corresponds to proenkephalin 211–228. In brain, some BAM18 is clearly processed into either metorphamide or Met-enkephalin; peptides corresponding to proenkephalin 218–228 and 221–228 have been detected by mass spectrometry, [68] indicating that cleavages of BAM18 occurs at the Arg–Arg adjacent to the Met-enkephalin sequence and also at the Arg downstream of the metorphamide sequence (Fig. 5.1).

In addition to the C-terminal amide present on metorphamide, proenkephalin undergoes several other post-translational modifications. There are two phosphorylation sites in the C-terminal region and both have been shown to be used in adrenal medulla [69]. In some species, a third phosphorylation site is present. Mass spectrometry of mouse brain only detected a singly phosphorylated form of this peptide (Fig. 5.1). A large peptide fragment corresponding to the middle portion of proenkephalin is glycosylated on an Asn residue [70, 71]. Because this modification adds several kilodaltons to the mass of the peptide, it is not detected by mass spectrometry under conditions optimized for smaller peptides. Likewise, the large N-terminal peptide named synenkephalin is

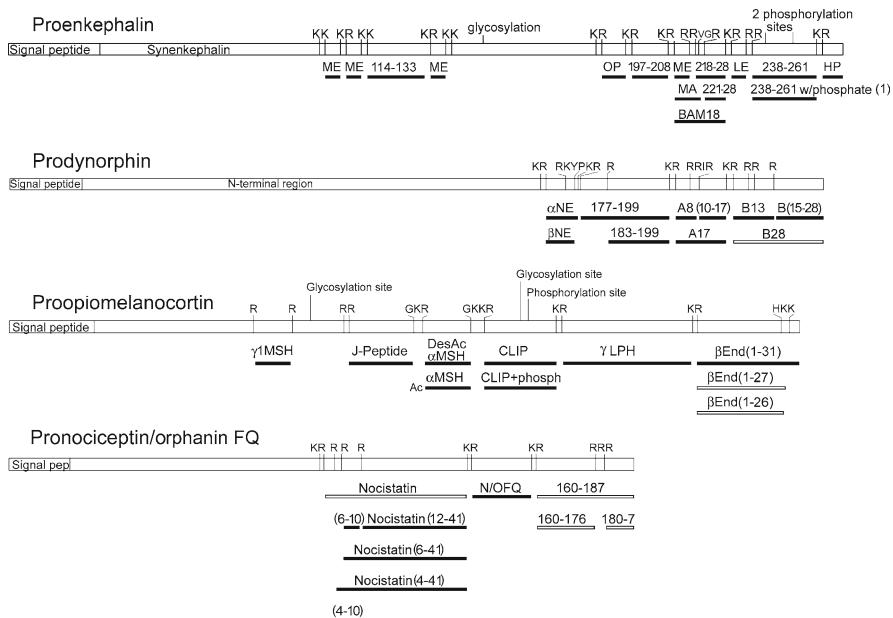


Fig. 5.1 Schematic diagram of the four precursors of opioid peptides and the major peptide products detected in mouse brain. Peptides extracted under conditions that reduce postmortem degradation and identified by mass spectrometry are indicated by *bold lines*. Peptides identified by radioimmunoassay (and in some cases, partial Edman sequencing and/or HPLC analysis comparing the elution time to standard peptides), are indicated by the *open boxes*. Basic amino acids (Lys, K; Arg, R) located in the cleavage sites used to generate the observed peptides are indicated above the precursor; in some cases additional amino acids near the cleavage site(s) are included; abbreviations use the single letter amino acid code. Numbers either refer to the position within the precursor if the peptide was not named (i.e., 177-208 in proenkephalin) or to the position within the peptide (i.e., βEnd (1-31) in proopiomelanocortin (POMC)); the latter are within *parentheses*. Other abbreviations: A8 dynorphin A8; A17 dynorphin A17; B13 dynorphin B13; B28 dynorphin B; BAM bovine adrenal medulla peptide; CLIP corticotropin-like intermediate lobe peptide; DesAc desAcetyl (meaning, without an acetyl group); End endorphin; HP heptapeptide; LE Leu-enkephalin; LPH lipotropin; MA metorphamide; ME Met-enkephalin; MSH melanocyte stimulating hormone; NE neoendorphin; N/OFQ nociceptin/ orphanin FQ; OP octapeptide; Phosph phosphate

also not seen by mass spectrometry, although this peptide has been detected with antisera [72].

Of the various opioid peptide receptors, Met- and Leu-enkephalin bind to the δ receptor with highest affinity [36]. Removal of the N-terminal Tyr eliminates binding, as does acetylation, which has been reported to occur in some brain regions [57, 73, 74]. The C-terminally-extended enkephalin peptides (such as metorphamide) bind with high affinities to all three opioid peptide receptor subtypes: μ, δ, and κ [36]. Thus, the extent of the processing of proenkephalin has a large impact on the resulting biological activity of the products.

5.2.2 *Prodynorphin*

Although prodynorphin contains three Leu-enkephalin sequences, each flanked by pairs of basic amino acids, this does not necessarily mean that prodynorphin is processed into Leu-enkephalin (Fig. 5.1 and Table 5.2). A variety of C-terminally-extended enkephalin-containing peptides have been identified, including α - and β -neoendorphin, dynorphin A8 and A17, and dynorphin B13 [4–7, 75, 76]. For all of these peptides, the intervening sequences between bioactive opioid peptides have been detected; for example, residues 10–17 of dynorphin A17 (Fig. 5.1). If prodynorphin were alternatively cleaved to generate Leu-enkephalin, this would

Table 5.2 Representative opioid peptides and other major bioactive fragments of the opioid peptide precursors found in mouse brain

Proenkephalin-derived peptides	
Leu-enkephalin	YGGFL
Met-enkephalin	YGGFM
Heptapeptide	YGGFMRF
Octapeptide	YGGFMRSL
Metorphamide	YGGFMRRV-amide
BAM18	YGGFMRRVGRPEWWMDYQ
Prodynorphin-derived peptides	
Dynorphin A8	YGGFLRRI
β -Neoendorphin	YGGFLRKYP
α -Neoendorphin	YGGFLRKYPK
Dynorphin B13	YGGFLRRQFKVVVT
Dynorphin A17	YGGFLRRIRPKLKWDNQ
Dynorphin B28	YGGFLRRQFKVVTRSQENPNTYSEDLDV
Proopiomelanocortin-derived peptides	
γ -1-MSH	KYVMGHFRWD
Des-acetyl-MSH	SYSMEHFRWGKPV-amide
α -MSH	Ac-SYSMEHFRWGKPV-amide
Diacetyl- α -MSH	DiAc-SYSMEHFRWGKPV-amide
J-peptide	AEEEAVWGDGSPEPSPRE-amide
CLIP	RPVKVYPNVAENESAFAFPLEF
Phosphorylated CLIP	RPVKVYPNVAENE-phosphoS-AEAFPLEF
β -Endorphin 1–26	YGGFMTSEKSQTPLVTLFKNAIIKNA
β -Endorphin 1–27	YGGFMTSEKSQTPLVTLFKNAIIKNAH
β -Endorphin 1–31	YGGFMTSEKSQTPLVTLFKNAIIKNAHKKGQ
γ -Lipotropin	ELEGERPLGLEQVLESDAEKDDGPYRV EHFRWSNPPKD
Pronociceptin/orphanin FQ-derived peptides	
Nociceptin/orphanin	FGGFTGARKSARKLANQ
Nocistatin	MPRVRSLVQVRDAEPGADAEPGADAEP GADDAEEVEQKQLQ

CLIP corticotropin-like intermediate lobe peptide; *J-peptide* joining peptide; *MSH* melanocyte-stimulating hormone. BAM18 is not an abbreviation, and is the name of an 18-residue peptide isolated from bovine adrenal medulla

produce different intervening sequences (e.g., residues 8–17 of dynorphin A17), as described above for the processing of proenkephalin into both metorphamide and Met-enkephalin.

Analysis of the peptides corresponding to the intervening sequences of prodynorphin suggests that processing of prodynorphin into Leu-enkephalin is not a major pathway, if it occurs at all, because the only detected intervening sequences reflect processing into the various dynorphin peptides and not Leu-enkephalin (Fig. 5.1). However, it is possible that one or more of the various dynorphin/neoendorphin peptides is processed into Leu-enkephalin outside of the cell after secretion; the transient formation of peptides in the extracellular environment is difficult to detect, and the observed forms of the peptides generally reflects the peptide stored within secretory granules. If processing does occur, it would dramatically change the biological activity of the peptide; the dynorphins and neoendorphins bind most potently to the κ opioid peptide receptor, whereas Leu-enkephalin is most selective for the δ receptor [36].

5.2.3 *Proopiomelanocortin (POMC)*

As with the dynorphins, the sequence of β -endorphin contains the 5-residue Met-enkephalin on its N-terminus (Table 5.2). This led to speculation that β -endorphin was the precursor of Met-enkephalin. However, there is no solid evidence to support this, and the major forms of β -endorphin are the full length form (residues 1–31) and two shorter forms that terminate with residues 27 and 26, although other forms appear after cleavage of the peptide in the extracellular environment [77].

Interestingly, while β -endorphin 1–31 is a potent agonist at μ and δ receptors, β -endorphin 1–27 is an antagonist and β -endorphin 1–26 is without effect [78, 79]. Acetylation of β -endorphin also eliminates its activity towards the opioid peptide receptors [74]. It is possible that the forms of β -endorphin that lack opioid activity have other functions [73].

5.2.4 *Pronociceptin/Orphanin FQ*

N/OFQ is produced from the precursor pronociceptin/orphanin FQ [13, 14]. In addition, several other peptides have been found to be produced from this precursor (Fig. 5.1 and Table 5.2), and some have been reported to have biological activity such as nocistatin [80] and the C-terminal peptide named N/OFQ 160–187 (numbering of the mouse precursor) [81]. Shorter forms of the C-terminal peptide have been detected and named OFQ2 (residues 160–176 of the mouse precursor) and N/OFQ 180–187 [82].

Shorter forms of nocistatin have been detected using a mass spectrometry-based peptidomics approach (Fig. 5.1), but these have not been tested for biological activity [68].

Although N/OFQ has a pair of basic amino acids in the middle of the peptide sequence, endogenous products resulting from cleavage at this site do not appear to be major products in brain [68]. A synthetic peptide corresponding to residues 1–7 of N/OFQ has been shown to be biologically active, [83] so if N/OFQ is cleaved at the internal pair of basic residues it is likely to be biologically active.

Pronociceptin/orphanin protein structure has high similarity between most mammalian species. However, mouse nocistatin contains a triple repeat of the AEPGAD sequence that is not found in rat or human; the function of this repeat is not known [84]. Despite a high structural homology to the opioid system, peptides derived from pronociceptin/orphanin have distinct properties from the other opioid peptides. The action of nociceptin/orphanin is mediated through the nociceptin opioid peptide receptor (NOR; also known as ORL-1) and not through the μ , δ , or κ opioid peptide receptors [13, 14].

Moreover, none of the opioid peptides from proenkephalin, prodynorphin, or POMC activate NOR with high affinity. In addition, the action of pronociceptin/orphanin-derived peptides appears to be more complex in terms of analgesia. There are contradictory data about the function of pronociceptin/orphanin-derived peptides in the nociceptive process. Depending on the brain area into which they are injected, N/OFQ and N/OFQ 160–187 have been reported to cause either analgesia or hyperalgesia [13, 14, 81–83, 85]. Other pronociceptin/orphanin derived peptides show anti-nociceptive effects [80–83].

5.3 Enzymes Involved in the Processing and Degradation of Opioid Peptides

The processing of the opioid peptide precursor has a large effect on the resulting biological properties of the peptide. Unlike conventional neurotransmitters, the processing of neuropeptides is not the same in all brain regions or tissues. Also, neuropeptide processing occurs within the cell and in many cases after secretion of the peptide. The extracellular peptidases that act on peptides can modulate activity, in some cases increasing the potency of a particular peptide towards a receptor, until ultimately further peptidase activity inactivates the peptide. Therefore, the processing enzymes are an important aspect of the opioid system. Many of the enzymes involved in the biosynthesis of the opioid peptides have been identified, as briefly described in Sect. 5.1.2. More details on these enzymes are provided in this section, and the effect of knock-out of several of the enzymes on peptide levels is described in Sect. 5.5.2.

5.3.1 Intracellular Peptide Processing Enzymes

Endopeptidase that cleave the opioid peptide precursors at specific sites containing basic amino acids include PC1 and PC [40, 42, 43]. These enzymes are members of a family that includes a total of nine members in mammals, all of which are

related to the bacterial serine protease subtilisin [40, 41, 86]. Of the nine mammalian PCs, seven cleave proteins at basic residues and are present in the secretory pathway; all of these are potentially able to process opioid peptide precursors into their products. However, some of these are not expressed in brain or other opioid peptide-producing tissues (such as PC4) and therefore it is unlikely that these contribute to this process [41, 86].

Although some, such as furin and PC7, are broadly expressed, these enzymes have neutral pH optima and are primarily localized to the *trans*-Golgi apparatus [87, 88]. Because the bulk of the peptide processing is thought to occur in post-Golgi secretory vesicles, furin and related Golgi enzymes are unlikely to contribute much to the overall processing. Therefore, only PC1 and 2 have the tissue, cellular, and subcellular distribution and enzymatic properties to be considered major opioid peptide-processing enzymes [40]. Both of these enzymes cleave opioid peptide precursors at basic amino acid-containing sites to generate smaller products that match the peptides observed in tissues. Furthermore, mice lacking PC2 activity show major defects in most opioid peptides, strongly supporting the major role for this enzyme in opioid peptide production (see Sect. 5.5.2).

Other endopeptidases are likely to be involved with the intracellular processing of peptides, although their identity is not clear. From the analysis of peptides found in various tissues, several have been detected that result from cleavage at nonbasic sites; these cleavages are not likely to be mediated by PC1 or PC2 [89]. However, it is difficult to be sure that observed peptides represent true endogenous peptides, and not post-secretion processing fragments. For example, enkephalin lacking an N-terminal Tyr has been detected in our peptidomics analysis of mouse brain (unpublished observation), but this is likely to be due to the cleavage of secreted enkephalin by extracellular aminopeptidases (described in the next section). Likewise, intermediate-sized forms of β -endorphin, named α - and γ -endorphin, may represent post-secretion processing of β -endorphin [67].

A third example is the peptide BAM22, which has received considerable recent interest based on the finding that this peptide is a potent agonist of several sensory neuron-specific G protein-coupled receptors (SNSRs) [90]. Specifically, BAM22 binds to SNSR3 and SNSR4 with EC₅₀ values of 13–16 nM [90]. However, the C-terminus of BAM22 contains a Gly, which is rarely found in peptides that are produced within the regulated secretory pathway except as a processing intermediate; C-terminal Gly residues are converted into C-terminal amide groups (discussed further below). Therefore, it is unlikely that BAM22 would be the major form of this peptide that is produced within the secretory pathway; instead, BAM22-amide should be the predominant form (and this form has not yet been reported). It is possible that BAM22 is produced outside the cell by cleavage of a longer C-terminally-extended peptide.

At least one example has been found in our peptidomics data that suggests an intracellular cleavage of an opioid peptide precursor at nonbasic residues; the cleavage of the P–Q bond located in the peptide corresponding to proenkephalin residues 197–208 (Fig. 5.1). In addition to finding the 197–208 peptide, we also detected a shorter peptide missing the N-terminal Ser-Pro (unpublished observation).

The shorter peptide has an N-terminal Gln residue that has been converted to an N-terminal pyroglutamate. Because the formation of pyroglutamate is catalyzed by an intracellular enzyme, [91] the presence of the shorter peptide with an N-terminal pyroglutamate residue implies that the endopeptidase activity to generate this peptide also occurred within the secretory pathway, rather than after secretion. The endopeptidase responsible for this conversion has not been conclusively identified, but several endopeptidases (other than the PCs) have been reported to be present in peptide-containing secretory vesicles including cathepsin L and endothelin converting enzyme 2 [92–95].

The major intracellular peptide processing carboxypeptidase is CPE, based on the distribution and properties of the enzyme, and the large changes in peptide processing observed in mice that lack CPE activity (discussed in Sect. 5.5.2). CPE was discovered in a search for an enkephalin-producing carboxypeptidase in bovine adrenal medulla and was initially named “enkephalin convertase” in publications [47]; when it became clear that this enzyme functioned in the biosynthesis of many neuropeptides and endocrine peptide hormones, in addition to enkephalin, the name CPE was used [96].

CPE is a member of a family of zinc metallocarboxypeptidase that includes 23 members in most mammals [97]. Several of these family members are digestive enzymes secreted from the pancreas, such as carboxypeptidases A1, A2, and B1. In addition to CPE, the only other member of this family that is thought to contribute to intracellular peptide processing is CPD, which is primarily found in the *trans*-Golgi network [49, 98]. CPD consists of three carboxypeptidase domains; the first two are enzymatically active, but the third domain lacks critical active site residues and does not show enzymatic activity. Interestingly, this feature of CPD has been conserved from *Drosophila* to humans, implying that the third “inactive” carboxypeptidase-like domain has a function [99].

The only other intracellular peptide processing enzyme that has been well-studied is PAM, the enzyme responsible for the formation of C-terminal amide groups [50, 51]. This enzyme consists of two independent enzyme activities, both of which are required for formation of C-terminal amide groups [51]. The monooxygenase activity functions first in the pathway, adding a hydroxyl group to the α carbon of C-terminal Gly residues by a copper and oxygen dependent process. Then, the carbons of the oxidized Gly are removed by a lyase activity, leaving behind the NH_2 group of the Gly as an amide of the C-terminus of the peptide. In mammals, a single gene encodes the precursor of both the monooxygenase and the lyase activities, and disruption of this gene is embryonic lethal.

Other post-translational modifications that occur in the secretory pathway include acetylation of N-terminal amines and/or other residues (Ser, Tyr), N-terminal pyroglutamylation, phosphorylation, sulfation, and N-linked and O-linked glycosylation [38]. However, either the enzymes responsible for these modifications have not been identified, or the modification is not important for opioid peptides; therefore, these are not further discussed in this chapter.

5.3.2 *Extracellular Peptide Processing and Degradation*

As described above, extracellular processing can lead to increased potency of the peptide towards a particular receptor, although there are relatively few examples of enzymes that activate the opioid peptides. One example is that of dynorphin A8, which is converted into Leu-enkephalin by endopeptidase 24.15 (EP24.15; also known as thimet oligopeptidase) and endopeptidase 24.16 (EP24.16; also known as neurolysin) [100]. It is unlikely that this conversion occurs within the cells prior to secretion; dynorphin is processed within the regulated secretory pathway, whereas EP24.15 is primarily localized to the nucleus of the cells, and EP24.16 is present in the cytosol of neuronal perikarya and processes [101]. However, EP24.15 and EP24.16 are known to be secreted from cells via an unknown mechanism that does not appear to involve the same secretory pathway used by dynorphin, and it is therefore possible that these endopeptidases convert dynorphin A8 into Leu-enkephalin in the extracellular environment [102, 103]. As described above, and in other chapters in this book, dynorphin A8 binds with highest affinity to κ opioid peptide receptors, whereas Leu-enkephalin is most potent at the δ receptors; therefore conversion of dynorphin A8 into Leu-enkephalin would decrease the κ specificity (i.e., “degradation”) but increase the δ specificity (i.e., “activation”).

Most of the well-studied extracellular peptidases are primarily involved in degrading the opioid peptides. A metallopeptidase that degraded Met- and Leu-enkephalin named enkephalinase was detected in brain [104–107] and an inhibitor of this enzyme was able to produce analgesia in animal models [108]. However, after purification and further characterization, it was determined that enkephalinase was identical to a kidney enzyme named neutral endopeptidase that was first described in 1968 [109]. Because of the name neutral endopeptidase, or NEP, the enzyme has been referred to as neprilysin, and is also known as endopeptidase 24.11 because of its enzyme classification number (EC3.4.24.11). Despite the fact that this enzyme is present at relatively high levels in kidney, in brain it does appear to function as one of the primary mechanisms for the degradation of enkephalin, cleaving YGGFL and YGGFM into YGG and the C-terminal dipeptide.

Another enzyme thought to play a major role in the degradation of enkephalin and other opioid peptides is aminopeptidase N, which was originally referred to as aminopeptidase M [110]. This metallopeptidase removes the N-terminal Tyr from enkephalin and other peptides, thus inactivating the opioid activity of these peptides; however, non-opioid activity of some of these peptides is unaffected by the removal of the N-terminal residue [90]. In addition to degradation of enkephalin, aminopeptidase N and neprilysin are also involved in the degradation of other opioid peptides such as N/OFQ [111]. Dual inhibitors of aminopeptidase N and neprilysin show stronger analgesic effects than inhibitors of either enzyme alone [112].

Another endopeptidase that has been found to act on opioid peptides is the metallopeptidase named angiotensin converting enzyme (ACE), but its function, like “enkephalinase” (neprilysin), is broader than the peptide for which it was named [113]. ACE converts the enkephalin heptapeptide into Met-enkephalin by removing

the C-terminal Arg-Phe [114]. Paradoxically, inhibitors of ACE potentiate the analgesic activity of the heptapeptide, even though these inhibitors would be expected to block the conversion of the heptapeptide into the pentapeptides enkephalin; this effect is presumably due to the observation that the ACE inhibitors block the degradation of the peptide [114]. Although there are some similarities in the cleavages mediated by ACE and neprylin, these two enzymes have distinct specificities [113, 115].

In addition to the enzymes described above, it is likely that additional enzymes function in the extracellular processing/degradation of opioid peptides. One additional candidate is carboxypeptidase A6 (CPA6), which is a member of the same metallocarboxypeptidase family as CPE, CPD, and the pancreatic digestive carboxypeptidases [116, 117]. CPA6 is secreted from cells and resides in the extracellular matrix [118]. This enzyme is enriched in the olfactory bulb and shows a similar distribution to enkephalin in this brain region [118]. Importantly, CPA6 removes the C-terminal Met or Leu from enkephalin, generating the tetrapeptide Tyr-Gly-Gly-Phe [118]. Previous studies found that enkephalin was converted into this tetrapeptide, along with the products of processing by an aminopeptidase, neprilysin, and ACE [119]. Thus, it is possible that CPA6, or other CPA-like enzymes, function also in the extracellular processing/degradation of peptides.

5.4 Distribution of Endogenous Opioid Peptides

5.4.1 *Distribution of Proenkephalin*

Proenkephalin-derived peptides are widely expressed throughout the brain and spinal cord. Met- and Leu-enkephalin immunoreactivity were detected in striatum (both caudate putamen and nucleus accumbens), hypothalamus, periventricular thalamus, lateral geniculate nucleus, locus ceruleus, hippocampus, amygdala, cerebral cortex, bed nucleus of stria terminalis, preoptic area, the superior and inferior colliculi, interpeduncular nucleus, substantia nigra, pons, nucleus tractus solitarius, medulla, and spinal cord [120, 121]. Moreover, terminals and fibers of these brain regions also showed enkephalin immunoreactivity, suggesting a role in a wide variety of CNS functions. Thus, the neuroanatomical data show that the proenkephalin system is located in brain regions that regulate extrapyramidal motor function, cardiovascular and water balance systems, eating, sensory processing, and pain perception.

The expression of proenkephalin mRNA is also observed throughout the CNS with highest levels in striatum and olfactory bulb (Fig. 5.2). Interestingly, it appears to be the most abundant when compared to expression of other opioid genes [122]. A good correlation between the distribution of the precursor mRNA and enkephalin peptides has been found.

Proenkephalin mRNA and peptides have been detected in neurons and in other cell types. For example, proenkephalin immunoreactivity has been detected

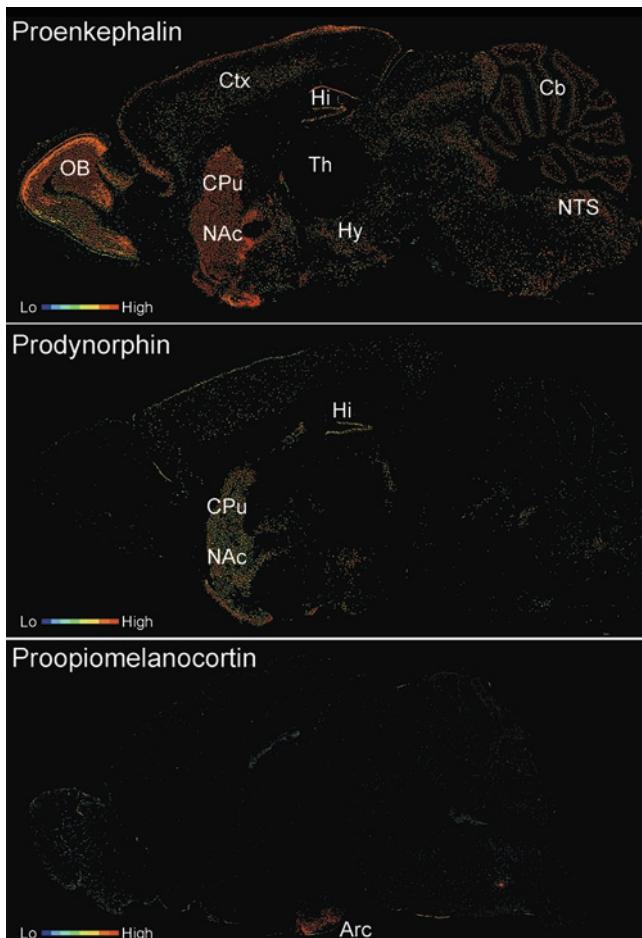


Fig. 5.2 Distribution of proenkephalin, prodynorphin, and proopiomelanocortin mRNA in mouse brain. Images were downloaded February 2010 from the *Allen Brain Atlas*, Allen Institute for Brain Science, Seattle, WA (©2004), available from: <http://www.brain-map.org> [168]. *Cb* cerebellum; *CPu* caudate putamen; *Ctx* cortex; *Hi* hippocampus; *Hy* hypothalamus; *NTS* nucleus of the solitary tract; *NAc* nucleus accumbens; *OB* olfactory bulb; *Th* thalamus

in subpopulations of neurons and astrocytes with the cerebellum, including astrocytes in both gray and white matter [123]. Antisera specific for mature enkephalin peptides do not react with the cerebellar astrocytes [123].

The expression of proenkephalin in a subset of astroglia is region-specific and increases with age [123–125]. Moreover, proenkephalin mRNA has been found to be transiently expressed during ontogenetic development of mesenchymal derivative tissues, implying the role of enkephalin peptides in normal development of these tissues [126].

5.4.2 *Distribution of Prodynorphin*

The distribution of dynorphin is, with a few notable exceptions, in the same nuclei as enkephalins [120, 121]. Immunoreactivity for prodynorphin-derived peptides has been observed in the supraoptic, paraventricular and suprachiasmatic nuclei of the hypothalamus (nuclei associated with the oxytocin/vasopressin/neurophysin systems), nucleus parabrachialis, nucleus tractus solitarius, and the posterior lobe of pituitary. Immunoreactive dynorphin cell bodies of neurons have been observed in several cerebral cortical areas, amygdala, hippocampus, striatum, supraoptic and paraventricular nuclei of the hypothalamus, midbrain periaqueductal gray, the parabrachial and spinal trigeminal nuclei, nucleus tractus solitarius, lateral reticular nucleus, and in the spinal cord dorsal horn. Fiber immunoreactivity was also found in many other areas of the brain (such as substantia nigra, raphe nuclei, globus pallidus, and others).

The distribution of prodynorphin mRNA is also observed throughout the CNS (Fig. 5.2). The highest levels were detected in the hippocampus (especially the dentate gyrus), supraoptic nucleus, parabrachial nucleus, striatum, the cerebral cortex, and spinal cord [120, 121]. This data suggest that prodynorphin system plays a role in a variety of CNS functions.

5.4.3 *Distribution of POMC*

The pituitary gland is a major site of POMC synthesis; within this tissue, POMC is expressed in intermediate lobe cells and within corticotrophs within the anterior pituitary [120, 121]. In brain, POMC mRNA is abundant in the arcuate nucleus of the hypothalamus (Fig. 5.2). POMC-derived peptides are present at relatively high levels in the arcuate nucleus of hypothalamus (with some cells scattered along the periarcuate medial–basal hypothalamus), and also present in other cells such as the caudal nucleus tractus solitarius and the commissural nucleus [120, 121].

In addition, POMC mRNA has been detected in peripheral tissues including skin, testis, thyroid gland, placenta, pancreas, gut, kidney, adrenal, and liver [120, 121, 127, 128]. POMC is also expressed in the immune system, including lymphocytes, macrophages, and mononuclear cells. In the periphery, opioid peptide β -endorphin was found in the immune system and placenta. The role of POMC-derived peptides in the periphery is not known. It was proposed that they participate in linking the immune, nervous and endocrine systems [129].

As described above in Sect. 5.1.1, POMC is cleaved in a tissue specific manner. Thus, the combination of POMC-derived peptides can differ between tissues, depending in which tissue the precursor is synthesized [127]. Because the various forms have different physiological roles, the functional outcome of the products of the same precursor differs between tissues.

5.4.4 *Distribution of Pronociceptin/Orphanin FQ*

Pronociceptin/orphanin FQ mRNA and peptides have been detected predominantly in the brain and spinal cord. This system is highly expressed in discrete neuronal sites with a pattern distinct from those of other opioid peptides [130–133]. A good correlation has been found between the distribution of the precursor mRNA and N/OFQ peptide [131, 132]. Both have been found in the following brain regions: cerebral cortex layers I–III (suggesting a role in local circuits in the cortex); dorsal horn and spinal trigeminal nucleus, caudal part (the sites that receive primary afferents mediating somatic sensations); and nuclei of the lateral lemniscus, superior olive and inferior colliculus (suggesting a role in acoustic information processing).

Moreover, pronociceptin/orphanin FQ mRNA and peptides are expressed in regions related to vision (lateral geniculate nucleus and superior colliculus), learning and memory (hippocampus), emotions (amygdala), autonomic control (hypothalamus), visceral function (nucleus of the solitary tract) and olfaction (olfactory bulb) [130–133]. These data indicate that the pronociceptin/orphanin FQ system plays a role in numerous systems, including nociception, modulation of the L-HPA stress axis, motivation and reward, learning and memory, gross motor control, balance and proprioception, sexual, aggressive, and investigatory behaviors, control of autonomic and physiologic functions, and integration of special sensory input.

5.5 Studies of Endogenous Opioid Peptides Using Knock-Out Mice

5.5.1 *Mice Lacking Peptide Precursors*

As described above, and in other chapters in this book, many of the different opioid peptides bind to the same receptors. One approach to learn the function of various peptides has been to examine the phenotype of mice with disruption of the gene encoding the peptide(s). In the simplest “knockout” approach the entire coding region of the opioid peptide precursor is deleted by the mutation. However, because multiple peptides are encoded by each of the opioid peptide precursors, it is not clear which of the deleted peptides contributes to the observed phenotype.

A more selective approach has been used to knockout specific peptides within the precursor; this was applied to the POMC peptide (described below). The comparison of results from mice lacking a particular peptide or an entire precursor with mice lacking a particular opioid peptide receptor is useful to help define which peptide/receptor system is functional *in vivo* in various behaviors. In some cases, the receptor knockout mice show the same phenotype as the peptide precursor knockout mice, but in other cases there are differences, suggesting that the system is more complex and that other peptides from the precursor and/or other receptor systems are involved.

The phenotype of mice lacking the various opioid peptide receptors is described in detail in another chapter in this book, and the discussion in this chapter is limited to the phenotype of mice with disruptions in the genes encoding the opioid peptide precursors (below) and in the genes encoding peptide processing enzymes (next section).

Mice lacking proenkephalin-derived peptides show normal pain threshold in the tail-flick assay, but exhibit a lower pain threshold in the hot-plate assay [134]. This discrepancy is presumably due to the role of endogenous opioids in the two systems; the tail-flick assay measures spinal analgesia whereas the hot-plate assay measures supraspinal analgesia.

Surprisingly, the proenkephalin knockout mice showed normal stress-induced analgesia [134]; it was assumed that the analgesia induced by stress (such as forced swim) was due in part to endogenous enkephalins. In addition to the lower pain threshold with the hot-plate assay, proenkephalin knockout mice show decreased horizontal locomotion, increased aggression, and increased anxiety [134–136]. The background strain of mouse influences the behavioral phenotype of the proenkephalin knockout mice, although nothing is known about the modifier genes that contribute to this effect [136]. The proenkephalin knockout mice did not show a depression-related phenotype using either the forced swim or tail suspension test [137]. Also, they are less sensitive to chemical pain and more sensitive to thermal pain. The finding that proenkephalin knockout mice do not show conditioned place preference to nicotine suggests that endogenous opioid peptides derived from proenkephalin are involved in the rewarding properties of nicotine [138].

Mice lacking prodynorphin-derived peptides have normal locomotion and sensitivity to thermal, mechanical, and chemical pain [139, 140]. They show a normal response to acute neuropathic pain stimuli but differ from wild type mice in their response to prolonged neuropathic pain [139]. A role for prodynorphin peptides in body weight regulation was previously suggested due to the localization and regulation of dynorphin, and this hypothesis is supported by the finding that prodynorphin knockout mice are skinnier (reduced fat mass) and lose more weight during fasting than wild type mice [141]. Prodynorphin knockout mice also show reduced spinal analgesia and conditioned place aversion after the administration of high doses of Δ -9-tetrahydrocannabinol, suggesting that the dynorphin system is involved with some of the physiological effects of this drug [140]. The dynorphin system also appears to be involved in the modulation of the aversive effects of nicotine [142].

Two different POMC knockouts have been generated: one mutant lacks the entire POMC coding region, [143] thus these mice are deficient in opioid as well as non-opioid POMC-derived peptides (such as MSH, ACTH, and others). Another mutant lacks only the β -endorphin-containing region of the protein; thus these mice are deficient just in the opioid peptide [144]. The mice lacking β -endorphin do not show naloxone-sensitive analgesia after swimming stress, unlike wild type mice which show a robust naloxone-sensitive analgesia after this stress [144]. This result suggests that β -endorphin contributes to stress-induced analgesia. The β -endorphin knockout mice exhibit normal behaviors in anxiety tests [144].

Although mice homozygous for the β -endorphin knockout allele consumed as much ethanol as wild type mice, mice heterozygous for the β -endorphin knockout

allele were found to consistently drink more than wild-type mice, suggesting that β -endorphin contributes to the response to ethanol [145]. Mice lacking β -endorphin were hyperphagic and obese, but the nonselective opioid antagonist naloxone produced an anorectic response in the knockout mice that is identical to the response in wild-type mice, suggesting that β -endorphin has effects on feeding that are distinct from other opioid peptides [146].

Mice with a disruption of the pronociceptin/orphanin gene show increased anxiety, elevated basal nociception and abnormal stress adaptation [147, 148]. Although these mutants have normal sensitivity to acute pain, they show stronger nociceptive responses upon chronic nociceptive stimulation [149]. This result suggests that N/OFQ contributes to endogenous pain control upon prolonged nociceptive stimulation, but not acute stimulation [149].

Endogenous N/OFQ also appears to be involved in the development of morphine tolerance; knockout mice injected with morphine (10 mg/kg/day for 3 weeks) do not develop tolerance, whereas wild type mice show profound tolerance to this dose after 10 days of treatment [150]. Mice deficient in N/OFQ also show delayed epileptogenesis, requiring a significantly greater number of kindling stimulations and a significantly longer time in electrical seizures to produce an epileptic response; this suggests that N/OFQ may play a pro-epileptogenic role [151].

5.5.2 *Mice Lacking Processing Enzymes*

In contrast to the studies on the mice lacking the opioid peptide precursors, which can give clues as to the function of the peptides, the mice lacking processing enzymes do not provide any information towards the function of individual peptides because the enzymes are involved in the production of large numbers of peptides, and not just the opioid peptides. However, analysis of mice lacking individual processing enzymes can reveal the function of each processing enzyme. This is especially important when multiple enzymes can perform the observed cleavage *in vitro* (such as PC1 and PC2, or CPE and CPD), and so analysis of the results of a disruption of one of the enzymes allows for identification of the *in vivo* function of each processing enzyme.

From the analysis of PC2 knockout mice, it is clear that this enzyme is physiologically involved in the biosynthesis of many opioid peptides [152–156]. Analysis by radioimmunoassay and/or quantitative mass spectrometry has found that mice lacking PC2 activity have substantial reductions in the levels of Dynorphin A-8, A-17, B-13, and a partial reduction in the level of α -neoendorphin [152, 155, 157].

Mice lacking PC2 activity also show substantial reductions of Met-enkephalin, Leu-enkephalin, and the enkephalin octapeptide, and a partial reduction of the enkephalin heptapeptide [155, 156]. The disruption of the PC2 gene also affects some of the POMC products, with dramatic reductions of α MSH and β -endorphin 1-27, and increases in ACTH and β -endorphin 1-31 [153–155].

A large decrease in N/OFQ was observed in the PC2-deficient mice, and increases were detected in processing intermediates [153, 155]. In contrast to the numerous studies reporting changes in opioid peptide levels in PC2-deficient mice, there are

no reports of altered opioid peptide processing in mice lacking PC1 activity. However, the processing of non-opioid neuropeptides is affected in PC1 null mice [158–164]. Studies co-expressing PC1 and opioid peptide precursors in cell lines, or incubating these precursors with purified PC1 show that this enzyme can process opioid peptide precursors [165, 166]. Taken together, these studies demonstrate that PC2 plays an important role in the biosynthesis of many opioid peptides, as well as many other neuropeptides, and that PC1 also contributes to peptide processing.

The major CP in the processing of the bioactive opioid peptides is CPE [68, 167]. Analysis of *Cpe^{fat/fat}* mice, which lack CPE activity, has shown a >70% decrease in the mature forms of Met- and Leu-enkephalin, octapeptide, metorphamide, BAM 18, dynorphin A-8, dynorphin A-17, dynorphin B-13, both α - and β -neoendorphin, N/OFQ, and several forms of nocistatin [68, 167].

The only bioactive opioid peptide detected in the analysis of *Cpe^{fat/fat}* mice that was not substantially affected by the absence of CPE activity was the enkephalin heptapeptide, but because this peptide is located on the C-terminus of proenkephalin, it does not require any CP for its production. Thus, CPD plays only a minor role in the production of opioid peptides, and CPE is the key carboxypeptidase involved with the biosynthesis of the opioid peptides as well as many other neuropeptides.

5.6 Conclusions

While a lot has been learned about endogenous opioids since the discovery of Met- and Leu-enkephalin in the mid-1970s, there are still many unanswered questions. For example, the physiological roles of many of the peptides produced from the four opioid peptide precursors are not fully known. In addition to the well-studied actions on analgesia, it is likely that opioid peptides contribute to a wide variety of other systems, based on the broad behavioral defects found in mice lacking the various peptide precursors. Another exciting direction for further research is on the hemorphins, the beta-hemoglobin derived peptides mentioned in Section 5.1.1. The recent finding that alpha- and beta-hemoglobin are synthesized in neurons and processed into other bioactive peptides, such as the hemopressins, raises the possibility that additional endogenous opioid peptide ligands exist [171–175]. As with most biological systems, an initially simple concept (e.g., the search for endogenous “morphine”) has revealed a highly complex system that will take many more decades of research to fully understand.

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Chapter 6

Molecular Biology of Mu Opioid Receptors

Ying-Xian Pan and Gavril W. Pasternak

Abstract The cloning of the Mu opioid receptor has led to the identification of a large series of splice variants. The gene is complex, with two independent promoters responsible for two distinct sets of splice variants. The primary promoter, associated with exon 1, encodes the majority of the variants, while a second promoter upstream of the first is associated with exon 11 (E11). The majority of the variants generated by the exon 1 promoter involve splicing at the C-terminus of the receptor, without any changes in the transmembrane domains or binding pocket. Most of the variants produced by the E11 promoter have unusual structures with six transmembrane domains. However, both sets of variants are functionally important, as shown by the actions of opioids in their respective knockout mice, providing a new perspective on understanding complex Mu opioid actions in animals and humans.

Keywords MOR-1 • Mu Opioid receptor • Alternative splicing • Cloning • Subtypes

6.1 Introduction

The concept of specific opioid recognition sites, or receptors, goes back many decades, based in large part on the rigid structure-activity relationships [1]. Soon afterwards, the existence of opioid subtypes was proposed by Martin in his proposal of receptor dualism [2], based on the interactions between morphine and nalorphine [3, 4]. When administrated together with morphine, he suggested that low nalorphine doses antagonized morphine analgesia, mediated through M receptors, while higher nalorphine doses were analgesic, presumably through an agonist action at N receptors. He then expanded this concept a number of years later to

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propose discrete Mu (morphine) and κ (ketocyclazocine) receptors [5], which was soon followed by δ receptors [6], the recognition site for the enkephalins [7–9].

These early suggestions of multiple classes of opioid receptors pre-dated the biochemical identification of the opioid receptors in 1973 [10–13]. However, the development of the receptor binding assays soon confirmed the existence of distinct receptors for morphine (Mu), the enkephalins (δ), and the dynorphins (κ). Initially, Mu opioids were defined by their selectivity in binding studies against ligands like ^3H -dihydromorphine and subsequently ^3H -DAMGO ([D-Ala²,MePhe⁴,Gly(ol)⁵] enkephalin) and by activity in bioassays, such as the guinea pig ileum [14–16]. However, these were supplanted by the use of the cloned Mu opioid receptor (MOR-1) (see below).

Although only a single gene for each of the three classes of opioids has been identified, pharmacological studies have raised the possibility of subtypes for all of them [17]. Mu Receptor subtypes were first proposed over 30 years ago [18–20], followed soon afterwards by suggestions of subtypes of δ [21] and κ receptors [22–24]. One of the κ subtypes, κ_2 , has been suggested to consist of a dimer between DOR-1 and KOR-1 [25], but little is known about the two U50-488H-sensitive sites [23, 24] or δ receptor subtypes at the molecular level. However, it is notable that δ ligands are still able to elicit analgesia in a DOR-1 knockout (KO) animal [26].

Mu Opioid receptors play a special role as analgesic targets since they mediate the actions of the vast majority of the drugs used clinically to relieve pain in patients. Clinicians have long observed that there are marked differences in Mu opioid responses among patients, both with regards to analgesic sensitivity and side-effects. Whereas some patients may respond better to one drug, other patients may find a different one more efficacious. In other circumstances, patients unable to take one Mu opioid due to intolerable side-effects may have little problem with a different Mu opioid. Finally, patients display incomplete cross tolerance, leading to the practice of *opioid rotation* in which patients highly tolerant to one Mu opioid can regain analgesic control after switching to another Mu opioid at doses far lower than would be expected based upon the tolerance to the first drug.

Many observations have been recapitulated in preclinical studies. For example, CXBK mice respond quite poorly to morphine, but retain normal sensitivity to other Mu opioids, such as methadone, fentanyl and heroin [27–29]. In addition, studies with the antagonists naloxonazine and naloxazone suggested two subtypes of Mu opioid receptors, Mu₁ and Mu₂ [17, 19, 20, 30–33]. Actions sensitive to naloxonazine or naloxazone were designated as Mu₁ while those insensitive were classified as Mu₂.

β -Funaltrexamine is a selective Mu antagonist that blocks all Mu actions, including both Mu₁ and Mu₂. Binding studies correlated the Mu₁ site with a binding component previously not seen [18, 19, 34]. Subsequent studies then suggested another naloxonazine-sensitive site through which morphine-6 β -glucuronide (M6G) worked. The differences pharmacologically between morphine and M6G have also been described at a molecular level, raising questions about the receptors responsible for these actions. Evidence now suggests that some of these differences appear to be due to alternative splicing of the MOR-1 gene.

6.2 Cloning MOR-1

6.2.1 *The MOR-1 Clone*

The MOR-1 was first cloned in 1993 [35–37], using information obtained from the δ receptor that had been reported several months earlier using expression cloning approaches [38, 39]. The κ_1 receptor (KOR-1) [40–42] and the orphanin FQ/nociceptin receptor (ORL-1) receptors [43–47] soon followed. Isolating these additional opioid receptors were facilitated by the highly conserved sequences among opioid receptor family that allowed isolating MOR-1, KOR-1 and ORL-1 cDNA clones by using homologous cloning or degenerate probes from the DOR-1 sequence. According to the GenBank database, MOR-1 cDNA clones have been isolated from at least eleven different species ranging from non-mammalian vertebrates such as white suckerfish and frog to mammalian species such as bovine, monkey, chimpanzee, mouse, rat, and human. MOR-1 has also been predicted from over 30 species using genome databases such as NICB and Emsembl.

6.2.2 *Receptor Structure*

Having the cDNA enables predictions of the molecular structure of the receptor. Like other members of the G-protein coupled receptor (GPCR) family, hydrophobicity analysis of the protein sequences deduced from the nucleotide sequence of the MOR-1 cDNA clones predicts a seven-transmembrane helix protein. GPCRs represent one of the largest gene families in the mammalian genome [48–50]. All the opioid receptors including MOR-1, DOR-1 and KOR-1, as well as ORL-1, belong to the rhodopsin-like receptor or class A family within the A–F classification system [50–52].

The seven transmembrane helices of MOR-1 are connected by three intracellular and three extracellular loops with an extracellular N-terminus and intracellular C-terminus. The three-dimensional (3D) structure of bovine rhodopsin at the 2.8 Å resolution published in 2000 [53] has served as basic model for all opioid receptors, in which the seven-helix transmembrane motif is organized in a counterclockwise manner to generate an β -helical pocket presumably designed for opioid ligand binding. All the opioid receptors contain several conserved motifs of the rhodopsin family, such as DRY motif at the boundary between TM 3 and the second intracellular loop, xBBxxB (B: basic amino acid residue) motif in the third intracellular loop, NSxxNPxxY motif in TM 7, and cysteine residues in the carboxyl terminal tail for potential palmitoylation site.

The predicted amino acid sequence of MOR-1 is 60–69% homologous to those of other opioid receptor families including ORL-1. The high homology is seen in transmembrane and intracellular loop regions, while the diversity is primarily within the N-terminus, C-terminus, and the second and third extracellular loops.

These features help define the functional domains, such as the ligand binding pocket and the G-protein coupling domain using chimera and mutagenesis approaches [54–56]. For example, Mu/δ and Mu/κ chimeras revealed that extracellular loops 1 and 3, TM2, TM6, and TM7 were implicated in the binding of Mu-selective ligands [57–64]. Further site-directed mutagenesis in these regions identified a number of important individual residues [65–70]. Similar mutagenesis approaches have mapped the domains and residues involved in G protein coupling, Mu agonist-induced receptor phosphorylation, internalization, and desensitization [54–56]. For example, a D164Q point mutation in the DRY motif yielded a constitutively active Mu receptor in the absence of Mu agonists, supporting the role of DRY motif in G protein coupling [71].

MOR-1 is glycosylated. Several N-linked glycosylation sites (NxS/T) are deduced from the N-terminus. The calculated molecular weight of the predicted MOR-1 sequence is approximately 45 kD. When expressed in cell lines, MOR-1 revealed diffused bands with molecular weight ranging from 55 to 98 kD on SDS PAGE depending upon the origin of the cDNA clones and the cell lines. These high molecular weight MOR-1 proteins represent N-linked glycosylation forms of MOR-1 since their molecular weights were reduced to their predicted level after treatment with N-glycanase [72]. The high molecular weight N-glycosylated MOR-1 was also detected in mouse and rat brain tissues [73]. Interestingly, differential N-glycosylation was observed in several brain regions, raising the potential functional relevance of the N-glycosylation in these regions [74].

6.2.3 Phylogeny

Opioid receptors appear to be expressed only in vertebrates with no evidence for their expression in invertebrate species. So far opioid receptor genes including MOR-1 (OPRM1), DOR-1 (OPRD1), KOR-1 (OPRK1), and ORL-1 (OPRL1) have been identified in over 30 vertebrate species by molecular cloning or by bioinformatic analysis of available genomic sequence data. It has been suggested that the various classes adapted from two rounds of genome-wide duplication (paleopoloidization) in vertebrate evolution [75–77]. A model for the evolution of vertebrate opioid receptor has been proposed in which a single ancestral opioid gene (unireceptor) was duplicated into two ancestral genes in the first-round duplication: ancestral DOR-1/MOR-1 and ORL-1/KOR-1 genes. The second-round duplication further generated DOR-1, MOR-1, KOR-1, and ORL-1, respectively [78, 79]. This model was supported by sequence analysis and chromosomal localization of opioid receptors [78, 80].

Phylogenetic analysis of the predicted MOR-1 protein sequences from 27 species reveals four major clades: (1) fish, (2) amphibians, (3) birds, and (4) mammals, which mimic the evolutionary tree of life with the same species (Fig. 6.1a). Sequence alignments of MOR-1 from multiple species showed the regions with the highest homology in the seven TMs and the three internal loops, the structures

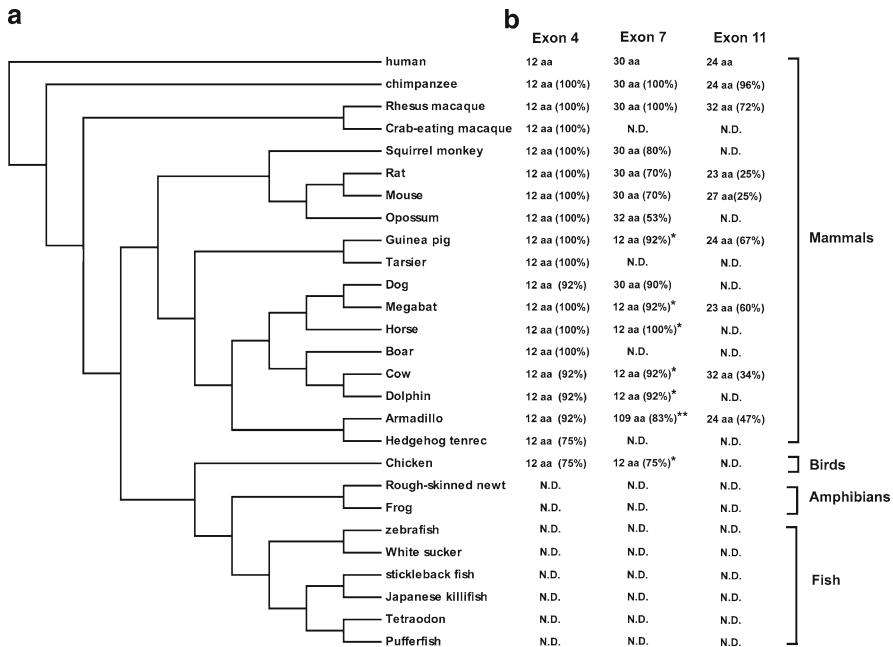


Fig. 6.1 Phylogenetic relations of the deduced MOR-1 protein sequences and comparison of amino acid sequences predicted from exons 4, 7, and 11 from 27 vertebrate OPRM1 genes. **(a)** Phylogenetic analysis of the deduced MOR-1 protein sequences. The MOR-1 sequences were downloaded from NCBI and Ensembl nucleotide and genomic databases. Phylogram was generated by the maximum parsimony (MP) method in MEGA4 [222]. **(b)** Comparison of amino acid sequences predicted from exons 4, 7, and 11. The amino acids predicted from human or mouse exons 4, 7, and 11 were blasted through the 27 vertebrate OPRM1 locus obtained from NCBI and Ensembl databases to identify the human or mouse homologs with criteria including splice junctions and exon locations. *aa* the number of amino acids were identified; % percentage of homology in prentices was calculated through comparing to human sequences; *N.D.* homologous sequences were not detected due to lack of the homologous sequences or incomplete genomic sequences in the OPRM1 locus; *single asterisk* percentage was calculated through comparing to the first 12 amino acids of the human sequences; *double asterisk* percentage was calculated through comparing the first 30 amino acids of armadillo with the 30 amino acids of the human sequences. Exon 7 was first identified in the mouse and its homolog in the human was assigned as exon O

important for Mu ligand binding and G protein coupling. The conservation of these structures during evolution further suggests that they are essential foundations for defining a MOR-1. However, the sequence alignments also revealed several regions, including the N- and C-termini and the third extracellular loops that were highly variable, providing the potential clues for how Mu opioid receptors are evolved.

The genomic structure of the OPRM1 genes also underwent evolutionarily changes [81]. In the early teleosts, the OPRM1 gene was composed of five exons in which the first two exons encoded TM1/TM2/TM3/TM4, a structure that has been conserved throughout evolution. However, starting in zebrafish during evolution

and extending to mammals, the two introns between the last three exons were lost, generating a single third exon that encodes the last three TM domains. This three-exon structure responsible for encoding all seven transmembrane domains is conserved in other opioid receptor genes including OPRD1, OPRK1, and OPRL1. Yet, only the OPRM1 gene, and not any other opioid receptor genes, further developed with emergence of additional exons in 3' and/or 5'-ends of the gene, starting with the chicken (Fig. 6.1b).

The appearance of these additional exons is concurrent not only with generation of multiple alternatively spliced MOR-1 variants (see below), but also with the development of opioid dependence, tolerance and addiction particularly in mammalian species. The major 5'-exon was exon 11 (E11) that was initially identified in the mouse OPRM1 gene, and later, found in an additional eight mammalian OPRM1 genes by homologous cloning or bioinformatics searches (Fig. 6.1b). Multiple alternatively spliced variants associated with E11 have been isolated from the mouse, rat, and human OPRM1 genes [82–84] (see below). The functional significance of these E11-associated variants has been demonstrated in our E11 KO mouse model in which M6G, fentanyl and heroin analgesia was greatly diminished, with normal morphine and methadone analgesia [85] (see below).

On the other hand, there were ten 3'-exons located downstream of exon 3 in the mouse, nine 3'-exons in the rat and six 3'-exons in humans (Figs. 6.3a, 6.4a, and 6.55a). Splicing from exons 1/2/3 to these 3'-exons produced multiple C-terminal variants which all share the exact receptor structure with the exception only at the very tip of the C-terminal tail in which the last 12 amino acids from exon 4 are replaced different sets of 3'-exons (Fig. 6.2) (see below). The most dominant 3' exon is exon 4 which encodes 12 amino acids located in the C-terminal tail of MOR-1 in the 19 species (Fig. 6.1b). The identical 12 amino acid sequence is present in at least 13 mammalian species, while 4 species have only one amino acid variation and 2 species have three variations.

Exon 7 was first identified in the mouse MOR-1C cDNA clone, with an additional eight splice variants subsequently identified in the mouse OPRM1 gene, including mMOR-1E, mMOR-1F, mMOR-1M, mMOR-1O, mMOR-1Q, mMOR-1U, mMOR-1V, and mMOR-1W [82, 86–89]. Of nine exon 7-associated variants, exon 7 is a coding exon in only four variants, mMOR-1C, mMOR-1M, mMOR-1O, and mMOR-1U, while exon 7 functions as 3'-untranslated region (UTR) in the other five variants due to early termination of translation in their upstream exons. The exon 7-associated splice variants are also present in the rat and human OPRM1 genes while exon 7 has been identified in an additional twelve mammalian OPRM1 genes through sequence alignments (Fig. 6.1b). Interestingly, there is an overlapping of 70 bp of the exon 7 sequence with exons in the interaction protein for cytohesin exchange factors 1 (IPCEF1) gene in mice, rats and humans in the opposite orientation [90]. This overlap is conserved in all the species containing exon 7. The predicted amino acid sequences from exon 7 also shares high homology among the species (Fig. 6.1b).

Receptor binding studies further illustrate the evolution of Mu opioid receptors. For example, MOR-1 clones from non-mammalian species including white suckerfish,

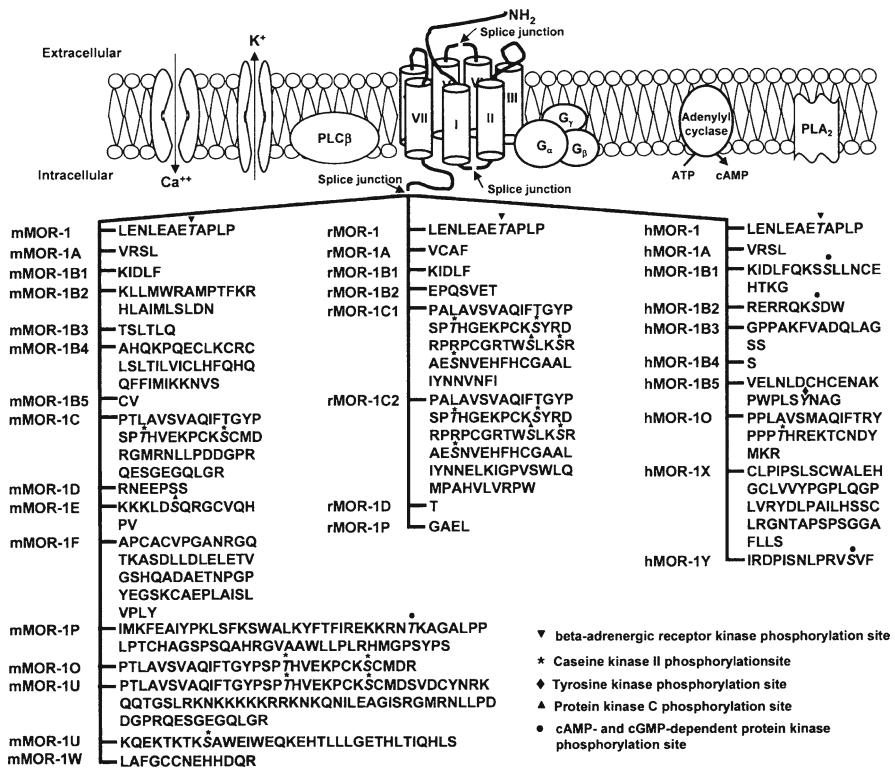


Fig. 6.2 Schematic of the OPRM1 carboxyl terminal splice variants' structures and amino acid sequences predicted from downstream exons of exon 3. The *top panel* displays the common structure of all the carboxyl terminal splice variants and adjacent proteins potentially coupled with the variants on plasma membranes. The seven transmembrane domains are showed by cylinders with Roman numbers. Splice junctions between exons are indicated by arrows. Ca⁺⁺ and K⁺ channels are displayed by opened channels across plasma membrane. PLA₂, phospholipase A₂; PLC_β, phospholipase C_β; G_α, G_β, and G_γ, G proteins. The *bottom panel* shows the amino acids deduced from the alternative exons downstream of exon 3 for mouse (mMOR), rat (rMOR), and human (hMOR) variants

frog, and rough-skinned newt had high affinities for [³H]naloxone, when expressed in mammalian cells [91, 92]. Although competition studies clearly show their Mu selectivity, they displayed lower affinities for Mu agonists such as DAMGO, as well in the inhibition of forskolin-stimulated cAMP accumulation and coupling to a G protein gated inward-rectifying potassium channel (GIRK1) [93]. With the evolution of MOR-1, the affinity of DAMGO for the receptor has increased even though the main structures responsible for the binding pocket, including the seven transmembrane domains, the three intracellular loops, and the first and second extracellular loops, are highly conserved throughout the evolution. More surprisingly, all the residues crucial for either Mu agonist or antagonist binding as determined by mutagenesis studies, such as D114 in TM2 [94–96], D147 in TM3 [97], and

W318 in EL3 [70, 98, 99], are identical among these species. These raise intriguing questions regarding the role of the nonconserved regions or residues on the affinity of agonists such as DAMGO. One obvious difference between non-mammalian and mammalian MOR-1 sequences is the lack of the last 12 amino acids in the C-terminal tails encoded by exon 4 in the non-mammalian species. It will be extremely interesting to investigate if these 12 amino acids, or the other C-terminal tails that have evolved in the mammalian species, would confer the higher affinity of DAMGO in these non-mammalian MOR-1 proteins seen in the mammalian MOR-1 clones.

6.2.4 Binding Studies

The cloning of MOR-1 provided an opportunity to characterize its binding in cell lines lacking opioid receptors, eliminating the ambiguity seen when looking at binding in the brain. When transiently or stably expressed in cell lines such as HEK 293 (Human Embryonic Kidney 293), COS-1 or COS-7 (African green monkey kidney fibroblast-like cell), and CHO (Chinese hamster ovary cell), the cloned MOR-1 from rat [35–37, 100], human [101–103] and mouse [86, 104] all displayed high affinity towards Mu-selective ligands such as DAMGO and morphine, and had much lower affinity to the δ -selective ligand, DPDPE, and κ -selective ligands, U50,488 and U69,593, establishing that they belonged to a Mu subtype of opioid receptor family.

Correlating the cloned receptor with the pharmacologically defined Mu_1 and Mu_2 subtypes is unclear. Based upon the high selectivity of MOR-1 for Mu ligands, it is likely to correspond to the Mu_2 subtype. However, the cloning of dozens of splice variants of MOR-1 has made any of these comparisons quite difficult. Furthermore, the subtle, but present differences in binding profiles in different cell lines make the comparisons even more ambiguous. In the decade following the initial cloning of the first opioid receptor, multiple alternatively spliced variants of the MOR-1 genes have been identified (see below), and homodimerization or heterooligomerization of MOR-1 with other members of opioid receptor family have been demonstrated [105–107]. It will be very interesting to explore the possibility that interactions of MOR-1 with other MOR-1 splice variants or with other opioid receptors contributes to the Mu_1 and Mu_2 subtypes.

6.2.5 Regional Expression of MOR-1

6.2.5.1 Northern Blot Analysis and RT-PCR

Soon after MOR-1 was cloned, expression of MOR-1 mRNA was determined by Northern blot analysis, revealing a major band of approximately 11–16 kb in mice

[82, 104], rats [36, 108] and humans [102, 103]. The coding region of MOR-1 only covers ~1.2 kb. Thus, the large size of the MOR-1 transcripts is composed primarily of 5' and/or 3' UTR. It is now confirmed that exon 4 of both mouse and human contained a poly(A) site and was expressed as a 10–13 kb large exon [109, 110], which contributed to the major transcript seen in Northern blots.

Northern blot analysis also revealed several bands in smaller sizes in mouse [82], rat [36], and human brains [82, 102], suggesting multiple transcripts from alternative splicing of the MOR-1 gene in these species (see below). Relative quantification of MOR-1 mRNA in the selected rat brain regions using ribonuclease protection assays [37] showed that the highest level of MOR-1 mRNA was seen in the thalamus and the lowest in the cerebellum, with moderate levels in the hypothalamus, brainstem, and spinal cord, results supported by solution hybridization analysis with more quantitative and reproducible measurements [111, 112].

6.2.5.2 In Situ Hybridization

Shortly after the cloning of MOR-1, the anatomical distribution of MOR-1 mRNA in the rat central nervous system was mapped by several labs using in situ hybridization [108, 113, 114]. Generally, the MOR-1 mRNA was widely distributed in brain and correlated with the distribution Mu opioid receptors previously defined. For example, MOR-1 mRNA was present in the periaqueductal gray (PAG), the locus coeruleus, and raphe magnus, all of which have been suggested to involve supraspinal analgesia of Mu agonists. MOR-1 mRNA was also observed in the somatosensory regions associated with pain perception, such as the dorsal root ganglia (DRG), the spinal trigeminal nucleus, dorsal horn of the spinal cord and the thalamus. One of the major side-effects of Mu agonists, respiratory depression, and regions associated with this action also express MOR-1 mRNA, including the nucleus of the solitary tract, the nucleus ambiguus, the parabrachial nucleus and the bed nucleus.

The distribution of MOR-1 mRNA mapped by in situ hybridization studies correlated with earlier autoradiography studies [115–118]. However, there were several mismatches. For example, MOR-1 mRNA was absent or at very low levels of expression in the cerebral cortex, in contrast to the higher level of the Mu opioid binding in the same regions. Similarly, there was no detectable MOR-1 mRNA in the dorsal raphe nucleus corresponding to the Mu opioid binding. The simplest explanation is that the receptors in these regions are presynaptic and localized on terminals far from the cell bodies.

Similar distributions of MOR-1 mRNA in the mouse brain were reported [104]. Human studies are more limited. However, in situ studies by Peckys and Landwehrmeyer revealed expression patterns of MOR-1 mRNA in most regions of the human central nervous system similar to those in the rat [119]. Interestingly, there were some discrepancies, such as the stronger labeling in human cortex and hippocampus than in the rat. On the other hand, the rat brainstem nuclei were labeled more intensely than the human.

6.2.5.3 Immunohistochemical Studies

To determine the distribution of MOR-1 at the protein level, many labs developed antibodies against various regions of MOR-1 including the N-terminus, extracellular loops and C-terminus. However, most antibodies against the N-terminus and extra-cellular loops proved to be ineffective in immunohistochemical studies, possibly due to the rigid N-terminal and extracellular structures or glycosylation at the N-terminus. Several antibodies against the C-terminus yielded specific and consistent labeling [120–123]. The anatomical distribution of the immunoreactivity was generally in agreement with the distribution of the Mu opioid binding identified autoradiographically, suggesting that these C-terminal antibodies represented the majority of the Mu opioid binding sites [120, 121, 123].

However, there were some discrepancies. In the cerebral cortex, the lateral and basolateral nuclei of the amygdala, and the medial geniculate nucleus of the thalamus, high levels of Mu opioid binding were observed and little or no immunoreactivity. This could be explained by the existence of MOR-1 isoforms or subtypes that are not recognized by the C-terminal antibodies, which recognizes variants expressing exon 4. It is important to note that all the full-length splice variants label opioid radiolabels as potently as MOR-1 itself. Conversely, several regions showing no Mu opioid binding still displayed MOR-1-like immunoreactivity (MOR-1-LI), including the dentate gyrus of the hippocampus, stratum oriens and radiatum of Ammon's horn, some pretectal nuclei, and the accessory facial nucleus [121, 123]. These variations may reflect differences in sensitivity between the two approaches or the antibodies may be recognizing nonfunctional receptors.

The distribution of the MOR-1-LI mapped through the C-terminal antibodies correlated very well with the distribution of MOR-1 mRNA determined by *in situ* hybridization in most brain regions [118, 120, 122, 123]. For example, similar to the MOR-1 mRNA, the MOR-1-LI was poorly detected in the cerebral cortex, and the lateral and basolateral nuclei of the amygdala, but showed higher expression in the hypothalamus and the medial preoptic area. Yet, there were mismatches. An example is the spinal cord where both MOR-1-LI and Mu opioid binding are present in the superficial layers I and II of the dorsal spinal cord whereas MOR-1 mRNA was hardly observed [120, 121, 123]. The most likely explanation is that much of the binding is presynaptic from neurons from the DRG or descending from the brain. Early studies had already documented that transection of the proximal root of the dorsal root ganglion lowered binding in the dorsal horn of the spinal cord.

Conversely, MOR-1 mRNA was observed in many neurons in the dorsal and ventral horns where MOR-1-LI was barely detectable. Again, these mismatches may reflect the transport of the receptors to the terminals in different regions or the existence of Mu isoforms detectable by *in situ* hybridization, but possessing different C-termini not recognized by the exon 4-based antibodies.

Overall, these studies provided the anatomical distribution of the cloned MOR-1 at both mRNA and protein levels with better resolution and more specificity

than those seen autoradiographically, where cross labeling of related, but different, binding sites was always a concern. While the specificity of in situ hybridization is very well established, there is always the question with immunohistochemistry of whether or not the antibody may also recognize other, unrelated epitopes. Thus, despite these advances, there are still questions that remain to be resolved.

6.2.6 *Developmental Expression of MOR-1*

Mu Binding was first detected in the mouse at embryonic day 12.5 (E12.5) using whole embryo homogenates [124]. The cloning of MOR-1 offered more sensitive probes to establish the spatial and temporal expression of the MOR-1. Using a ^{33}P -labeled MOR-1 probe for in situ hybridization studies, the expression of MOR-1 mRNA was observed in the facial-vestibulocochlear preganglion complex as early as E10.5, 1 day later than the expression of KOR-1 mRNA in the gut epithelium (E9.5) and 2 days earlier than the expression of DOR-1 mRNA in peripheral tissues (E12.5) [125]. By E17.5, the distribution of MOR-1 mRNA in most regions resembled that in adult brain [125]. Paralleling the MOR-1 mRNA expression, Mu agonist-induced G protein activation, determined by ^{35}S -GTP γ S binding autoradiography, was observed in the caudate-putamen as early as E12.5. The temporal and spatial expression patterns of MOR-1 mRNA during ontogenesis also suggested the involvement of Mu opioid receptors in early stages of neurogenesis [126]. Interestingly, MOR-1 mRNA appeared in the spinal cord (E11.5) earlier than in the DRG (E13.5) while DOR-1 mRNA was detected earlier in the DRG (E12.5) than in the spinal cord (E15.5). KOR-1 mRNA appeared later than the others (E15.5 in the spinal cord and E17.5 in the DRG) [125].

Clearly, MOR-1 mRNA was detected earlier by in situ hybridization (E10.5) as compared than receptor binding (E12.5), illustrating the different sensitivities of the approaches. Using more sensitive approaches such as RT-PCR, MOR-1 mRNA was detected at even early embryonic stages, E8.5 or E9.5 [127, 128]. However, immunohistochemical approaches to examine MOR-1 expression at the protein level during ontogeny have not been systematically carried out.

In situ hybridization studies in the rat revealed the expression of both MOR-1 and KOR-1 mRNAs as early as E13 [129]. Since no earlier stages of embryos were used in this study, it is not clear whether or not both mRNAs were present even earlier. Interestingly, DOR-1 mRNA was detected much later at E21. MOR-1 mRNA was initially observed in the striatal neuroepithelium and cortical plate at E13. At later stages (E14–E19), the MOR-1 mRNA in striatal anlage was homogeneously labeled. By E20–E21, the homogeneous distribution of the MOR-1 mRNA became patchy, which was coincident with the development of striatal compartmentation [129].

6.3 MOR-1 (OPRM1) Gene

6.3.1 Chromosomal Mapping and Overall Structure

With the cloning of MOR-1, its gene was quickly mapped in both mice and humans. Using *in situ* hybridization, the human Mu opioid receptor gene (OPRM1) was mapped to 6q24-25 [130] while the mouse OPRM1 gene was allocated to proximal chromosome 10, a region containing a human counterpart of chromosome 6q, by linkage analysis using different interspecific backcrosses [104, 131, 132]. Interestingly, quantitative trait locus (QTL) in mice have also mapped morphine analgesia, hypothermia and consumption to the same location in proximal chromosome 10, supporting the association of these functional traits with the OPRM1 gene [133, 134].

Subsequent sequence analysis of the genome databases from both public and private sources confirmed the chromosomal locations of the OPRM1 gene from the previous studies, but also revealed their precise chromosomal location. The mouse OPRM1 gene is located at 3.27–3.61 Mb of chromosome 10, and the human OPRM1 gene, at 154.3–154.7 Mb of chromosome 6. The sequence analysis of the available genome databases shows the chromosomal location of the OPRM1 gene in over 20 different species.

The chromosomal mapping studies and the sequence analyses reveal a single chromosomal locus, suggesting that a single copy of the OPRM1 gene is conserved across species. Similarly, single gene copies exist for the other opioid receptor families including DOR-1 (OPRD1), KOR-1 (OPRK1) and ORL-1 (OPRL1). Although the single OPRM1 gene is located in different chromosomes in various species, their adjacent gene loci are quite conserved among mammalian species. For example, synteny analysis indicated that in mammalian species, the OPRM1 gene locus is flanked by a regulator of G protein signaling 17 (RGS17) gene and subunit 5 of the splicing factor 3b (SF3b5) gene with similar distances at the 5' and 3' regions, respectively [81]. As mentioned before, the IPCEF-1 gene overlaps with the OPRM1 gene at the 3' ends with opposite orientations in several mammalian species [90], raising the question how these two genes are regulated during transcription.

The gene structure of the OPRM1 genes was initially defined by four coding exons and three introns based upon MOR-1 cDNA sequences. The isolation of multiple additional exons further extended the OPRM1 genes at both 5' and 3' regions. For example, the mouse OPRM1 gene now contains 19 exons spanning over 270 kb. Similarly, the human OPRM1 gene covers a region over 210 kb including 12 exons.

6.3.2 Promoters

6.3.2.1 Exon 1 Promoter

Shortly after MOR-1 was cloned, several groups characterized the promoter controlling expression of MOR-1 mRNA from immediately upstream of exon 1 (E1) in the mouse, rat, and human OPRM1 genes using genomic clones containing the

5'-flanking region of E1 [135–139]. This E1 promoter, located to a ~1.5 kb region upstream of E1, is conserved from rodent to human at the nucleotide level and contains two active regions, leading to a dual-promoter model in both mouse and human [140–142]. In this model, the proximal and distal regions of the promoter are separated by approximately 500 bp. The activity of the proximal promoter was much stronger than that of the distal promoter, as determined by *in vitro* reporter assays and RT-PCR [127, 141, 142]. Multiple transcriptional start points have been identified in the E1 promoters through ribonuclease protection assays and RT-PCR [135, 137, 138]. The E1 promoter lacks a TATA box, but has cis-acting elements with high GC content such as Sp1 and Ap1. Thus, the E1 promoter exhibits many characteristics of a “housekeeping” gene.

Of the several mammalian E1 promoters that have been examined, the mouse E1 promoter has been most extensively studied. Various cis-acting elements, such as the Sp binding element, NF- κ B, CRE, Oct-1, IL-4, Sox, STAT6 and NRSE been identified in the proximal or distal regions [143–149]. The interactions of these cis-acting elements with their corresponding trans-acting factors regulate E1 promoter activity. For example, the neuron-restrictive silencer factor (NRSF) binds to a 21 bp cis-acting NRSE element in the proximal promoter region to suppress the promoter activity in non-neuronal cells [144]. A cis-acting element can interact with different trans-acting factors to produce opposing effects. For example, the binding of Sp1 and Sp3 to a 10 bp Sp binding element in the proximal promoter stimulated promoter activity. However, the same Sp binding element interacted with the M1 and M2 isoforms of Sp3 to suppress the promoter activity [150]. Several trans-acting factors were identified with a DNA affinity purification coupled with MALDI-TOF mass spectrometric approach. For example, using a 24 bp poly(C) fragment as bait, Choi et al. isolated two proteins, α CP3 and poly(ADP-ribose) polymerase-1 (PARP-1), that acted as repressors of the E1 promoter when bound to the poly(C) sequence [151, 152].

Cytokines released from immune cells, such as the interleukins (IL), tumor necrosis factor (TNF), and interferon (IFN)- γ , induce or modulate MOR-1 expression in both immune cells and neural cells [153]. The cytokines induce expression of certain trans-acting factors that in turn trans-activate the E1 promoter through their corresponding cis-acting elements. For example, IL-4 induced de novo synthesis of MOR-1 transcripts in a number of immune cells, such as primary and Jurkat T lymphocytes, Raji B lymphocytes, primary polymorphonuclear leukocytes, and dendritic cells, through the interaction of a STAT6 cis-acting element with the induced STAT6 factor [154]. In another example, TNF induced nuclear factor κ B (NF- κ B) which, in turn, interacted with three NF- κ B binding sites in the human E1 promoter, accounting for the TNF-induced de novo synthesis of MOR-1 transcripts in a number of immune cells [145].

Epigenetic control of the mouse MOR-1 gene has recently been explored. The level and pattern of DNA methylation and histone modification in the E1 promoter region account for silencing and activating the gene in undifferentiated and differentiated P19 cells, respectively [155], as well as the unique spatial expression of MOR-1 mRNA in some brain regions [156]. For example, the E1 promoter in the cerebellum was hypermethylated, consistent its lower mRNA there. Specifically,

methyl-CpG-binding protein 2 (MeCP2) plays an important role in epigenetic control of MOR-1 expression through its association with other chromatin remodeling factors including Brg1 and Dnmt1 [156].

6.3.2.2 Exon 11 Promoter

Soon after the identification of eight E11-associated variants from the mouse OPRM1 gene (see below), we identified the E11 promoter which is located in the 5' UTR of E11 approximately 30 kb upstream of E1 [157]. The E11 promoter was characterized in several cell lines using a 6 kb genomic fragment containing E11 constructed with a secreted form of human placental alkaline phosphatase (SEAP) reporter and found to be more active in neuronal cells such as NIE-115, a mouse neuroblastoma cell line, and SHSY-5Y, a human neuroblastoma cell line, than in CHO cells. Both 5' and 3' deletion assays further defined a basal core region, a negative region and a positive region. The basal core region contains a putative TATA box that was confirmed by an *in vitro* gel shifting assay with a recombinant TATA-binding protein (TBP). Functional mutation analysis suggested that the TATA box was crucial for E11 promoter activity. Several other cis-acting elements, including NF-1 and cMyc/Max sites near the TATA box, interact with their trans-acting factors and play a modulatory role in E11 promoter activity. A basic core model of E11 promoter has been hypothesized, in which a pre-initiation complex was assembled through binding TBP to the TATA box and subsequent recruiting other general transcription factors to the basal core region, while the cMyc-Max-like and NF-1-like proteins further regulate the assembling of the transcription complex and initiation [157]. The E11-associated splice variants and the E11 promoter have also been identified in the rat and human OPRM1 genes (unpublished observation) [83, 84], suggesting conservation of the E11 promoter and its associated variants during evolution.

The E11 promoter clearly differs from the E1 promoter in the following aspects. First, the E11 promoter contains a TATA box, whereas the E1 promoter does not. Second, the E11 promoter contains a single major transcription start point, contrasting with the multiple transcription start points in the E1 promoter. Third, the E1 promoter has several GC-rich cis-acting elements like Sp1 and AP-2 that are absent in the E11 promoter. These GC-rich cis-acting elements are involved in transcriptional regulation of TATA-less promoters. Therefore, the E11 promoter favors a eukaryote class II promoter mode associated with RNA polymerase II, while E1 promoter mimics a “house-keeping” gene mode. Lastly, each promoter controls the expression of a different set of splice variants (see below). The E1 promoter directs transcription of over sixteen variants in the mouse, while E11 promoter drives the expression of nine other variant transcripts. Intriguingly, three E11-associated variants generate the same MOR-1 protein that is also transcribed by the E1 promoter. The OPRM1 gene now has two distinct promoters that are apart from each other ~30 kb, raising questions of “when,” “where,” and “how” these two promoters are activated and regulated.

The temporal expression of E11 mRNA transcribed through the E11 promoter in C57BL/6J mice during ontogeny was studied using a semi-quantitative

RT-PCR approach [128]. Expression of the E11 transcript began at embryonic day 13.5 (E13.5), 4 days later than that of E1 promoter-driven transcript (E9.5). The different onset of the exons 11 and 1 promoters suggested differential regulation and function of these two promoters during mouse embryonic development. The spatial expression of the exons 11 and 1 promoters was explored by using a transgenic mouse model [128]. The transgenic mouse model was generated through a ~20 kb transgenic construct, in which a 3.7 kb E11 promoter and a 8.9 kb E1 promoter drove expression of tau/LacZ and tau/GFP reporters, respectively. Although both exons 11 and 1 promoter activities as revealed by X-gal staining and GFP imaging, respectively, were widely distributed in the central nervous system, their differential expression was clearly observed in some brain regions such as the hippocampus and substantia nigra, suggesting region-specific promoter regulation [128].

6.3.3 Single Nucleotide Polymorphisms

The development of many diseases and various responses to pathogens, chemicals, and drugs in human are related to genetic variations. Single nucleotide polymorphisms (SNP) are the most common genetic variation, with an estimated ten million SNPs in the human genome. More than 1,000 SNPs have been identified in the human MOR-1 gene based upon the International HapMap Project database [158]. Serving as the primary target for most clinical opioids, the Mu opioid receptor (OPRM1) gene has been considered as one of the most prominent candidate genes contributing to vulnerability of opioid addiction, abuse and dependence [159–163]. A number of SNPs in the human MOR-1 gene have been associated with vulnerability to dependence to opioids [164–167] and other substances of abuse, including alcohol [166, 168–170] and nicotine [171, 172]. Among these SNPs, the most extensively studies is the A118G SNP, which some believe is associated with heroin addiction. Functionally, this A118G SNP was reported to increase the affinity of MOR-1 for β-endorphin [173, 174] or lower the expression of MOR-1, probably through altering the secondary mRNA structure [175].

Recently, a mouse model carrying an A112G mutation mimicking the human A118G SNP was generated to study the potential function of the human SNP in mouse [176]. The A112G mouse displayed a lower level of mMOR-1 mRNA and protein expression, reduced morphine analgesia, and sex-specific reductions in the morphine-induced rewarding properties.

Two SNPs, R265H and S268P, significantly alter basal G protein coupling and calmodulin binding and Mu agonist-induced receptor signaling [107, 177, 178]. Several SNPs in the E1 promoter region influence promoter activity [149, 167, 179]. For example, one SNP weakened the promoter activity by altering a STAT6 binding site [149]. Interestingly, a A to C polymorphism in the E1 promoter in CXBK mice is responsible for decreased MOR-1 transcripts through lowering Sp1 binding, suggesting a potential molecular explanation for the reduced morphine analgesia phenotype in CXBK mice [180].

6.4 Alternative Splicing of the OPRM1 Genes

Pharmacological studies had long suggested subtypes of Mu opioid receptors, which was hard to reconcile with a single-copy of the OPRM1 gene. One hypothesis is that alternative splicing creates MOR-1 diversity at mRNA and protein levels, leading to the effort over the past few years to isolate potential OPRM1 splice variants. We now know that the OPRM1 gene undergoes extensive splicing, as demonstrated by the identification of multiple splice variants from the OPRM1 gene in various species. Conservation of alternative splicing in OPRM1 gene from rodent to human suggests that alternative splicing plays an important role not only in evolution but also in the function and regulation of the OPRM1 gene.

Within the opioid receptor family, only the OPRM1 gene shows extensive and highly conserved alternative splicing. Indeed, the complexity of the splicing far exceeds the suggestions raised by the earlier pharmacological studies. The functional importance of these alternatively spliced variants has been suggested by differences in their region-specific and cell-specific expressions, agonist-induced G protein coupling and receptor phosphorylation and internalization, as well as *in vivo* functions mediating the actions of various Mu opioids.

6.4.1 *The Mouse OPRM1 Gene*

Although the first two splice variants, hMOR-1A and rMOR-1B, were initially isolated from the human [181] and rat OPRM1 genes [182], respectively, extensive alternative splicing has been mostly extensively documented in mice and confirmed in rats and humans [90].

6.4.1.1 Full Length Variants (C-Terminal Variants)

The mouse OPRM1 gene undergoes extensive 3' splicing, which mainly involves splicing from exon 3 to different sets of downstream exon to generate a series of full length splice variants (Figs. 6.2 and 6.3). These full-length variants were isolated primarily using a modified 3' RACE strategy coupled with a nested PCR approach. These variants include mMOR-1A (homologous to hMOR-1A), mMOR-1B1 (homologous to rMOR-1B), mMOR-1B2, mMOR-1B3, mMOR-1B4, mMOR-1B5, mMOR-1C, mMOR-1D, mMOR-1E, mMOR-1F, mMOR-1O, mMOR-1P, mMOR-1U, mMOR-1V, and mMOR-1W [86–90, 183, 184]. All these variants contain exons 1, 2, and 3, but alternative splicing from exon 3 to different downstream exon generated the variations of the transcripts at the 3' ends. Thus, all these variants have the same protein structure predicted from exons 1–3, which encodes the N-terminus, all seven transmembrane (TM) domains, both extracellular and intracellular loops, and much of the intracellular

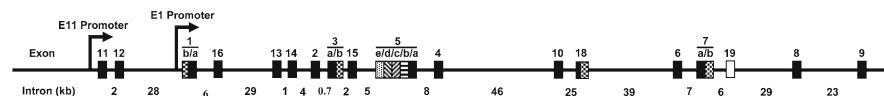
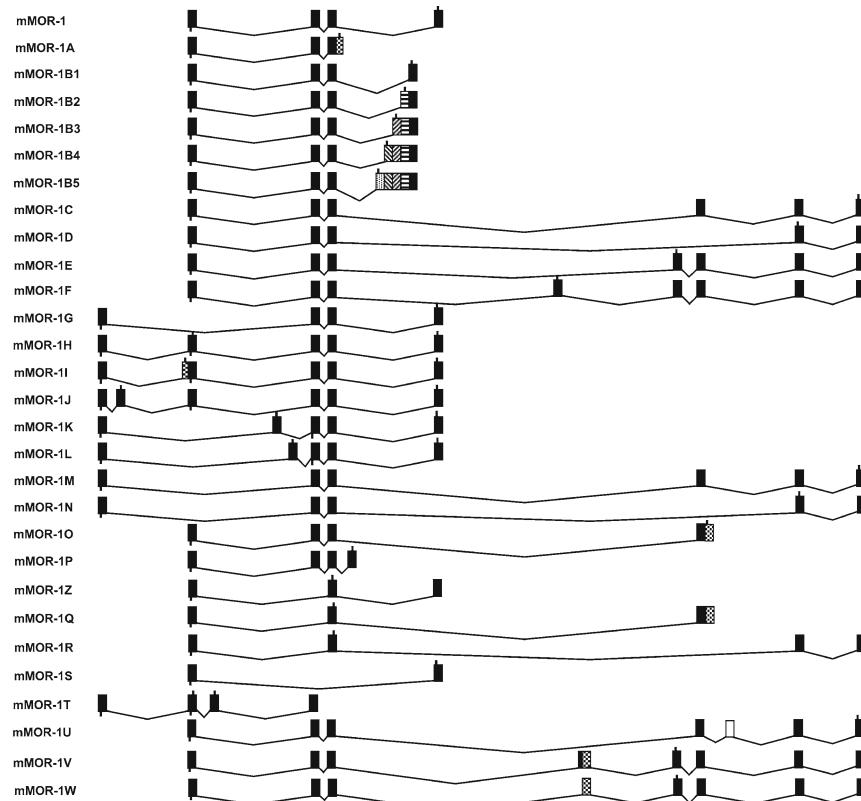
a Genomic structure**b Alternatively spliced variants**

Fig. 6.3 Schematic of the mouse OPRM1 gene structure and alternative splicing. (a) Genomic structure of the mouse OPRM1 gene. Exons and introns are indicated by boxes and horizontal lines, respectively. Intron size is indicated below the introns as kilobases (kb). Promoters are shown by arrows. Exons are numbered based upon the published data. (b) Alternatively spliced variants of the mouse OPRM1 gene. Exon composition for each alternatively spliced variant was indicated by appropriate exon boxes. The lines between exons are introns that are spliced out during splicing. Translation start and stop points are shown by bars below and above exon boxes, respectively

carboxyl terminus. Thus, it would be expected that they each would have identical binding pockets for the ligand. The differences were restricted to the tips of the C-terminus (Fig. 6.2), leading to their designation as C-terminal splice variants. To date, a total of fifteen C-terminal splice variants have been isolated from the mouse OPRM1 gene.

The deduced amino acids from the C-terminal tails encoded by different combinations of exons downstream from exon 3 all were distinct and varied widely in length (Fig. 6.2), ranging from only two amino acids in mMOR-1B5 to 88 in mMOR-1U. Some of these C-terminal tails contain potential phosphorylation sites for protein kinase C, cAMP- and cGMP-dependent protein kinase and Caseine kinase II. The functional significance of these C-terminal tails has been suggested in Mu agonist-induced receptor phosphorylation, internalization, post-endocytic sorting, and G protein coupling (see below).

The 3' splicing involved splicing patterns that included intron-retention, exon inclusion/skipping, and alternative 3' splicing (Fig. 6.3). For example, mMOR-1A and mMOR-1O were intron-retention variants, in which the donor splice sites at exons 3a and 7a were silent, respectively [90]. Therefore, introns downstream of exons 3a and 7a were retained and now defined as exons 3b and 7b, respectively. Splicing at exon 5 was another example of alternative 3' splicing, with five alternative splice sites within exon 5. Splicing from exon 3 to these alternative sites generated five C-terminal variants, including mMOR-1B1, mMOR-1B2, mMOR-1B3, mMOR-1B4, and mMOR-1B5 [183]. Both mMOR-1V and mMOR-1W were also generated through alternative 3' splicing within exon 18 [88]. Even though many of the splice variants share the same spliced exons, their amino acid sequences were all unique due to frame shifts and early termination.

6.4.1.2 Truncated Variants (Single TM and Six TM Variants)

In addition to the 3' splicing that generates a series of C-terminal variants, the mouse OPRM1 gene also undergoes extensive 5' splicing, primarily involving splicing from E11 to downstream exons [82, 90]. Initially, E11 was isolated through a modified 5' RACE approach with primers located in exon 2. Further RT-PCRs identified additional three exons: exons 12, 13, and 14, and nine E11-associated splice variants, all of which had E11 as the first coding exon. Exons 11 and 12 were located at ~30 kb and ~28 kb upstream of E1, respectively, whereas exons 13 and 14 were mapped at ~35 kb and ~36 kb downstream of E1, respectively (Fig. 6.3).

Of nine E11 splice variants, three variants, mMOR-1G, mMOR-1M, and mMOR-1N, had the identical splicing pattern at the 5'-end, involving direct splicing from E11 to exon 2, skipping E1 [82, 90]. Since exons 2 and 3 encode only six TM domains (TM2–TM7) and E11 did not predict any TM domain, all three variants predicted a protein structure containing six TM domains, but with different C-termini encoded by exon 4 in mMOR-1G, by exons 7/8/9 in mMOR-1M, or by exons 8/9 in mMOR-1N, respectively (Fig. 6.3).

Western blots with an E11-specific antibody suggested that these variants were expressed in mouse brain [82]. Two other E11-associated variants, mMOR-1K and mMOR-1L, also excluded E1. However, a small exon (exon 13 in mMOR-1K and exon 14 in mMOR-1L) was inserted between exons 11 and 2. Translation from the E11 AUG predicted short peptides (<10 kD) due to early translation termination within exon 13 and 14, respectively. On the other hand, there was a methionine residue

predicted at the beginning of the exon 2. Translation from this methionine also predicted a protein with six TM domains, which was identical to that of mMOR-1G except for lack of the E11 coding sequence. Thus, five different E11-associated variants predict proteins with six TM.

Three variants, mMOR-1H, mMOR-1I, and mMOR-1J, contained the same coding exons 1, 2, 3, and 4 as the original mMOR-1 in addition to their upstream exons [82]. Translation from the E1 AUG in these three variants generated a protein identical to mMOR-1 (Fig. 6.3). Thus, the identical mMOR-1 protein can be generated by four different transcripts. On the other hand, translation from the E11 AUG in these variants only produced short peptides (<10 kD) because of early termination of translation within its downstream exons (Fig. 6.3) [82]. In vitro transcription coupled translation followed by immunoprecipitation with an E11-specific antibody confirmed that both mMOR-1 and the short proteins can be produced [82]. However, it is not clear whether these peptides are expressed endogenously and what their potential functions are.

Another E11-associated variant, mMOR-1T, had a similar exon composition to mMOR-1H, but an additional exon (exon 16) was inserted between exons 1a and 2 [90]. Therefore, translation from the E1 AUG was terminated within exon 16, leading to a single TM protein in which 95 amino acids composing of the N-terminal and TM domain were encoded by E1 and followed by 20 amino acids of a carboxyl tail predicted from exon 16. On the other hand, translation from E11 AUG predicted a short protein with 84 amino acids, which was identical to that of mMOR-1H.

Four additional single TM variants were isolated from the mouse OPRM1 gene, mMOR-1Q, mMOR-1R, mMOR-1S, and mMOR-1Z, and presumably transcribed through the E1 promoter (Fig. 6.3) [90, 184, 185]. All the four variants were exon-skipping variants: mMOR-1Q, mMOR-1R, and mMOR-1Z were exon 2-skipping variants, while mMOR-1S skipped exons 2/3. In mMOR-1Q, mMOR-1R, and mMOR-1Z, translation from the E1 AUG ended within exon 3 due to switching to a different reading-frame in exon 3. Thus, all these three variants predicted a single TM protein with the same N-terminus and TM domain as mMOR-1T encoded by E1 and followed by a carboxyl tail containing 127 amino acids predicted from exon 3. Although they had different exons downstream of exon 3, all these exons were considered within the 3' UTR.

mMOR-1S was alternatively spliced from E1 to exon 4 by skipping exons 2 and 3, also predicting a single TM protein. Translation from the E1 AUG in mMOR-1S terminates immediately when it reaches exon 4 because of a reading-frame shift in exon 4. Thus the majority of the protein sequence was encoded by E1. To date, a total of five single TM variants have been isolated containing the same N-terminus and transmembrane domain encoded by E1 with variable C-terminal tails encoded by different downstream exons.

Interestingly, all these single TM variants structurally complemented the six TM variants that lacked this same first TM. To date, there has been no evidence that the single TM or the six TM variants alone function alone as an opioid receptor. Although unpublished data indicated that the single TM variants can dimerize with the six TM variants when co-expressed in CHO cells (YX Pan and GW Pasternak, unpublished observations), it is not known whether they can constitute a functional receptor.

6.4.2 The Rat OPRM1 Genes

Since the first rat splice variant, rMOR-1B, was reported in 1994 [182], 15 additional splice variants have been isolated in the rat [84, 90, 186]. rMOR-1B was a C-terminal variant in which an alternative exon 5 replaced exon 4 to encode an different C-terminal tail. Exon 5 is conserved between rodents and humans, with rMRO-1B homologs identified in mouse (mMOR-1B1) and human (hMOR-1B1). We named rMOR-1B as rMOR-1B1 since other variants using different splice sites within exon 5 were designated as rMOR-1B2–rMOR-1B5.

Similar to hMOR-1A and mMOR-1A, rMOR-1A was an intron-retention variant. rMOR-1C1 had an identical exon composition as mMOR-1C and the deduced 30 amino acids from rat exon 7 shared high homology with those of mouse exon 7 (83%) and human exon O (67%) [186].

However, the rat exon 8 differed from the mouse exon 8. Thus the rat exons 8 and 9 in rMOR-1C1 predicted 35 amino acids as compared to 22 amino acids from mouse exons 8 and 9. rMOR-1C2 had the same exon composition as rMOR-1C1 except that splicing from exon 8 to an alternative site upstream of exon 9a resulted in insertion of exon 9b [186]. Therefore, both variants had the same protein sequences up to exon 8, but different C-terminal tails encoded by exon 9a (four amino acids) in rMOR-1C1 or exon 9b (21 amino acids) in rMOR-1C2. rMOR-1D had a splicing pattern resembling mMOR-1D [186]. However, translation of the rat exon 8 from exon 3 in rMOR-1D only predicted one threonine residue. rMOR-1B2 and rMOR-1P had similar splicing patterns as mMOR-1B2 and mMOR-1P, respectively [90].

Using available rat genome databases, we mapped a mouse E11 homolog in the rat OPRM1 gene locus to ~21 kb upstream of E1, a distance similar to that of the mouse E11 (~30 kb) and the human E11 (~28 kb). Subsequent RT-PCR cloning isolated seven rat E11-associated variants, including rMOR-1G1, rMOR-1G2, rMOR-1H1, rMOR-1H2, rMOR-1i1, rMOR-1i2, and rMOR-1i3 (Fig. 6.4) [84].

rMOR-1G1 and rMOR-1G2 had a similar splicing patterns. However, an alternative 5' splice site in E11 differentiated these two variants. rMOR-1G1 contained exons 11a/11b/2/3/4. If the AUG in exon 11a was used, rMOR-1G1 predicted a peptide with only seven amino acids since the stop codon predicted from exon 11b terminated its translation. However, rMOR-1G1 also predicted a six TM protein when using the first AUG from exon 2 as the translational start codon, a similar situation seen in mMOR-1K, mMOR-1L, hMOR-1G1 and the Mu₃ receptor (see below). Similar to mMOR-1G and hMOR-1G2, rMOR-1G2 encoded a six-TM protein when using exon 11a AUG as the translational start codon.

The other five variants, rMOR-1H1, rMOR-1H2, rMOR-1i1, rMOR-1i2, and rMOR-1i3, contained the same exons 11a, 1a, 2, 3, and 4, but alternative splicing among exons 11a, 11b, 1a, 1b, and 1c generated different transcripts [84]. Although having different 5' sequences, rMOR-1H1, rMOR-1i1, rMOR-1i2, and rMOR-1i3

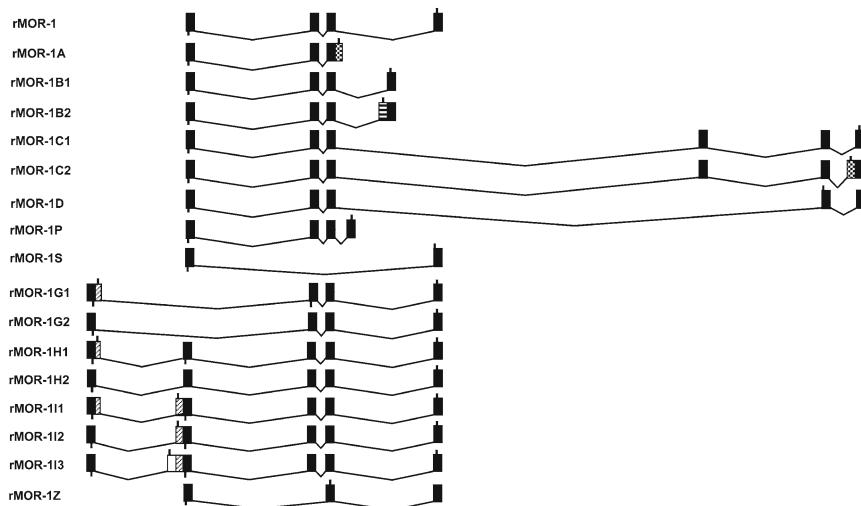
a. Genomic structure**b. Alternatively spliced variants**

Fig. 6.4 Schematic of the rat OPRM1 gene structure and alternative splicing. (a) Genomic structure of the rat OPRM1 gene. Exons and introns are indicated by *boxes* and *horizontal lines*, respectively. Intron size is indicated below the introns as kilobases (kb). Promoters are shown by *arrows*. Exons are numbered based upon the published data. (b) Alternatively spliced variants of the rat OPRM1 gene. Exon composition for each alternatively spliced variant was indicated by appropriate exon *boxes*. The lines between exons are introns that are spliced out during splicing. Translation start and stop points are shown by *bars* below and above *exon boxes*, respectively

all predicted the same protein sequence as the original rMOR-1 when using AUG in exon 1a as translational start codon, while translation from the AUG of exon 11a in these variants predicted only short protein sequences due to early translation termination. Therefore, there were four E11-containing transcripts that encode the exact rMOR-1 protein in addition to the original rMOR-1 generated from the E1 promoter. This mimics the mouse OPRM1 gene where we observed three mouse E11-containing variants, mMOR-1H, mMOR-1I, and mMOR-1J.

Translation of rMOR-1H2 from the exon 11a AUG predicted a novel N-terminal variant in which an additional 50 amino acids were fused to the N-terminus of the original rMOR-1 [84]. In vitro transcription coupled translation studies suggested that the exon 11a AUG was preferentially used in rMOR-1H2 compared to the exon 1a AUG. When expressed in CHO cells, the 50 amino acids significantly altered agonist-induced G protein activation with little effect on opioid binding [84] (see below). Similar to mMOR-1S and mMOR-1Z, rMOR-1S, and rMOR-1Z encoded a single TM protein.

6.4.3 The Human ORPM1 Gene

The first human splice variant reported was hMOR-1A, an intron-retention variant [181]. The homolog of hMOR-1A was also isolated from the mouse (mMOR-1A) and rat (rMOR-1A) OPRM1 genes. Isolating multiple splice variants in the mouse OPRM1 gene led us to further explore additional splice variants in the human OPRM1 gene. To date, a total of eighteen splice variants have been isolated in the human OPRM1 gene [83, 103, 187–190], in addition to hMOR-1A. Most human splice variants were identified in human neuroblastoma cell lines such as SK-N-SH, its subclone SK-N-SHSY-5Y and SK-N-BE(2)-C, all of which expressed Mu opioid receptors.

Alternative splicing patterns of the human OPRM1 gene were similar to those of the mouse and rat OPRM1 genes, consisting of both 3' and 5' splicing, intron-retention and exon skipping (Fig. 6.5). Of eighteen splice variants, eight variants were full length C-terminal variants generated through alternative 3' splicing, including hMOR-1B1, hMOR-1B2, hMOR-1B3, mMORR-1B4, hMOR-1B5, hMOR-1O, hMOR-1X, and hMOR-1Y [103, 187]. Similar to the mouse exon 5, the human exon 5 had five alternative 3' splice sites (5a, 5b, 5c, 5d, and 5e). Alternative splicing from exon 3 to these 3' splice sites within exon 5 produced five C-terminal variants (hMOR-1B1–hMOR-1B5). Although the human exon 5a is highly homologous to the mouse and rat exon 5a, the deduced amino acids from the human exon 5a in hMOR-1B1 contained an extra thirteen amino acids beyond the five amino acids that were identical to those predicted from mouse and rat exon 5a. On the other hand, the nucleotide sequences and predicted amino acid sequences from human exons 5b, 5c, 5d, and 5e in hMOR-1B2, hMOR-1B3, hMOR-1B4, and hMOR-1B5 were quite different from those of the exons 5 in the mouse homologs.

Human exon O, a homolog of the mouse exon 7a and the rat exon 7, is mapped ~128 kb downstream of exon 4, a distance very close to the ~131 kb and ~120 kb between exons 4 and 7 in the mouse and rat OPRM1 gene, respectively (Figs. 6.3–6.5). The predicted 30 amino acids from human exon O shared ~67% and ~63% identities with those from exon 7a in mMOR-1O or mMOR-1C, and exon 7 from rMOR-1C1, respectively [86, 103, 186]. In the mouse and rat OPRM1 genes, there are two exons downstream from exon 7, exons 8, and 9, which are expressed in several mouse variants such as mMOR-1C, mMOR-1D, mMOR-1E, and mMOR-1F, and rat variants such as rMOR-1C1, rMOR-1C2, and rMOR-1D. However, these downstream exons have not been identified in human OPRM1 gene so far.

The human exon X is ~2 kb downstream of exon 3, a distance similar to that of mouse exon 15 [187]. Although exon X shared certain homology with the mouse exon 15 at nucleotide level, the predicted amino acids from exon X in hMOR-1X differed significantly from exon 15 in mMOR-1P. hMOR-1Y had the same exon composition as hMOR-1B3 except that an exon Y was inserted between exons 3 and 5c (Fig. 6.5) [187].

The human OPRM1 gene also underwent 5'splicing. A mouse E11 homolog in the human OPRM1 gene was initially identified ~28 kb upstream of E1 by searching

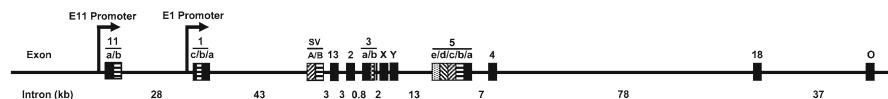
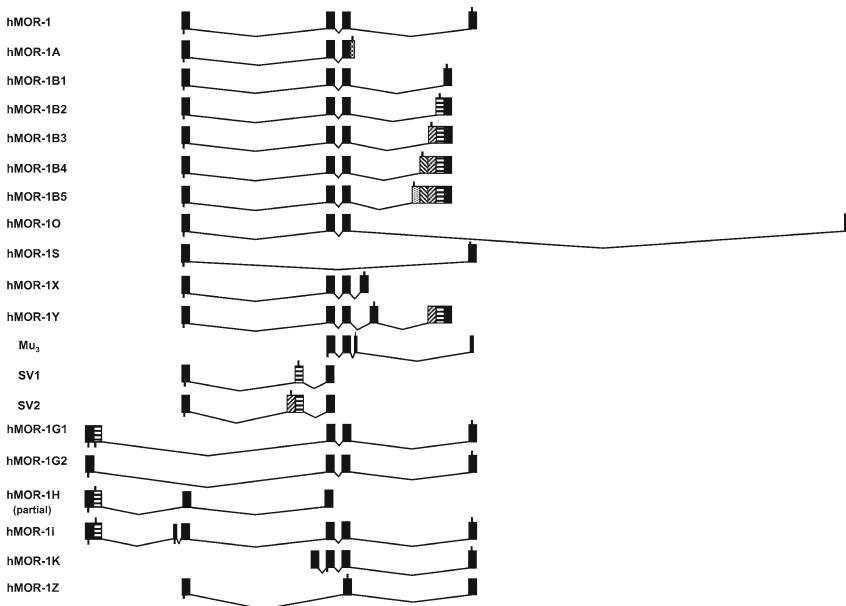
a Genomic structure**b Alternatively spliced variants**

Fig. 6.5 Schematic of the human OPRM1 gene structure and alternative splicing. (a) Genomic structure of the human OPRM1 gene. Exons and introns are indicated by boxes and horizontal lines, respectively. Intron size is indicated below the introns as kilobases (kb). Promoters are shown by arrows. Exons are numbered based upon the published data. (b) Alternatively spliced variants of the human OPRM1 gene. Exon composition for each alternatively spliced variant was indicated by appropriate exon boxes. The lines between exons are introns that are spliced out during splicing. Translation start and stop points are shown by bars below and above exon boxes, respectively

the human genome databases using the mouse E11 sequence. We then isolated three full-length variants, hMOR-1G1, hMOR-1G2, and hMOR-1i, and one partial length variant, hMOR-1H, by RT-PCR cloning [83]. Similar to the rat E11, there were two alternative 5' splice sites in human E11 (exons 11a and 11b) (Figs. 6.4 and 6.5). Alternative splicing from these two splice sites directly to exon 2 generated two six TM variants, hMOR-1G1 and hMOR-1G2, that lacked E1 which is responsible for encoding the first TM.

Similar to mMOR-1G and rMOR-1G2, translation from exon 11a in hMOR-1G2 was in-frame with the reading-frame used in hMOR-1, and predicted a six-TM variant. Although the translation from exon 11a in hMOR-1G1 was terminated in exon 11b, using the AUG from exon 2 also predicted a six-TM protein, a similar scenario seen in rMOR-1G1, hMOR-1K, and human Mu₃ (see below).

hMOR-1i had an unique splicing pattern in which a 105 bp sequence (exon 1b) was spliced out, unlike the continuous E1 in mMOR-1I [83]. Thus, translation from the AUG from exon 1c predicted a protein with a novel sequence of 93 amino acids fused to the N-terminus of hMOR-1. Similar to rMOR-1H2, these extra N-terminal sequences did not contain any predicted transmembrane domains, suggesting that they still belonged to the 7-TM GPRC family, but with an elongated extracellular N-terminus. On the other hand, translation from the AUG in exon 11a in hMOR-1i predicted a short peptide due to early termination of translation in exon 11b. The usage of the AUG from exon 1c in hMOR-1i was confirmed using an in vitro transcription coupled translation system. When expressed in CHO cells, the extra 93 amino acids in hMOR-1i had little effect on opioid binding, but significantly changed agonist-induced G-protein activation [83].

Another two variants, Mu₃ [188] and hMOR-1K [190], predicted six TM proteins. Mu₃ contained exons 2, 3, a new exon ~263 bp downstream of exon 3 and a portion of exon 4. Translation from the AUG of exon 2 predicted a six TM protein identical to hMOR-1G1 except that 12 amino acids encoded by exon 4 in hMOR-1G1 was replaced by 26 amino acids encoded by the new exon. There was a consensus splice site upstream of the new exon, but it is not clear where the splice sites between the new exon and the exon 4 are located due to ambiguous sequences. When expressed in COS-1 cells, the Mu₃ clone showed only moderate binding affinity toward morphine (K_i : 29 nM), naltrexone (K_i : 31 nM) and naloxone (K_i : 39 nM) using a [³H]dihydromorphine binding assay, and displayed pharmacological properties similar to that of a Mu₃ receptor early reported [188]. Recently, a mouse exon 13 homolog was identified in the human OPRM1 gene, which led to the isolation of hMOR-1K [190]. Human exon 13 was located at ~3 kb upstream of exon 2. However, there was no predicted coding sequence in exon 13. Thus, using the AUG of exon 2 in hMOR-1K predicted a 6-TM protein identical to that of hMOR-1G1. Interestingly, a SNP located upstream of exon 13 contained a conserved internal ribosome entry site and influenced expression of hMOR-1K at both the mRNA and protein levels. Together, we now have a total of four 6-TM variants in the human OPRM1 gene, including hMOR-1G1, hMOR-1G2, hMOR-1K, and Mu₃.

Four single TM variants, hMOR-1S, hMOR-1Z, SV1, and SV2, were also isolated [189, 191]. Like their rodent homologs, hMOR-1S was an exons 2/3 skipping variant, whereas hMOR-1Z was an exon 3 skipping variant. hMOR-1S was the first single TM variant initially identified in a human neuroblastoma SHSY-5Y cell line [191]. The expression level of hMOR-1S mRNA was quite abundant as compared to that of hMOR-1 mRNA in the cell line. Similar to mMOR-1Z and rMOR-1Z, skipping exon 2 in hMOR-1Z led to translation termination in exon 3. SV1 and SV2 variants were identified in human NMB cells, in which an exon B or exons A/B was inserted between exons 1 and 2, respectively [189]. However, translation from the E1 AUG was terminated in exon B or A, generating a single TM protein in both variants. The function of SV1 and SV2 was indicated to modulate hMOR-1 binding when co-expressed in NMB cells.

6.4.4 Expression and Function of Splice Variants

6.4.4.1 Regional Distribution of Variant mRNAs

To examine expression of the variant mRNAs, Northern blot analysis was initially performed using individual exon probes and RNAs from whole brain. Banding patterns of different sizes and intensities were detected [36, 82, 102], further supporting alternative splicing of the OPRM1 genes. However, since most of the exons are associated with more than one variant, it is difficult to analyze expression of a single variant mRNA. Furthermore, the low abundance of the mRNA requires a large amount of RNA for Northern blot analysis, restricting its use in small brain regions where the amount of RNA is limited. Thus, a semi-quantitative RT-PCR approach has been used to explore regional distributions of variant mRNAs in a number of dissected brain regions, including cortex, thalamus, striatum, hypothalamus, hippocampus, brain stem, cerebellum, and spinal cord [82, 83, 86, 87].

Although expressed at low levels, remarkable differences of the regional distribution of the variant mRNAs were seen, in contrast to the relatively universal expression of MOR-1 mRNA. For example, in mouse brain, mMOR-1C mRNA was highly expressed in the thalamus, where there was little expression of mMOR1D and mMOR-1E mRNAs [86]. mMOR-1E mRNA was highly expressed in the striatum and hypothalamus, whereas expression level of mMOR-1D mRNA was higher in the cortex, brain stem and PAG [86]. In human brain, hMOR-1G2 was highly expressed in the prefrontal cortex, piriform cortex, nucleus accumbens and pons, but not in the temporal cortex and spinal cord [83]. These results further suggested region-specific RNA splicing. However, semi-quantitative RT-PCR has disadvantages such as saturation of amplification at the point of detection and sensitivity and variation of ethidium bromide staining, limiting its ability to accurately quantify mRNA and pointing the need for more accurate, reliable and sensitive assays, such as real-time PCR and ribonuclease protection assays.

6.4.4.2 Regional Distribution of Variant Proteins

Several antisera against individual exon coding sequences were used to determine the distribution of variants at the protein level, recognizing the limitations of the approach due to antibody specificity. The results showed dramatic differences in the regional distribution of the exon-containing variant proteins [192–195]. For example, the distribution of exons 7/8-like immunoreactivity (LI) revealed with an antisera against a peptide from mouse exons 7/8 clearly differed from that of exon 4-LI using an antisera against a peptide from exon 4 in several regions, such as the medial eminence, thalamic nuclei, and nucleus ambiguus [193]. Even when expressed in the same regions, the two antisera labeled different cell populations, as demonstrated in laminae I–II of the spinal cord [193]. An antisera raised against an exon 8 peptide labeled uniquely in the dentate gyrus, the mossy fibers of the

hippocampal formation and the nucleus of the solitary tract [192], while an antisera raised against exon 3/5a hybridized predominantly in the olfactory bulb [196].

It should be pointed out that an epitope from an exon or exons for generating antisera is commonly shared by several variants. Thus, exon 4-LI would represent at least five variants in mouse including mMOR-1, mMOR-1H, mMOR-1i, mMOR-1J, and mMOR-1G, all of which contained the identical twelve amino acids encoded by exon 4. Similarly, exon 7/8-LI would correspond to two variants, mMOR-1C and mMOR-1M, whereas exon 8-LI would represent mMOR-1D and mMOR-1N. It will be challenging to distinguish each individual variant at protein level when an exon-specific antisera is used.

These studies also revealed a mismatch between mRNA and protein levels for some of the variants. For example, the expression levels of both mMOR-1C and mMOR-1M mRNA was much lower than that of mMOR-1 mRNA [82, 86]. However, the immunostaining revealed by the exon 7/8-specific antisera appeared similar to that by the exon 4-specific antisera [193]. Although the sensitivities of these antisera may differ, it raises the question of the relationship of mRNA and protein levels. These studies further suggested not only region-specific and cell-specific splicing and/or receptor targeting, but also potential distinct pharmacological functions of the variants.

The localization of variant proteins was also examined at the ultrastructural level [197]. For example, in the superficial laminae of the spinal cord, the exon 7/8-LI was distributed predominantly presynaptically and co-localized with calcitonin gene-related peptide (CGPR) whose antibody labels unmyelinated primary afferents, in contrast to the exon 4-LI which displayed an equal distribution between presynaptic and postsynaptic membranes and did not co-localize with CGPR [197].

6.4.4.3 Receptor Binding

Among the cloned splice variants, most are full-length variants containing identical seven TM, varying only at the tip of the C-terminus or N-terminus. To address whether these C-termini or N-terminal changes altered receptor binding, CHO cell lines stably expressing the full-length variant cDNA clones were established, and opioid binding assessed [83, 84, 86, 87, 103, 183, 186]. All these full-length variants had similar, high affinities for [³H]DAMGO, a Mu agonist. Competition studies further confirmed the Mu binding selectivity for all the full-length variants, with Mu-selective drugs potently competing binding, while δ -selective and κ_1 -selective ligands displaying very poor affinity. This was not surprising since the binding pocket has been proposed to be defined by the 7-TM regions, which are identical among them.

However, there were some subtle but significant differences of the binding profiles for the endogenous opioid peptides such as dynorphin A and β -endorphin. For example, both β -endorphin and dynorphin A competed [³H]DAMGO binding in mMOR-1D over four to fivefold more potently than in mMOR-1 [86]. On the other hand, hMOR-1 had over twofold higher affinity than hMOR-1X [103]. While the

reasons for this are not clear, it is possible that the different carboxy termini may lead to slight alterations in receptor structure that are most easily seen with the larger ligands.

6.4.4.4 μ Agonist-Induced G Protein Activation and Adenyllyl Cyclase Activity

The intracellular loops and carboxyl terminus play a major role in the association of the receptor with its transduction system, including G proteins and other associated proteins. One possible consequence of the C-terminal differences, therefore, may involve differences in transduction. Looking at receptor activation using [³⁵S]GTP γ S binding assays revealed dramatic differences of agonist-induced G protein activation in both their potency, determined by the EC₅₀ values, and efficacy, indicated by the maximal stimulation, among the C-terminal variants from mouse, rat and human [183, 186, 187, 198].

Most intriguing, the relative efficacy of some of the drugs to each other varied markedly among the variants. For example, dynorphin A and β -endorphin displayed widely varying efficacies among the human C-terminal variants, while DAMGO was a full agonist for the most of them [187]. Second, the efficacies for the different drugs varied in each specific splice variant. For example, morphine was more efficacious in hMOR-1A than in hMOR-1B5, contrasting to dynorphin A [187]. Third, there was little correlation between binding affinity (K_d) and potency (EC₅₀). The best example was β -endorphin, which was over 20-fold more potent in rMOR-1 than in rMOR-1C1 in [³⁵S]GTP γ S binding assay despite having similar binding affinity for both variants in opioid binding assay [186]. This implies differences in their intrinsic activities since maximal [³⁵S]GTP γ S binding can be seen at different receptor occupancies. Fourth, there was no obvious correlation between potency and efficacy. For example, DAMGO was less potent, but more efficacious, than morphine in hMOR-1B1, hMOR-1B3, hMOR-1B4, and hMOR-1B5 expressing cells [187]. These studies suggested that minor changes at the tip of the C-terminus greatly influence agonist-induced receptor-G protein coupling and possibly downstream signal transduction pathways.

The different N-termini in the full-length N-terminal variants, including hMOR-1i and rMOR-1H2, also showed significant differences in agonist-induced G protein coupling using [³⁵S]GTP γ S binding [83, 84]. For example, β -endorphin, dynorphin A and M6G were less potent in hMOR-1i than in hMOR-1 despite having similar binding affinities for the two receptors. It is intriguing to consider how the variation at the tip of the N-terminus can modulate receptor-G protein coupling which many would have predicted would be more dependent upon intracellular structures. Dissociation of opioid binding affinity with receptor-G protein coupling potency and efficacy among these C-terminal and N-terminal variants also provides new insights to understanding different opioid responses in animals and humans. It also opens up new areas in drug design, suggesting a dissociation between the structure-activity relationships of binding affinity and efficacy.

Mu Opioid receptors primarily couple to inhibitory G-proteins, such as $G_{\alpha i}/G_{\alpha o}$, and have been shown to inhibit adenylyl cyclase and decrease intracellular cAMP levels. The effect of the different C-termini on agonist-induced inhibition of forskolin-stimulated cAMP accumulation was examined using a whole cell assay in CHO cells stably expressing one of several human C-terminal variants [187]. Again, marked differences in both their potency determined by IC_{50} values, and efficacy indicated by percentage of maximal inhibition were observed. For example, dynorphin A was 150-fold more potent in hMOR1B2 than hMOR-1B1 even though they had similar efficacies. Morphine was much less efficacious in hMOR-1B3 than other C-terminal variants [187]. However, there was little correlation between [^{35}S]GTP γ S binding and adenylyl cyclase assays results. For example, β -endorphin had the greatest potency among all the drugs in [^{35}S]GTP γ S binding assays, but not in adenylyl cyclase assays [187]. On the other hand, the efficacy of β -endorphin in adenylyl cyclase assays was far greater than in [^{35}S]GTP γ S binding assays. These discrepancies may be attributed to different assay conditions: isolated membranes were used for [^{35}S]GTP γ S binding, whereas intact live cells were used for the adenylyl cyclase assay. Adenylyl cyclase, however, is only one of many downstream effectors of G proteins activated through the Mu receptors, such as their regulation of calcium and potassium channels. Further work is needed to define the role of the various C-terminal and N-terminal variants influence on other transduction pathways.

6.4.4.5 μ Agonist-Induced Receptor Phosphorylation, Internalization and Post-Endocytic Sorting

Agonist-induced MOR-1 phosphorylation is thought to be involved in receptor desensitization and internalization, as well as the development of opioid dependence and tolerance [199–202]. The majority of phosphorylation sites are located within the intracellular loops and C-terminus in regions encoded by exons 2 and 3. In addition, consensus sequences have suggested potential phosphorylation sites for β -adrenergic receptor kinase, protein kinase C, caseine kinase, tyrosine kinase and cAMP- and cGMP-dependent protein kinases, in the spliced exons downstream from exon 3 (Fig. 6.2) [86, 88, 89, 103, 182, 186, 187].

For example, a threonine 394 located at the C-terminal tip encoded by exon 4 in rMOR-1 is a major residue for DAMGO-induced receptor phosphorylation. A T394A mutation abolished 90% of DAMGO-induced receptor phosphorylation [203], which was correlated with the elimination of DAMGO-induced adenylyl cyclase superactivation [204]. When expressed in HEK 293 cells, mMOR-1D and mMOR-1E displayed robust morphine-induced receptor phosphorylation and internalization, in contrast to the nonresponsiveness of mMOR-1 and mMOR-1C to morphine [205]. However, when given intracerebroventricularly, morphine induced marked mMOR-1C internalization in the mouse lateral septum [206]. These discrepancies suggested that morphine-induced receptor phosphorylation and internalization are highly dependent upon which cell type is being examined. Yet, different carboxyl termini greatly influenced morphine-induced receptor internalization.

Unlike mDOR-1, mMOR-1 undergoes rapid recycling back to the plasma membrane following agonist-induced endocytosis. A sequence termed the MOR1-derived recycling sequence (MRS) responsible for the efficient mMOR-1 post-endocytic sorting was identified at the tip of the C-terminus encoded by exon 4 [207]. Several C-terminal variants including mMOR-1B, mMOR-1D and mMOR-1E lacking the MRS due to alternative 3' splicing showed inefficient receptor recycling after DAMGO-induced endocytosis in HEK 293 cells, suggesting a functional role of the C-terminal splice variants in directing the post-endocytic sorting fate of G protein coupled receptors [208]. It will be interesting to further investigate the structural and functional relationships between the endocytic and post-endocytic processes and the development of opioid dependence and tolerance in these C-terminal variants.

6.4.4.6 In Vivo μ Opioid Analgesia

Antisense approaches utilizing short oligonucleotides have been successfully used to correlate the cloned receptors with opioid pharmacology in animal models [209]. Antisense targeting individual members of the opioid receptor family efficiently blocked the analgesic actions of the agonists specifically for each class of the opioid receptors [210–214]. Antisense mapping in which oligodeoxynucleotides are targeted to specific exons has been used to explore the role of splice variants in opioid action. In MOR-1, antisense targeting exons 1 and 4 blocked morphine analgesia in both mouse and rat, but failed to decrease M6G analgesia, implying the existence of alternative exons in other potential alternatively spliced transcripts that are responsible for M6G action [215, 216].

Following the discovery of the many splice variants and their new exons, antisense mapping implicated these newly identified exons, and therefore the new splice variants, in Mu analgesia. However, all Mu drugs were not affected equally. For example, when given intracerebroventricularly, antisense probes targeting exons 6, 7, 8, 9, and 10 all blocked supraspinal morphine analgesia in mice, but not M6G analgesia, suggesting a functional role of these exons and their associated variants in mediating the analgesic action of morphine [86, 87].

Gene targeting studies have more explicitly revealed the pharmacological functions of the Mu opioid receptors *in vivo*, as discussed later in this volume. At least five KO mouse models of the OPRM1 gene have been established with various gene targeting strategies, including exon 2 disruption [217], deletion of the coding regions of E1 [218], deletion of both the coding and promoter regions of E1 [219], deletion of both exons 2 and 3 [220], and deletion of E11 [85].

Morphine analgesia was completely lost in all the KO models except the one targeting E11, suggesting that these exons are crucial for morphine analgesia. However, the analgesic activity of both heroin and M6G were retained in the mouse generated by Pintar, in which the coding region of E1, but not its promoter region, was deleted, suggested that the analgesic actions of heroin and M6G are mediated through a receptor mechanism different from morphine analgesia [218].

On the other hand, the E11 KO model displayed diminished heroin and M6G analgesia, without altering morphine analgesia [85]. Of the E11-associated variants, a number contain E1. Since they would be lost in both the E11 and the E1-targetted KOs, they cannot be responsible for the heroin and M6G responses lost in the E11 KO mice. Thus, these studies strongly argue for a role of only the six TM variants, mMOR-1G, mMOR-1M, and mMOR-1N which lack E1 and are still expressed in the Pintar E1 KO mouse, were responsible for the residual heroin and M6G analgesia in the Pintar mice and the diminished response in the E11 KO model.

However, it has been reported that deleting both E1 and its promoter eliminates both heroin and M6G responses [221]. Unfortunately, it is not known whether these animals, which have lost a far larger region of the gene, still express the six TM variants. This is a concern since the loss of a longer stretch of the gene might influence the overall splicing of gene, leading to a loss of variants lacking E1.

6.5 Summary

Although Mu opioid receptors were identified pharmacologically and biochemically in binding studies over 30 years ago, their structure, function, and true complexity have only emerged after the cloning of MOR-1 in 1993. Since then, efforts from many laboratories have greatly advanced our understanding of Mu opioid receptors, ranging from their anatomic distributions to cellular and molecular mechanisms, from transcription to post-translational modification, and from cell lines to *in vivo* models. The initial concept of multiple MOR-1 subtypes suggested by the pharmacological studies 30 years ago have now been confirmed, reinforced and complicated by the molecular identification of multiple alternatively spliced variants. The functional significance of these multiple splice variants and promoters implicated in a series of *in vitro* and *in vivo* studies provides a new perspective on understanding complex Mu opioid actions in animals and humans and developing new drugs for better controlling pain and drug of abuse.

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Chapter 7

Delta Opioid Receptors

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Abstract Of the opioid receptors, the μ opioid receptor is the most well known as it mediates the important physiological states of analgesia and addiction. By contrast, the delta (δ) opioid receptor is not as well recognized and its role remains, for the most part, elusive. Drawing from recent studies, this chapter examines new information highlighting specific roles of this receptor that have begun to clarify its role. We briefly discuss how the δ receptor was first cloned and the little we do know of its phylogeny. The unique manner in which this receptor is processed and then signals once on the cell membrane is then described. Finally, we discuss the specific behaviors in which the δ receptor plays a role highlighting possible pharmaceutical benefits.

Keywords Delta opioid receptor • Constitutive activity • Analgesia • Mood • Substance abuse • Inflammation

7.1 A Brief History of Delta Opioid Receptors

Delta (δ) opioid receptors, also called DOR or delta opioid peptide (DOP) receptors, are members of the rhodopsin G-protein coupled receptor family comprising about 670 of the approximately 30,000 genes expressed by the human genome. Multiple prior reviews have detailed the landmark research leading to the identification of δ -receptors and their closely related family members, μ , κ and ORL-1 receptors [1] thus only a very brief reiteration will be provided here.

As a distinct opioid receptor type, δ -receptors were first proposed during the mid 1970s, soon after the discovery of stereospecific opiate binding sites. The concept that opioid receptors were not of one homogeneous type was evolving and key

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pharmacological studies demonstrated convincingly that not all opioid-like drugs elicited the same in vivo pharmacological profiles [2]. Martin et al. initially proposed three classes of interactions namely; μ , κ , and σ – a δ -receptor class was not designated at this time. In the search for selective opioid receptors for the newly discovered endogenous opioid peptides, methionine- and leucine-enkephalin, Kosterlitz and colleagues first described δ -receptors [3]. The studies used ligand binding assays combined with muscle-twitch preparations (guinea pig ileum and mouse vas deferens) to convincingly characterize a receptor type with a distinct pharmacology from either μ or κ receptors.

In the early days of δ -receptor research [D -Ala², D -Leu⁵]enkephalin or DADLE (enkephalin protected from peptidases by D -amino acids substituted at positions 2 and 5) was the “selective ligand” that distinguished δ -receptors from other opioid receptors. In truth DADLE has only about a tenfold selectivity for δ - over μ -receptors, similar to the selectivity and affinity profile of the endogenous enkephalins. More selective drugs that could be administered peripherally were clearly needed. The considered importance of δ -opioid receptors as therapeutic targets has over the years vacillated widely, and paralleled efforts of synthetic chemists to develop more selective agonists and antagonists. As of today synthetic chemists have created an excellent “ligand toolbox” to probe δ -receptors and provided both alkaloid and peptide agonists (internalizing and noninternalizing), partial agonists, neutral antagonists and inverse agonists to probe δ -receptor signaling, trafficking and function.

One of the early outcomes of this “ligand toolbox” was the identification of constitutive activity of δ -receptors – the ability of δ -receptors to signal without agonists. The δ -receptor in 1989 was one of the earliest G-protein coupled receptors to be associated with ligand-independent activation of signaling [4]. A second concept that resulted from the ligand toolbox has been heterogeneity in δ -pharmacology. Subtypes of δ -receptors, designated Delta1 and Delta2, were initially distinguished based upon in vivo actions of the agonists [D -Pen^{2,5}]enkephalin (DPDPE) and Tyr- D -Ala-Phe-Glu-Val-Val-Gly-NH₂ (Deltophin II) that could be blocked respectively by the antagonists [D -Ala², Leu⁵ Cys⁶] enkephalin and naltrindole 5' isothiocyanate [5].

As the story progressed, one explanation put forward was that Delta2 receptors represented δ -receptors associated with μ -opioid receptors. The concept of μ/δ interactions also has an extensive history but is muddied by reliance on agonists with unclear selectivity when injected in vivo [6]. The awareness of heteromeric receptor complexes with distinct pharmacology from the individual receptor complexes has provided a molecular explanation for at least some of the μ/δ observations [7]. More recently there is an emerging story for differentiating peptide and alkaloid agonists at δ -receptors with the suggestion that alkaloid agonists may trigger different signaling desensitization pathways than peptide ligands [8]. The cloning of the first opioid receptors just over 15 years ago [9, 10], can also be credited to the δ -ligand toolbox. The δ -opioid receptor was cloned using δ -selective analogs of enkephalins to probe a cDNA expression library created from a mouse

neuroblastoma/rat glioma hybrid cell line (NG108-15 cells) long known to express δ -receptors. The other members of the opioid receptor family were readily cloned by homology to the δ -receptor. Cloning of the receptor family has provided the tools to significantly progress our knowledge of the molecular, cellular and behavioral aspects of opioid systems.

Initially the rationale for developing δ -therapeutics was that they were considered analgesics, not unlike μ -agonists, but importantly with low or no propensity for addiction. The finding in the early 1990s that δ -agonists induced convulsions in several animal models [11] and more recently that much of the acute μ -like analgesic effects of ICV injected δ -preferring drugs could be explained by cross-reactivity with μ -receptors [6] tempered the rush to develop δ -drugs. However, δ -mediated therapeutic actions on mood, chronic inflammatory pain models, the immune system and cardiac control have invigorated the search for new δ -agonist and antagonist drugs [11–14].

The purpose of this chapter is to review the highlights and very recent advances concerning δ -opioid receptors. Those wishing to revel in details and pathways to our current understanding of δ -receptors are referred to a relatively comprehensive book titled, *The Delta Opioid Receptor*, published in 2004 by Marcel and Dekker, New York.

7.2 Phylogeny of Delta Receptors

Although there are reports of opioid systems in invertebrates [15], genomic evidence has not been forthcoming. δ -receptors have not been convincingly demonstrated in any invertebrate and genomic sequence of the sea squirt, a close precursor of vertebrates, has no clearly opioid-related genes encoding either opioid peptide precursors or opioid receptors [16]. Molecular analysis of the cloned δ -receptor genes shows that the protein-coding regions are constituted by three exons, which appear conserved across vertebrate species. Complete receptors have been cloned and analyzed from several vertebrate species besides the mouse and human, such as the rat (*Rattus norvegicus*), zebrafish (*Danio rerio*), the rough-skinned newt (*Taricha granulosa*), and the frog (*Rana pipiens*); see Fig. 7.1.

Areas of sequence heterogeneity among the vertebrate δ -receptors are the N-terminus, the third and fourth extracellular loops and the C-terminus. Interestingly this pattern of conserved to nonconserved regions among the vertebrate δ -receptors parallels the regions of heterogeneity among different members of the opioid receptor family (δ , μ , ORL-1 and κ receptors). The different sequences of the vertebrate opioid receptors provide important insights concerning structure-function relationships for ligand binding, trafficking, and signaling.

The most primitive vertebrates known today, the first ones to be separated from the common evolutionary line, are the jawless vertebrates (Agnatha) (Fig. 7.2). Even though their notochord persists through life, they are considered vertebrates

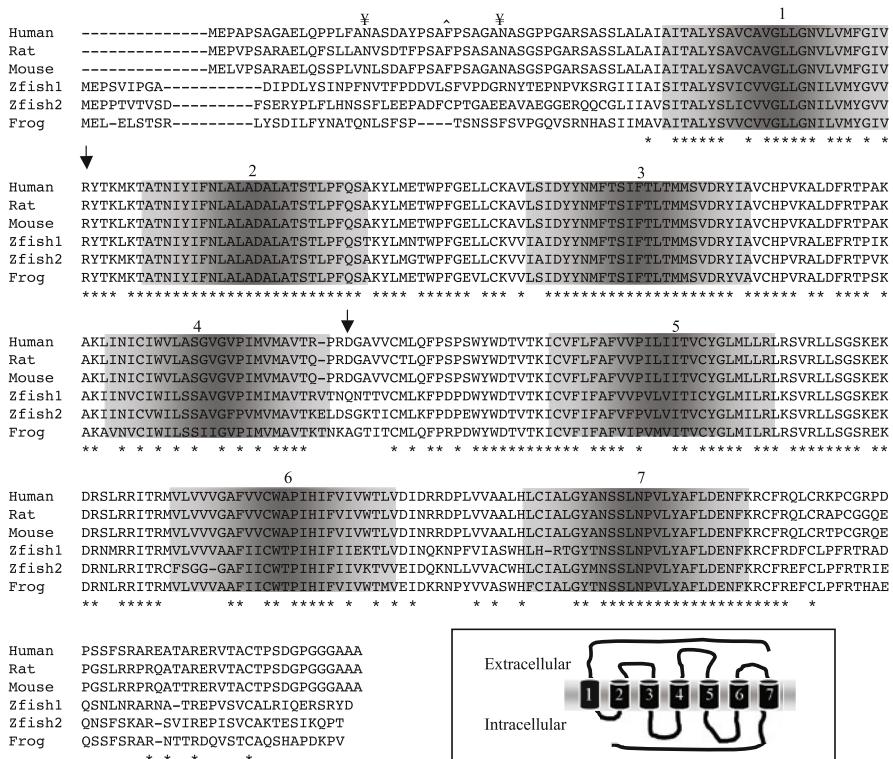


Fig. 7.1 Homology of δ-opioid receptors from different vertebrates. Sequences were obtained from Genbank (accession numbers: human [NM-000911]; rat [U00475]; mouse [L11064]; zebrafish1 [NM-131258]; zebrafish2 [NM-212755]; frog [AF530572]) and aligned using the clustal format. Identical amino acids are indicated (*). Extracellular N-linked consensus glycosylation sites (N any amino acid, then S or T) are shown for the human sequence (¥). Predicted transmembrane domains are shaded and numbered 1–7. The phenylalanine (F) in the first extracellular domain of the human δ-and denoted by ^ is a polymorphism site; in some populations this is a cysteine residue (compare accession numbers U10504 and NM-000911). Arrows designate conserved intron/exon splice junctions among all receptors. The boxed inset shows the predicted membrane topography of the δ-receptor with transmembrane domains 1–7 labeled

because they share a number of other important characteristics (they have a skull, a chambered heart, a central nervous system, specialized sense organs, a muscular system, a lateral line system used for hearing, one semicircular canal, etc). The only members of this group that have survived are the lampreys and the hagfish and multiple opioid receptor like sequences have been identified in pacific hagfish [17]. The presence of opioid receptors in hagfish suggests they originated in the genome over 500 million years ago but it remains a mystery how the opioid system was created given the lack of existence of either opioid receptors or precursors in the sea-squirt and other pre-vertebrate lineages [16].

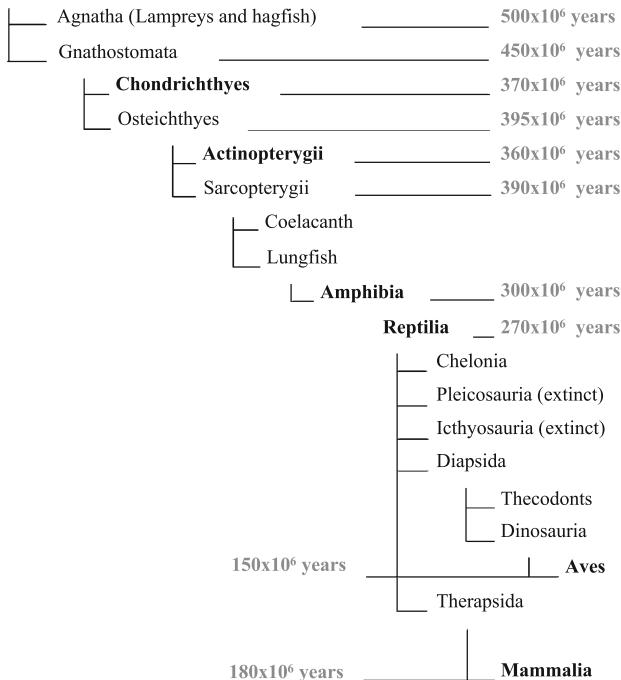
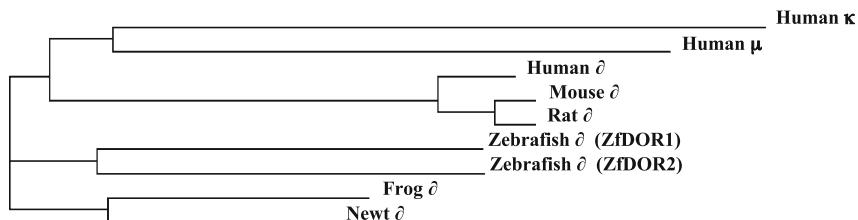
a**Vertebrata****b**

Fig. 7.2 Phylogeny of δ -receptors. (a) Cladogram showing vertebrate evolution. (b) Phylogram generated using ClustalW2 (<http://www.ebi.ac.uk/>) and showing the homology relationships among vertebrate δ -opioid receptors in relation to human μ and κ receptors. The branch length is proportional to the amount of homology and inferred evolutionary change

The first members of the superclass Gnathostomata to appear in evolution were the achantodians, or spiny sharks, and it is believed that achantodians gave rise to the modern bony fishes about 395 million years ago, during the Devonian Period. The bony fishes are divided in two classes, Actinopterygii, which are the dominant group of fish, and Sarcopterygii. The zebrafish, a member of the Actinopterygii class, presents a number of characteristics that makes it suitable as a model organism to study the opioid system.

Barrallo et al. cloned the first opioid receptor in zebrafish in 1998, a δ -like opioid receptor named ZFOR1 or ZfDOR1 [18]. Since then five opioid receptors (ZfMOR, ZfDOR1, ZfDOR2, ZfKOR and ZfORL) which include a duplicate δ -receptor ZfDOR2 [19] and six opioid precursors (ZfPOMC, ZfPOMC-like, ZfPENK, ZfPENK-like, ZfPNOC and ZfPDYN) with gene duplication of both the pro-enkephalin and proopiomelanocortin precursors have been cloned and characterized. An interesting feature of the zebrafish genome is the existence of two duplicate zebrafish δ -opioid receptors. Both zebrafish duplicates (Figs. 7.1 and 7.2) are considered to be δ -receptor like as they share greater homology with the human δ -opioid receptor than with the other opioid receptors (Fig. 7.2b).

It is notorious that the zebrafish δ -duplicates are not as closely related with each other as might be anticipated (about 71% identity in the case of ZfDOR1 and ZfDOR2). Competition assays have shown that neither of the duplicates show affinity towards specific mammalian δ -agonists, such as DPDPE or SNC80, but several endogenous opioid peptides such as enkephalins and Met-enkephalin RGL are able to displace [3 H]Diprenorphine from ZfDOR2 with nM Ki's [19]. A new endogenous opioid peptide has been discovered in the zebrafish proenkephalin gene, consisting of seven amino acids (YGGFMGY), and named MEGY [20]. MEGY showed high affinity towards both δ -receptor duplicates, although it is a better displacer at the ZfDOR2 than at the ZfDOR1.

With more comprehensive analysis of the opioid receptors from different species a fuller understanding of structure-activity relationships among opioid receptors will become apparent and perhaps we will gain insights as to the original function of opioid receptors that retained them in the mammalian genome.

7.3 The Life History of δ -Receptors

Although it is difficult to draw an accurate and full comparison between the different opioid receptors, it appears that the δ -receptor has a unique and intriguing “life history” – from transcription and translation to exocytosis then endocytosis and finally degradation via lysosomes. This has been summarized in Fig. 7.3, and each aspect of this life history will be discussed in further detail below. It is important to note that for this review data has been extracted from a number of different model systems and it is inevitable that what pertains in one system may not hold for another. Acceptance for individual models to provide different life journeys for δ -receptors in different cellular environments must be anticipated.

Transcription and translation: Even 15 years following their cloning, the entire structure of δ -receptor transcripts remain unknown because they are extremely large messages with extensive 3' untranslated regions. While the protein coding region of δ -opioid receptor transcripts spans just over 1.1 kb, the predominate transcript forms are over 10 kb [9] consisting mainly of 3' untranslated RNA with an unknown function (Fig. 7.3). The three protein coding exons of the δ -receptor span 30.8 kb in the mouse genome [21] and 50.9 kb in the human genome. However, the extent of the promoter as well as areas of the genome contributing this large 3'

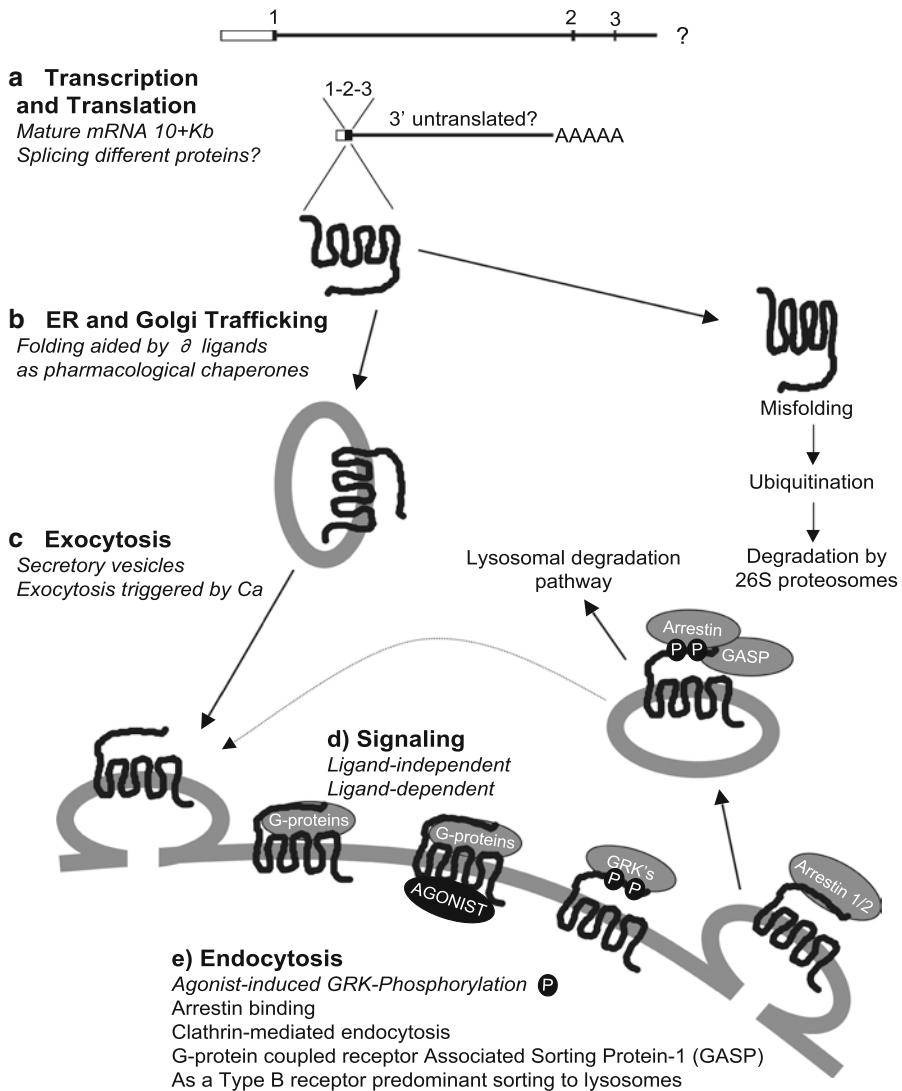


Fig. 7.3 The life history of δ -receptors

untranslated region in the transcripts remain undetermined and the size of the murine and human δ -genes is likely to be substantially greater than 30 and 50 kb respectively. The complex genomic structure of the δ -receptor has commonalities with the μ -receptor gene and speaks to the likely importance of regulatory mechanisms, other than promoter activity, controlling μ - and δ -receptors at the transcriptional level [21]. Although the μ -receptor gene has a series of complex splice variants generated via alternative splicing at all intron/exon boundaries [22], analogous complexity has not been observed in δ -receptor transcripts.

The promoter of the δ -receptor has many putative transcription factor binding sites including those regulated by cAMP, growth factors and cytokines. Thus it is not surprising that δ -receptor transcripts are regulated by many treatments including NGF, retinoic acid, forskolin and alcohol [21]. The structure of the δ -opioid receptor protein (only one variant of the protein coding region has been identified to date) has been reviewed exhaustively [16, 23] and models deduced from homology with other G-protein coupled receptors and mutation studies are evolving [24].

Folding and processing of δ -receptors in the Golgi and endoplasmic reticulum (ER): The precursor δ -receptor protein is transformed to its mature form after various stages of trimming and processing of the core-glycosylated protein and addition of N- and O-linked glycans in the ER before leaving the trans-golgi network to appear on the cell membrane. δ -receptor processing appears to undergo strict regulation, at least in some cellular environments, with more than half of the precursor protein being withheld in the endoplasmic reticulum [25, 26]. Misfolded or incompletely folded, receptor is retained on the cytoplasmic side of the ER, ubiquitinated and then degraded by 26S proteasomes. Interestingly, such strict quality control may be relaxed by pharmacological chaperones, either membrane permeable δ -agonists or antagonists, that increase the release of δ -receptors from the ER [27]. These membrane permiable δ -ligands presumably bind and stabilize the receptor structure even in the ER.

Intracellular δ -receptor localization: In contrast to other opioid receptors the δ -receptor is localized within a sub-membranous compartment associated with the ER or large dense core vesicles in different cell types, including neurons of the dorsal root ganglia and peri-aqueductal gray matter [28–32]. In addition to this sub-membranous population, a smaller population of receptors is found on the cell surface. Diverse stimuli increase the relative contribution of this population to the total δ -receptor population, possibly a result of enhanced exocytosis of the sub-membranous population [31]. This suggests that the intracellular localization of the δ -receptors on or close to the cell membrane is dynamic and influenced by external stimuli. Furthermore this localization may be affected by the sequence or folding of the receptor, for example, tagged δ -receptors, expressed in a recombinant background become membrane-associated in contrast to those that are untagged and associated with large dense core vesicles [33]. Another note of caution is that many of these studies have been conducted with polyclonal rabbit antibody immunocytochemistry to identify δ -receptors and issues with specificity of many of the available anti-opioid receptor antibodies are continually being questioned.

δ -Receptors exocytosis: Specific, physiological stimuli increase δ -receptor exocytosis, and for the most part, enhance δ receptor function [34, 35]. At the cellular level, acute stimuli such as δ -receptor activation, ATP, capsaicin, bradykinin and P2xR1 [31, 32, 36] increase δ -receptor exocytosis. In vivo the δ -receptor may be recruited to the cell membrane of neurons of the peri-aqueductal gray matter following a forced swim test [30] or after more prolonged stimuli such as chronic morphine or chronic inflammation [37]. The cell surface levels of δ -receptor are increased under many of these conditions, implying that δ -receptor function is closely linked with cell surface expression of the receptor.

Signaling of δ-receptors: The δ-receptor is a classical G-protein coupled receptor and for the most part, couples with the Gi/Go family of G-proteins to modulate signaling within diverse second messenger systems such as adenylyl cyclase, phospholipase C, MAP-kinase and ion channel function. This modulation is well-documented for δ-opioid receptors and the information below has been reviewed in depth elsewhere [38].

Acute δ-opioid receptor activation in most cell types attenuates adenylyl cyclase activity resulting in decreased intracellular cAMP levels. This is mediated by the G α subunit of either a Gi/Go G-protein or in some cases via the Pertussis toxin-insensitive G α subunit Gz. There are a few reports of δ-mediated Gs and β/γ-mediated stimulation of adenylyl cyclase. Diversity and complexity of cyclase signaling is, at least in part, attributable to the different adenylyl cyclase forms (1–9) expressed in different cellular backgrounds. Chronic (hours) as opposed to acute (minutes) activation of δ-opioid receptors causes a superactivation of both basal and stimulated adenylyl cyclase activity that has long been considered a potential cellular adaptation underlying tolerance and dependence [39]. Interestingly, the signaling mechanisms underlying this compensation of the cyclase system have remained somewhat elusive.

Although activation of the ubiquitous MAP kinase cascade has been extensively examined for the μ-opioid receptor, there is little information on how the δ-receptor may activate this cascade. Phosphorylation of ERK1/2, one of the terminal kinases of the cascade, appears to require δ-receptor internalization, suggesting that a post-internalization and possibly β-arrestin dependent mechanism is involved. However, other studies in different systems have demonstrated that non-internalizing agonists and even inverse agonists can activate ERK1/2 in a Pertussis toxin sensitive manner [40]. This is reminiscent of other G-protein coupled receptors that activate this cascade through β-arrestin [41], or G-protein dependent mechanisms allowing for different time courses and extent of activation [42]. Further studies are required to examine how δ-receptors modulate this complex cascade and undoubtedly there will be several activation mechanisms clearly identified in the future. In addition other kinase cascades can be modulated by δ receptors including Akt, JNks, STAT3, P38 involving Src, Ras, Rac, Raf-1, Cdc42 RTKs.

Multiple channels can be regulated by δ-receptors. Modulation of either the voltage-dependent Ca²⁺ or inwardly rectified K⁺ channels by δ-receptor agonists, inhibits neurotransmission through different mechanisms. In the case of the voltage dependent Ca²⁺ channels, δ-receptor activation inhibits the Ca²⁺ channels, decreasing the probability of depolarization. This effect is less efficacious than other second messenger systems such as δ-receptor modulation of adenylyl cyclase, but is a rapid response suggesting a close association between the receptor, Gβ/γ subunit, and ion channel complex. There is considerable diversity in the Ca²⁺ channels involved, and N, P, L and Q, may be regulated by different β/γ subunits expressed in different cellular backgrounds.

In contrast, δ-receptor modulation of the inwardly rectified K⁺, GIRK or Kir3, channels activates these channels to hyperpolarize the cell, to prevent depolarization, a post-synaptic mechanism. This has been shown in locus coeruleus and substantia

nigra neurons and involves the tetrameric G-protein gated Kir3.1-3.3 channels in a G $\beta\gamma$ -dependent manner. Finally, coupling of the δ -receptor with phospholipase C stimulates the formation of IP₃ and the mobilization of intracellular Ca²⁺. This appears to be a G α , G $\beta\gamma$ interaction activating the PLC β subunit, particularly β 3.

Such ligand-dependent signaling by the δ -receptor is complemented by a ligand-independent, or constitutive activity, of the δ -receptor [4]. A number of antagonists have been shown to be “inverse agonists” (ICI-174,864 being the prototype) blocking δ -activation of constitutive GTPase activity [4], GTPgS binding [43], several signaling pathways including cAMP inhibition [40]. Other antagonists appear to be neutral antagonists (e.g., naltrindole or MR2266) blocking agonist-induced activation but not constitutive activity. However, as implicated above not all inverse agonists block all forms of signaling and although ICI-174,864 may be inverse agonist for some signaling pathways, e.g., cAMP inhibition, it is an agonist for others such as MAP-kinase activation [40]. It remains unclear if the signaling and trafficking of constitutively-activated and ligand-activated δ -receptors will differ but this appears likely given the demonstration of agonist-selective signaling for δ -receptors and that different complexes induce trafficking of agonist-activated as opposed to constitutively active μ -receptors [44].

The bottom line is that there is a multiplicity of signaling pathways that the δ -receptor can modulate and increasing evidence points to signaling specificity via δ -receptors depending upon the ligand occupying the receptor. A dramatic example is the inverse agonist ICI-174-864, which like agonists, activates MAP-kinase pathways yet inhibits δ -mediated inhibition of adenylate cyclase [40]. More subtle differential signaling desensitization is observed between alkaloid and peptide agonists [8]. Given that there are nearly 30 different endogenous opioid peptides it is tempting to speculate that the different endorphins and enkephalins may encode differences in signaling capacities at the δ -receptor and other opioid receptor types.

Endocytosis and agonist-regulated trafficking of δ -receptors: Once on the cell membrane, the δ -receptor may be removed from the cell surface, either as a result of receptor protein turnover (perhaps variable for constitutive and non-constitutively active receptors) or in response to activation by agonists [43]. Degradation of the receptor occurs through ubiquitination and proteosomal pathways [45, 46] resulting in a ~15 h half-life of the fully matured receptor [47], and slightly elevated compared to the μ -receptor [48]. δ -receptor ligands increase such degradation five-fold [46], interestingly the μ -receptor is degraded only twice as fast in the presence of a ligand [48].

Such rapid ligand-induced internalization correlates with decreased δ -receptor function *in vivo* suggesting that, unlike the μ -receptor, δ -receptor internalization and desensitization are closely linked [49]. This may be a result of the degradative, as opposed to recycling fate, of the agonist-bound, internalized, δ -receptor. The internalized δ -receptor associates with G-protein coupled receptor associated sorting protein-1 (GASP-1) [50, 51] leading to degradation in an ubiquitination-independent manner [52]. The fate of the δ -receptor may also be determined by its interaction with β -arrestin 1 which has greater affinity for the δ - than μ -receptor [53], possibly a result of the multiple phosphorylated serines and threonines in its Carboxyl-tail.

This enhances the receptor- β -arrestin 1 association targeting the receptor to a degradative rather than a recycling trafficking fate [54, 55]. Such stable β -arrestin complexes are a hallmark of the Class B receptors that remain as an intact arrestin-receptor complex through the internalization process. In contrast, Class A receptors, such as the μ -receptor, dissociate rapidly from β -arrestin and may be recycled back to the cell surface. These findings suggest that the ligand-activated δ -receptor is exclusively degraded once internalized. Interestingly a recent study has shown that this degradative fate of the δ -receptor may be converted to a recycling fate by inhibiting c-Src suggesting a dynamic role of this ubiquitous tyrosine kinase in modulating δ -receptor trafficking and function [56].

7.4 Distribution of δ -Receptors

Prior to the cloning, receptor autoradiography using selective radiolabeled ligands showed discrete patterns of expression of opioid receptor types in the rodent and human nervous system. The development of GTP γ S binding in slices [57] provided a localization methodology for detection of agonist-activatable δ -receptors. The GTP γ S binding technology is particularly pertinent for δ -receptors since several studies noted above suggest a large pool located in intracellular vesicles [34]. The cloning provided additional reagents for localization, including nucleic acid probes for in situ hybridization analysis which, when compared to autoradiographic analysis, provided some insights into areas of synthesis and targeted receptors [58].

The cloning also provided the potential to generate antibodies. The use of antisera for identification of opioid receptors has unfortunately become increasingly problematic. For example the monoclonal antisera to the ORL-1 receptor cross-reacts with an unknown antigen [59] and multiple other published studies using antisera to opioid receptors are suspect for many reasons, including antisera showing the same patterns of staining in the receptor knockout and wild-type mice. Antisera lacking target selectivity appears to be a common issue plaguing receptor identification. A consortium study in the nicotinic receptor field found that none of the many commercial nicotinic receptor antisera could be relied upon [60]. There is clearly a need to apply increased stringency for specificity prior to publishing antibody obtained data.

Very recently a δ -receptor tagged with green fluorescent protein (GFP) has been knocked in to replace the endogenous mouse δ -receptor providing additional distribution information in the mouse nervous system [49]. The δ -receptor GFP-knock-in mouse does slightly overexpress GFP-tagged δ -receptor transcripts and protein such that some δ -agonist functions and sites of expression could possibly be unrepresentative of the wild-type, but the model has immense power in studying receptor localization with respect to function.

Besides the extreme caution in interpretation of the antibody-based localization studies there are four major points that are noteworthy regarding the δ -receptor distribution studies.

1. Expression of δ -receptor mRNA and δ -ligand/GTPgS-stimulated binding appears early in development, and indicate widespread expression of functional δ -receptors throughout the central nervous system [61, 62]. However, the weak GTPgS-stimulated binding in sections, that many δ -receptors appear localized internally, the fast agonist-induced pathway to lysosomal degradation (type B receptor), that large and complex transcripts are required for receptor synthesis are suggestive of a system not optimized for fast turnover.
2. There are significant species differences in opioid receptor distribution which may translate to different opioid functions among species and questions rodents as a good model for translational studies of opioids in humans [63]. In the case of δ -opioid receptors this has been noted in the distribution of receptors in the spinal cord and dorsal root ganglia during comparative *in situ* studies in rodent, nonhuman primate, and human samples [64].
3. All receptor transcript and protein identification techniques that are available have detection limitations and it remains a question of how many receptors are required to elicit a neuronal response that can influence behavior. Locations expressing highest receptor levels may not necessarily be those most important for δ -receptor modulation of behavior.
4. Given the discrepant distribution of endogenous opioid peptides and δ -receptors [58] an important question remains as to whether ligand-independent signaling via δ -receptors is of functional significance. As yet there has been no methodology to map constitutively active δ -receptors *in vivo* and detection of ligand-independent signaling in *ex-vivo* preparations has been reported for μ but not δ -receptors.

Given that the G-protein coupled receptor family is so extensive, many receptors inevitably share signaling pathways with other receptors. What makes δ -receptors unique therapeutic targets is not their signaling and trafficking but their distribution throughout the CNS and the importance of accurate localization studies cannot be overstated.

7.5 Function of δ -Receptors

This section will focus on the novel roles of the δ -receptor, either alone or in combination with other molecules, in modulating diverse behaviors and cellular functions.

Analgesia: As the δ -receptor is widely expressed in regions of the peripheral and central nervous system known to modulate analgesia, that the δ -receptor may have an antinociceptive function, is not surprising. Indeed both non-peptidergic agonists, such as BW373U86 and its derivative SNC80, and peptidergic agonists, DPDPE and DELT II, enhance analgesia, effects which are, for the most blocked by the δ -antagonists such as Naltriben, TIPP and Naltrindole [5, 65]. In addition mice lacking the δ -receptor show decreased inflammatory and neuropathic pain suggesting a δ -mediated nociceptive “tone” [12, 66]. However, the μ and δ -receptor

knockout animals has placed some doubt on δ -specific drugs as effective analgesics in classical acute pain assays such as the tail immersion and hot plate assays [6].

More recently the δ -receptor has been found to play a modulatory role in nociception, particularly in nociceptive paradigms known to influence the μ -receptor. These include chronic morphine, painful stimuli such as capsaicin, and neuropathic pain associated with chronic inflammation. In many of these, an upregulation of δ -mediated analgesia occurs, this phenomenon is dependent on the μ -receptor and is associated with an increased number of δ -receptors on the cell surface of neurons within the nociceptive pathway, perhaps those that have been recruited from a sub-membranous location [35, 37]. Although the cellular mechanism of this μ/δ -interaction is as yet unknown, a shift in the ratio of the different opioid hetero and homo-dimer populations present on the cell membrane, as well as a change in glial-neuronal communication [67] are distinct possibilities.

Mood: As the opioids are well known to affect euphoria, it is perhaps not surprising that mice lacking the μ and the δ -receptors show alterations in mood states. However, that the phenotypes in μ and δ -receptor knockouts are opposing may be surprising [13]. Unlike the μ -opioid receptor, deletion of the δ -receptor increases anxiety and depressive-like systems in mice [13], a similar phenotype to mice lacking proenkephalin, the precursor for enkephalin which is an endogenous δ -ligand [68]. Similarly, enhancing the levels of endogenous opioids by inhibiting their degradation results in an anti-depressant like response in animal models of learned helplessness, a close correlate of depression. The anti-depressant-like and anxiolytic effects of δ -agonists and their reversal by δ -antagonists has been intensively studied leading to various proposals that the δ -receptor may be a suitable target for a novel anti-depressant with a shorter clinical lag-time than the commonly used tricyclic antidepressants [69].

Continuing the theme of a δ -mediated enhanced mood state, the δ -receptor has also been implicated in alcohol abuse. Using a specific operant alcohol-training paradigm, mice lacking the δ -receptor self-administered more alcohol than their wild-type littermates [70]. Interestingly this increase in alcohol consumption was associated with a decrease in anxiety, suggesting the δ -receptor is involved in the connection between alcohol abuse and anxiety states. However, these data are in stark contrast to a number of other studies showing that δ -antagonists decrease alcohol consumption [71], raising concerns of ligand specificity and different interpretations and limitations of the behavioral paradigms used.

Substance abuse: Although δ -agonists alone cannot produce the same reinforcing behaviors as known drugs of abuse [72], a recent study has shown an interesting link between the anxiety associated with withdrawal from cocaine and the δ -receptor [73]. A chronic cocaine administration paradigm decreased δ -receptor function in specific regions of the rat central nervous system and increased the anxiogenic phenotype during the early stages of withdrawal. The latter behavior was reversed, in a dose-dependent manner, by the δ -specific agonist, SNC80. From this study and that investigating alcohol abuse [70] it is interesting to note that although the δ -receptor may not play a principal role in either abuse paradigm, the δ -receptor appears to be linked with an abuse-associated anxiety state.

Cellular functions: The δ -receptor is involved in diverse cellular functions in different cell types ranging from neuro- and cardiac-protection, to modulation of the immune system. For example, δ -agonists are able to attenuate disruption of ionic homeostasis that occurs following ischemia-induced neuronal injury. This suggests a potential therapeutic benefit of δ -agonists in stroke patients [74, 75]. The δ -receptor is also highly expressed in the sino-atrial node of the heart, positioned to modulate vagal transmission [14] and accordingly has been shown to mediate the cardiac protective effect of ischemic preconditioning [76]. The three opioid receptors are expressed in cells of the immune system, such as T cells, neutrophils, monocytes, and macrophages and are known to modulate antibody responses, cell-mediated immunity, phagocytic activity and chemotaxis [77]. This interaction may underlie the neuro-inflammatory response to pain, for example the expression of the opioid receptors and their endogenous agonists in the sensory nerve endings and immune cells of the joint synovium is elevated in various types of arthritic patients [78]. However, the immunomodulatory role of the δ -receptor, as distinct from those of the μ - or κ receptors, has not been clarified, except for a recent study showing an interesting interaction between the δ -receptor and the most prevalent chemokine receptor, CXCR4, which is required for hematopoiesis, lymphocyte trafficking, HIV entry and tumor cell metastases. The δ -receptor forms a complex with this chemokine receptor and DPDPE-mediated activation of the δ -receptor, silences CXCR4 through heterologous desensitization [79].

These diverse roles of the δ -receptor have been shown in various adult cell types. As the expression of the δ -receptor is temporally and spatially regulated during development [62], it is not surprising that the δ -receptor may also influence cellular differentiation. Hippocampal progenitor cells express both the μ and the δ -receptors, and inhibition of both receptors decreases proliferation and stimulates neurogenesis yet inhibits astroglial- and oligodendro-genesis [80]. Such affects on differentiation may be linked with the presence of a binding site for NF κ B, a terminal kinase of the NGF/P13K/AKT signaling sequence involved in NGF-mediated differentiation of PC12 cells, within the δ -promoter [81]. The involvement of the δ -receptor in cellular differentiation is further shown by the delay in wound healing in mice lacking the δ -receptor [82].

7.6 Summary

It will be clear from this review that the δ -opioid remains a little bit of a mystery opioid receptor. The receptor has intriguing signaling and trafficking characteristics with functions that are worthy of consideration as a target for therapeutic drug development. In rodents and other animal models δ -receptor modulation of chronic pain, immune responses and mood, suggest multiple therapeutic targets however, the disparity in opioid receptor distribution among species is a concern with assigning a function for δ -receptors in humans. An exciting concept emerging rapidly in the field is that different opioid agonists will be able to trigger the formation of, or

recognize, different receptor protein complexes thereby modulating different signaling cascades [83].

The implication is that every δ -ligand will possess a unique signaling profile and the continued development of δ -drugs should be encouraged to assess multiple aspects of δ -signaling and function to identify selectivity of action. As with the hope of developing μ -analgesics with reduced tolerance, dependence and modulation of reward circuitry, the hope would be to develop δ -therapeutics with reduced deleterious effects such as seizure-inducing activity. Identification for a potential behavioral role for constitutively active receptors has not been forthcoming, but given that inverse agonists are agonists for some δ -receptor signaling pathways perhaps the “ligand toolbox” is lacking appropriate ligands to address this. Though the δ -opioid receptor has taken backseat to the μ -opioid receptor given its unclear role in analgesia and lack of reward modulation, the δ -receptor has led the way to many discoveries in the opioid system and likely has a promising future as a therapeutic target.

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Chapter 8

Kappa Opioid Receptor Gene and the Regulatory Mechanisms of Its Protein Expression

Li-Na Wei and Horace H. Loh

Abstract Molecular studies of kappa opioid receptor (KOR) and its gene (*Oprk1*) were conducted following the cloning of its cDNA and genomic DNA. The KOR gene is mapped to human chromosome 8 and to mouse chromosome 1. Its genomic structure and coding sequences are highly conserved among the various animal species examined, and most molecular studies have focused on the mouse clone. The mouse KOR gene spans a distance of approximately 16 kb in length, contains three introns, and can produce at least six mature mRNA species due to the use of alternative promoters, splicing, and polyadenylation sites. Transcription of the mouse KOR gene is controlled by two promoters that are subjected to regulation by various signals such as retinoic acid and nitric oxide, transcription factors, chromatin remodeling complexes, and epigenetic events. In addition, the expression of endogenous KOR in primary neurons is tightly regulated by RNA-based post-transcriptional mechanisms such as RNA stability and transport, and translational regulation. The regulation of KOR protein function has been examined, primarily, in heterologously expressed cell systems, and can involve receptor desensitization, internalization and down-regulation. However, the pharmacological and physiological relevance of these findings remain to be validated.

Keywords κ Opioid receptor • Transcriptional regulation • Post-transcriptional regulation • RNA transport • Translation

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8.1 The Kappa Opioid Receptor Gene

8.1.1 Cloning and Mapping of the KOR Gene

Following the cloning of δ opioid receptor (DOR) cDNA in 1992 independently by Kieffer's [1] and Evans' [2] groups, the cDNAs for κ opioid receptor (KOR) from various species were reported in the following year [3–7]. Using KOR cDNA as the probe, the KOR gene (*Oprk1*) was subsequently cloned from different species including mouse [8], rat, [9] and human [10, 11], and was found to be highly conserved in terms of genomic structure and DNA sequence. The KOR gene is mapped to q11.12 in human chromosome 8 [12, 13] and to mouse chromosome 1 [14–16]. With the available KOR cDNAs and genes as the molecular tools, experiments have been launched by various groups to investigate the expression, regulation, and function of the KOR gene and its protein at the molecular level.

8.1.2 The KOR Gene Structure

The structure of the KOR gene was first suggested by the identification of its introns [14], and completely determined for the mouse gene. The mouse KOR gene utilizes multiple transcription initiation sites and alternative polyadenylation signals [8, 17, 18]. Unlike the μ opioid receptor (MOR) and the DOR genes, both the mouse [8] and the rat KOR genes [9] encode an additional intron in its 5'-untranslated region (5'-UTR). Further, two functional polyadenylation (PA) sites can be used by the mouse KOR gene, named PA1 and PA2 [18].

By comparing to the genomic structures of the MOR and the DOR genes, it appears that intron 2 of the mouse KOR gene aligns to the same Arg residue where intron 1 of the MOR and the DOR genes resides, and that intron 3 of KOR gene aligns to a position close to intron 2 of the MOR and DOR genes [8]. With its promoters mapped and PA sites determined, it is concluded that the mouse KOR gene spans a distance of approximately 16 kb in length, and can produce at least six mature mRNA species resulting from alternative promoters, differential splicing in its 5'-UTR, and termination at either one of the two polyadenylation sites at its 3'-end [19] (Fig. 8.1). The presence of alternative spliced and polyadenylated KOR mRNA variants prompted a series of studies focusing on its post-transcriptional regulation (see Sect. 8.3), in addition to vigorous studies of its transcriptional regulation (see Sect. 8.2).

8.1.3 Distribution of KOR mRNA and Protein

Using KOR cDNA as the probe, the distribution of KOR mRNA in the brain has been systematically examined. It is detectable in many areas of the brain including the claustrum, striatum, olfactory tubercle, and several thalamic and hypothalamic

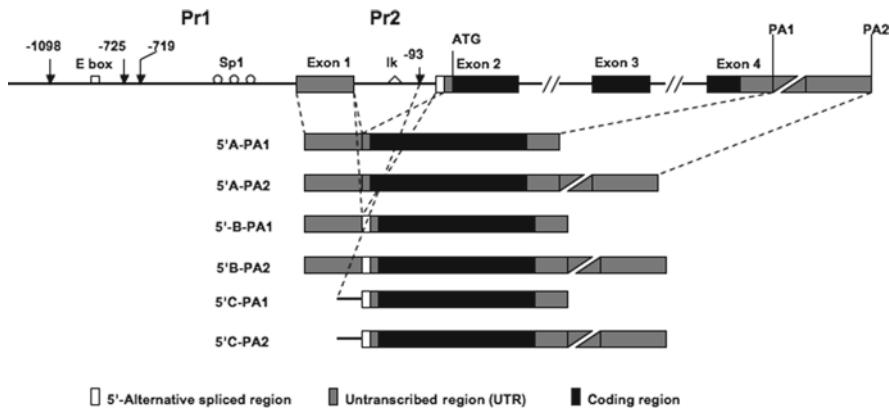


Fig. 8.1 The genomic structure, splicing pattern, and regulatory elements of mouse KOR gene. The mouse KOR gene utilizes two promoters (Pr1 and Pr2), encodes four exons (1–4), and can terminate at two polyadenylation sites (PA1 and PA2). Alternative splicing can occur in intron I where Pr2 also resides, to generate 5'-UTR isoforms A and B that are initiated from Pr1. The 5'-UTR isoform C is initiated from Pr2. As a result, a total of six mRNA isoforms can be produced from the mouse KOR gene, including 5'A-PA1, 5'A-PA2, 5'B-PA1, 5'B-PA2, 5'C-PA1, and 5'C-PA2. Established transcription factor binding sites include the E box, Sp1 site, and Ik site

nuclei and locus coeruleus [20–22]. In terms of its protein distribution, immunohistochemical studies have also revealed positive KOR immunoreactivity in many areas of the brain including the ventral forebrain, hypothalamus, thalamus, posterior pituitary, and midbrain.

At the subcellular level, KOR immunoreactivity can be found in both axonal and somatodendritic compartments, but the majority of staining seems to reside in the somatodendritic compartment, implying a major postsynaptic function [23, 24]. By comparing KOR immunoreactive areas and the distribution pattern of endogenous opioids, it was initially suggested that KOR was deployed, primarily, to post-synaptic membranes to mediate the effects of dynorphin and enkephalin. Recently, KOR mRNA has been clearly and abundantly detected in the pre-synaptic compartment of primary neurons of dorsal root ganglia (DRG) [25]. Further, KOR immunoreactivity and specific ligand-binding activity in the axons of DRG neurons can be stimulated by KCl-induced local depolarization and by a growth factor/axon guidance cue, Netrin-1 [26, 27], suggesting a presynaptic role for KOR and its potential function in axon growth or neurite extension, probably in developmental stages.

8.2 Regulation of KOR Gene Transcription

8.2.1 Development/Cell Type-Specific Regulation

Initial studies of endogenous KOR mRNA expression have suggested a temporal and tissue-specific expression pattern that is enriched in the developing central

nervous system [28]. In more mature embryos, the level of KOR transcripts is reduced, but then elevated, in post-natal stages. Studies of an embryonal carcinoma cell line that can be induced for neuronal differentiation, P19, reveal that the KOR gene is constitutively transcribed in the precursor cells before neuronal differentiation, and that its transcription is repressed during neuronal differentiation until later stages when mature neurons are born and KOR transcription is then reactivated [29]. Thus, a developmental and cell/tissue type-specific regulatory pathway is likely to account for differential accumulation of KOR mRNA in animals and cultured cells. Based upon results gathered from studying these two systems, it is concluded that the KOR gene is constitutively transcribed in neuron precursors, repressed in differentiating cells, and then reactivated later in more mature neurons. Importantly, the biphasic activation of the KOR gene involves extensive chromatin remodeling and is subjected to epigenetic regulation (see Sect. 8.2.3)

Using the KOR-lacZ reporter system, the functional role for KOR promoters in animals has been confirmed [28]. In this transgenic animal model, the first neuro-endocrine connection has been demonstrated at the gene level for the regulation of KOR expression. It appears that KOR mRNA expression is elevated in animals deficient in vitamin A, or its active ingredient retinoic acid (RA) [29]. Regulation of KOR gene transcription by RA has been validated, mechanistically, using the P19 neuronal differentiation system (see Sects. 8.2.2 and 8.2.3).

In addition to vitamin A, the gas-signaling molecule, nitric oxide (NO), is also an effective regulator of KOR transcription. It appears that KOR transcription can be repressed by NO through its ability to inactivate NF- κ B that acts on the c-Myc binding site in promoter 1 of the mouse KOR gene (see Sect. 8.2.2). At present, it can be concluded that KOR gene transcription is regulated, in part, by vitamin A and NO [30]. However, these regulatory signals appear to be more important for KOR expression in developmental stages, or during neuronal differentiation. While it remains to be confirmed whether KOR gene transcription can be regulated in adult animals, and in mature neurons, recent studies have suggested epigenetic regulation may play a role in this regard (see Sect. 8.2.3).

8.2.2 *Transcription Factors*

Identification of transcription initiation sites for the KOR gene has facilitated the dissection of detailed transcriptional regulatory mechanisms for KOR mRNA expression. The mouse KOR gene utilizes two functional promoters both of which are TATA-less but regulated by different transcription factors [30]. Additionally, the transcripts driven by promoter 1 can also be regulated by alternative splicing occurred at intron 1 junction. For the two functional KOR promoters, multiple positive and negative regulatory elements have been identified and their cognate transcription factors confirmed, including binding sites for Sp1 [31], c-Myc [32], and Ikaros (Ik) [33]. The known regulatory DNA elements of the mouse KOR gene are depicted in Fig. 8.1.

Validation of the functionality of these transcription factors has been obtained from studying, primarily, the P19 model that endogenously expresses all these components and responds to proper hormonal regulation in terms of KOR expression. In this model, RA initially activates Ik that in turn suppresses KOR gene transcription by recruiting histone deacetylases to condense the chromatin of KOR promoters [29, 33]. The effect of RA is further extended to its chromatin remodeling ability mediated by recruiting chromatin-remodeling complexes to reactivate the KOR gene (see Sect. 8.2.3).

This underlines the initial, repressive effect of RA on KOR transcription during neuronal differentiation. The physiological significance has been validated by the finding that the level of KOR transcripts is dramatically elevated in embryos carried by mothers under a hypovitaminosis A status [29]. It is tempting to speculate that the nutritional factor vitamin A in pregnant mothers may modulate the formation, or maturation, of specific KOR-positive neurons, thereby affecting the analgesic or other physiological effects of opioids in the offspring. This remains to be explored. Another interesting signaling molecule is NO gas that can suppress KOR transcription by inactivating NF- κ B [34], an activator of c-Myc for KOR transcription [32]. Since c-Myc is also known to recruit histone-modifying enzymes such as histone acetyl transferases, it is highly possible that the NO signal is also capable of triggering chromatin remodeling on the KOR gene promoter during the course of cellular differentiation. However, the physiological or pharmacological relevance of NO to KOR transcription in animals remains to be determined.

Most mechanistic studies of KOR transcriptional regulation have been conducted with regards to the regulation of its promoter 1, the major promoter. An initial interesting observation is that promoter 2 remains silenced until later stages of differentiation in the P19 system, and during later developmental stages in animals. Further, this second promoter seems to be activated by epigenetic regulation that requires the inputs of certain neurotrophic factors produced only in later stages of development. Recently, we have only begun to understand how this promoter can be activated by neurotrophic factors in differentiating cultures where receptors for neurotrophic factors are expressed (see Sect. 8.2.3).

8.2.3 Chromatin-Remodeling and Epigenetic Regulation

The mouse KOR gene is constitutively transcribed from its promoter 1 in the stem/precursor cells in the P19-neuronal differentiation model. RA provides a negative signal to silence KOR gene transcription in the initial phase of cell differentiation process. Consistently, in P19 stem cells, the chromatin structure of KOR promoter 2 is organized into a regularly spaced nucleosomal array (and hence suppressed) whereas that of promoter 1 is disorganized, i.e., lacking nucleosomal organization (and hence activated). Importantly, RA indeed can trigger the formation of regular nucleosomes on the otherwise disorganized promoter 1 in differentiating P19 cells [35]. This provides the molecular details for the suppressive effects of RA on KOR

transcription in the initial phase of RA-induced P19 differentiation. However, a pharmacological dose of RA (10^{-6} M) can transiently activate KOR gene expression, mediated by rapid dephosphorylation of Sp1 due to the blockage of the ERK pathway by a transient surge of RA concentration [31]. Therefore, RA, at the physiological concentrations, is likely to provide a silencing factor for KOR transcription in differentiating stem cells by inducing chromatin remodeling. A pharmacological dose of RA, however, may contribute to a rapid up-regulation of KOR gene in the more mature, committed cells where KOR gene promoters already exist in an open configuration and are ready to be further induced.

As introduced earlier, KOR gene activation occurs in two phases during the process of neuronal differentiation: the first phase being constitutive activation proceeding neuronal differentiation, which is followed by chromatin condensation and gene suppression, and the second being re-activation in later stages of neuronal differentiation. The delayed re-activation of KOR gene, especially for promoter 2, in more differentiated neurons, is under epigenetic regulation, which involves nerve growth factor (NGF)-activated transcription factor activation protein 2 (AP2) that binds to its target site within promoter 2 [36, 37]. As a result of NGF-activated AP2 binding to promoter 2, the activating chromatin marks, such as lysine 9 demethylation and lysine 4 dimethylation on histone 3, appear on the KOR promoter region. According to results obtained from studies using the P19 neuronal differentiation model, it is likely that KOR gene transcription, in developing animals, can be regulated by both hormones, such as RA, and growth factors, like NGF, which elicit extensive chromatin-remodeling events through coordinating the activities of various transcription factors and chromatin remodeling complexes. This represents one form of epigenetic regulation triggered by the surge/availability of certain hormones/growth factors that modify transcription factors and chromatin remodelers, thereby remodeling specific chromatin segments of the KOR gene and activating/suppressing its transcription, possibly at different developmental stages and in different brain regions.

8.3 Post-Transcriptional Regulation of KOR Protein Expression and Activity

8.3.1 Alternative Splicing and Polyadenylation, and Tissue Specificity

The splicing pattern of KOR gene has been examined mostly for the mouse gene. The mouse KOR gene undergoes alternative splicing at intron 1 which resides in its 5'UTR, generating mRNA 5'-UTR isoforms A and B followed by the same coding region [38]. Due to the retention of additional 30 nucleotides from intron 1, KOR mRNA isoform B is slightly longer. By initiating from an alternative promoter present in intron 1, mRNA 5'-UTR isoform C contains the shortest 5'-UTR

(93 nucleotides) among the three 5'-UTR mRNA isoforms. Isoform C also contains the identical coding region. In addition, the mouse KOR gene can use either one of the two functional polyadenylation signals (PA1 and PA2) to generate two types of 3'-UTR varying in size.

The longer 3'-UTR contains approximately 3.5 kb and utilizes PA2, whereas the shorter 3'-UTR contains less than 1 kb and utilizes PA1. Therefore, the mouse KOR gene can potentially generate a total of six mature mRNA isoforms with the same coding region flanked by various combinations of 5'- and 3'-UTRs, i.e., isoforms 5'A-PA1, 5'A-PA2, 5'B-PA1, 5'B-PA2, 5'C-PA1 and 5'C-PA2 (Fig. 8.1). The presence of all the six KOR mRNA isoforms has been validated in studies of animal tissues (Wei, unpublished). Since the same coding region is retained intact in all the six KOR mRNA isoforms, in theory, only one type of KOR protein is generated. Therefore, it is tempting to speculate a physiological need for the use of extensive and complicated UTRs to express KOR, through differential transport and/or translation of these mRNA isoforms (see Sects. 8.3.2–8.3.4).

In fact, studies of animal tissues and cultured cells have validated differential distribution of some of these KOR mRNA isoforms [29]. 5'-UTR Isoforms A and B, both initiated from promoter 1, are more ubiquitously expressed in the central nervous system and in earlier developmental stages (beginning on gestation day 9), whereas isoform C is restricted to brain stem and only in post-natal animals [29]. In addition, in RA-induced P19 neuronal differentiation model, isoforms A and B, but not C, can respond to RA-triggered repression (RA). The functionality of PA1 and PA2 has been validated [18] but its regulation remains to be examined.

8.3.2 mRNA Stability

The production of KOR mRNA isoforms has prompted the examination of potential post-transcriptional regulation via these mRNA isoforms [38]. It appears that the steady-state level of three 5'-UTR KOR mRNA isoforms vary in the P19 model. Isoform A is most stable, with a half-life of 12 h, whereas isoform B has a half-life of 8 h. This is consistent with the relatively higher level of isoform A as compared to isoform B detected in the P19 cells. The half-life of isoform C could not be determined because of the lack of expression of this isoform in the undifferentiated cells [39]. In terms of the 3'-UTR, KOR mRNA using PA2 is significantly more stable than that using PA1 [18].

The physiological relevance of the expression of these KOR mRNA isoforms has been explored in a mechanical allodynia animal pain model. While the total KOR mRNA level can be significantly reduced in the contralateral DRG relative to the side of nerve injury only in animals that experience pain, 5'-UTR isoforms B and C are not significantly affected [40]. This suggests an extremely interesting possibility that production or processing of KOR mRNA 5'-UTR isoform A can be altered in animals experiencing pain following nerve injury. This may be due to the physiological response of the animals to the feeling of pain that elicited certain

events to change RNA processing thereby altering the expression of specific nociceptive receptors in order to alleviate the pain. Alternatively, recent results have demonstrated that KOR mRNA 5'-UTR isoforms can be differentially transported [25, 41] which suggests a high probability that various KOR mRNA isoforms may differentially respond to pain and then move to different areas. Interestingly, recent studies have shown mobilization of KOR mRNA to stress granules in stressed cells (see Sect. 8.3.3)

8.3.3 mRNA Transport

KOR mRNA isoforms are differentially distributed not only in animal tissues but also in different subcellular compartments of primary neurons. For instance, in the isolated axons and somas of adult trigeminal ganglia plated on two-layered sandwich cultures, 5'-UTR isoform A is evenly distributed in both axons and somas, whereas B and C are present mostly in the soma. Similarly, in the *in vitro* differentiated P19 neuron culture, isoform A is more evenly distributed in both soma and processes but isoforms B and C are preferentially detected in the somas. These observations have prompted the proposition of a hypothesis that KOR mRNAs may be differentially transported to different neuronal compartments, which appears to be correct.

This hypothesis has been tested in rigorous molecular studies. Initial studies have employed a phage RNA-binding protein motif MS2-tagged RNA tracing system, that contains two components, one with the MS2-binding RNA sequence fused to the KOR mRNA in its 3'-UTR and the second containing a nuclear GFP-fused MS2 (MS2-GFP). In cells where both KOR-MS2 and MS2-GFP are present, the extra-nuclear KOR-MS2 mRNA can be decorated by MS2-GFP, i.e., KOR mRNA can be traced by following the movement of the otherwise nuclear GFP granules. In cells that contain no MS2-tagged mRNA, the MS2-GFP granules stay in the nuclei. Using this system, it has been demonstrated that KOR isoform A-PA2 is most efficient in mobilizing nuclear MS2-GFP to P19 neuronal processes and to the axons of primary DRG neurons, followed by isoforms B, and then C.

It is further concluded that the 3'-UTR of KOR mRNA is essential for axonal transport, whereas the 5'-UTR of isoform A plays a facilitating role [25, 41]. This is in consistence with the result of earlier studies where the distribution of isoform A can be most significantly affected in the DRG of animals exhibiting pain following nerve-injury. It is tempting to speculate a role for mRNA transport (at least for KOR) in regulating or eliciting animals' response to pain.

Transport of mRNA to neuronal processes has been reported mostly for the lower animal species. In terms of mRNA transport to the axons of sensory neurons in higher organisms, only mRNAs of structural proteins have been found to be transported, particularly in growing/turning axons [42, 43]. Recently, transport of KOR mRNA to the axons of sensory neurons has been established [25], and further validated by the cloning of a component required for the transport process, the

Copb1 subunit of COP1 vesicles. This is supported by the observation that the process can be blocked by colchicines, a microtubule-disturbing agent [27]. Therefore, it is concluded that KOR mRNA can be transported not only to dendrites as observed for other post-synaptically distributed mRNA species, but also to axons of sensory neurons.

This suggests a novel RNA-based regulatory process for the production of KOR in different neuronal compartments. The classical dogma describes that, for axons (pre-synaptic compartments) proteins are synthesized in the soma and carried by cargos to these remote areas where no mRNAs, except mRNAs for structural proteins, are believed to be present. The demonstration of KOR mRNA transport to DRG axons via Copb1-containing granules suggests a previously ignored role for axonal transport of mRNAs in certain physiological processes. While axonal mRNA transport in the mammalian sensory neurons has been discovered first for the KOR mRNA [25, 27, 41], this phenomenon has also been found in recent studies of other mRNAs. Therefore, studies of this particular mechanism, with regards to the control of protein targeting to subcellular compartments of neurons are being rigorously carried out.

With regards to mobilization of KOR mRNA, it has first been found that, in stressed cells/neurons, KOR mRNA is mobilized, together with those of house-keeping proteins, to a subcellular structure named “stress granule” (SG) [44]. Cells, including the axons of DRG neurons, form multiple SGs which store/protect crucial mRNAs under a stressful condition such as injury, heat shock, or arsenite treatment [45, 46]. It is interesting that KOR mRNA is constitutively bound by a specific RNA binding protein named *growth factor receptor bound proteins 7* (Grb7) which arrests KOR’s translation (see Sect. 8.3.4).

Further, mobilization of Grb7-KOR mRNA to SGs in stressed cells appears to require a motor protein Dynein [46]. This suggests that in stressed cells, mobilization of KOR mRNA, perhaps within a short distance, may require Grb7 (for suppressing its translation) and Dynein (for mobilization). Of potential relevance is a recent report showing that KOR activation in the raphe nucleus can mediate the aversive effects of stress in animals [47]. Another surprising yet interesting study has reported that KOR mRNA nuclear-cytoplasmic export in both P19 and DRG neurons is coordinated with its translation, which is activated by epidermal growth factor (EGF) [48]. This is mediated by the actions of multiple players and requires specific, sequential dephosphorylation and re-phosphorylation of the specific KOR mRNA binding protein Grb7 in the nucleus and the cytoplasm, respectively, as well as another RNA-binding protein, Hu-antigen R (HuR) [48].

8.3.4 Translational Regulation

Using in vitro translation and translational reporters in the P19 system, it is found that the KOR 5'-UTR isoforms A, B, and C differ in their translation rates. Specifically, the 5'-UTR isoform A is least efficient in translation, approximately

twofold lower than that of isoforms B and C [39]. In a yeast three-hybrid screening experiment, specific KOR RAN-binding proteins have been cloned. One protein, Grb7, is confirmed as a translational repressor for KOR 5'-UTR isoform A, which binds and competes out the Cap-binding protein, thereby repressing KOR translation [49]. Upon phosphorylation by its upstream kinase FAK, phospho-Grb7 loses its RNA binding ability and releases KOR mRNA for active translation.

Interestingly, one of the axon guidance cues, Netrin-1, appears to be an effective activator for FAK and KOR translation in both primary neurons and P19 neurons [26]. Additionally, EGF can also activate FAK, thereby stimulating KOR translation [48]. Therefore, it seems that KOR mRNA, especially the most predominant isoform A, is constitutively repressed and requires stimulation for translation by growth factor/axon guidance cue such as EGF and Netrin-1. It remains to be investigated whether EGF and Netrin-1 plays a role in KOR pharmacology.

8.3.5 *KOR Protein Activity*

The activity and regulation of KOR protein has been examined, primarily, using transfected cells of various kinds, such as Chinese hamster ovary (CHO) cells, human embryonic kidney (HEK) 293 cells, monkey kidney cells COS-7, NG108-15 mouse/rat hybrid cells, AtT-20 pituitary tumor cells, *Xenopus* oocytes, etc. Pitifully, none of these functional studies have been examined in a relevant, neurobiological context and the results are often contradictory [50, 51]. Several seemingly more agreeable conclusions are: (a) the C-terminal sequence, in particular S369 of rat KOR, may affect receptor desensitization assayed in oocytes and AtT-20 cells [52, 53]; (b) agonist induces phosphorylation of KOR [46, 54]; (c) agonist induces internalization of human and rat, but not mouse, KORs [55, 56]; (d) there are also agonist and species differences in terms of agonist-induced receptor down-regulation [55, 56]. Due to the lack of studies using physiologically relevant experimental systems, it remains unclear how the molecular features of KOR protein, as demonstrated in these heterologous systems, may contribute to its pharmacological and/or physiological activities.

8.4 Future Perspectives

Based upon the gene structures, it is believed that the three opioid receptor genes evolve from the same ancestral gene, yet their expression patterns and the identified regulatory events for their transcriptional controls are quite different. However, they seem to share one common feature in terms of the expression of their proteins, i.e., their mRNAs are mostly silent and the expression of their proteins requires stimulation [38, 55].

Studies of their transcriptional control have generated fundamentally important information in terms of the understanding of how the genomic architecture of these genes in different neurons is molded as their precursor cells undergo differentiation. However, studies of post-transcriptional events, which ultimately determine the specific neuron phenotypes or functions (production of specific receptor proteins), can be more informative in terms of the production of functional opioid receptor proteins.

Classical pharmacology has examined, almost exclusively, the behavior of receptor proteins, such as receptor desensitization, internalization, down-regulation and recycling, etc. With recent demonstration of de novo production of KOR proteins in both the post- and pre-synaptic compartments, it has become an even more complex issue in terms of how the distribution/production of receptor proteins can be regulated. For instance, the local environment surrounding a specific neuronal junction or synapses is likely to modulate not only the behavior of existing receptor proteins but also the production of new receptors, by regulating targeting and translation of mRNAs for the specific receptor proteins.

This is strongly supported by results obtained from studies of KOR post-transcriptional regulation [57]. The fact that these post-transcriptional events are regulated by growth factors, such as EGF and axon guidance cue netrin 1 [48, 49], would strongly suggest the highly plastic nature of the production/location of KOR protein, and the potential integration of KOR signaling with many other physiological processes. However, it cannot be over-emphasized that appropriate experimental systems must be adopted for future studies in order to obtain physiologically relevant information that can advance our understanding of these important receptors in a specific physiological/pharmacological context.

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Chapter 9

Opioid Receptor Signal Transduction Mechanisms

Ping-Yee Law

Abstract The myriad functions of morphine and its congeners are the consequences of the drug interacting with the three opioid receptors. In order to develop a perfect analgesic compound that mediates its function via these receptors, the mechanisms by which signaling occurs and the regulation of the signals must be fully elucidated. Since opioid receptors are members of the G protein-coupled receptor (GPCR) superfamily, many of their signaling processes mimic those of other GPCRs. However, it is apparent from recent proteomic and other studies that opioid receptors, similar to several GPCRs in the rhodopsin subfamily, exist in signaling complexes. These signaling complexes include the oligomerization of the opioid receptors with each other, with other GPCRs, or with other cellular proteins, such as β -arrestin or regulators of G protein signaling (RGS) that could alter or modulate the final receptor signals. In addition, accumulating evidence points to the presence of an agonist-selective signaling process with the opioid receptors. In this chapter, we will review the classical signaling mechanisms of opioid receptors, the various effectors that are regulated by opioid receptors, and their possible roles in the in vivo functions of drugs, and cellular regulators that could influence the amplitude and duration of the signals. We will examine recent data that support the existence of opioid receptor signaling complexes, or “receptosomes,” in the transduction of opioid receptor signals. Finally, evidence for ligand-selective signaling and its implication in future drug development will be discussed.

Keywords GPCR • Heterotrimeric G protein • Regulation of signaling • Receptosomes • Receptor oligomerization • Agonist-biased signaling

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9.1 Introduction

The actions of the opioid alkaloids have long been postulated to be mediated via the high-affinity stereoselective binding sites located at the synaptic plasma membrane. The existence of these sites was demonstrated by the pioneering studies reported by Snyder, Simon, Terenius and their co-workers, based on the paradigm defined by Goldstein [1–4]. Although the distribution of the opioid binding sites and the binding affinities of various alkaloid drugs closely parallel the *in vivo* sites of the actions and potencies of these drugs, a single opioid receptor type cannot account for the diverse actions of the morphine congeners.

Martin and his colleagues clearly demonstrated the existence of multiple opioid receptors by their classical pharmacological studies with the chronic spinal dog model [5]. They defined their pharmacological findings to correspond to the activation of μ -, κ - and δ -opioid receptors. This was quickly followed by the discovery of the δ -opioid receptor [6], the result of the isolation and characterization of the endogenous opioid peptides enkephalin, β -endorphin and dynorphin [7–11]. These multiple opioid receptors subsequently were substantiated with biochemical binding assays [12–14], cross-tolerance studies [15], and the development of receptor-selective antagonists such as β -funaltrexamine and CTOP for the μ -, nor-binaltorphimine for the κ -, and TIPP ψ for the δ -opioid receptor [16–20].

From the beginning, these multiple opioid receptors have been considered to belong to the superfamily of membrane receptors, the G protein-coupled receptors (GPCRs). Blume and his co-workers were the first to demonstrate the absolute requirement for GTP and Na^+ in opioid receptor signaling [21]. Later, using pertussis toxin (PTX) that specifically ADP-ribosylates the Gi/Go subunit to block opioid activities, several laboratories established that G proteins are the transducers for opioid receptor signals [22–27]. The unequivocal classification of these multiple opioid receptors as members of the GPCR family was not accomplished until the cloning of the δ -opioid receptor by Evans, Kieffers, and their co-workers [28, 29], which was followed closely by the cloning of μ - and κ -opioid receptors by several laboratories, based on the reported sequence of the δ -opioid receptor [30–32].

The cloned opioid receptors, henceforth abbreviated as MOR, DOR, and KOR for μ -, δ - and κ -opioid receptors respectively, all have the putative structure of seven transmembrane domains for GPCRs, with an extracellular *N*-terminus containing multiple glycosylation sites, intracellular loops with multiple amphiphatic α -helices, and the fourth intracellular loop formed by the putative palmitoylation sites at the carboxyl tails [32].

On the whole, these receptors are about 60% identical to each other, with most of the identicalness found in the transmembrane domains (73–76%) and intracellular loops (86–100%). The areas of greatest divergence are found in the *N*-terminus (9–10%), the extracellular loops (14–72%) and the *C*-terminus (14–20%) [32]. Having the NPXXY motif in transmembrane 7 and the DRY motif at the border between transmembrane 3 and intracellular loop 2, opioid receptors are classified as being in the G group of the rhodopsin subfamily [33]. They are products of distinctive genes, with the murine chromosomal location of 1, 4, and 10 for MOR, DOR, and KOR, respectively [34, 35].

Regardless of how the opioid receptors are classified, since they are members of the GPCR family, these diverse gene products have one feature in common, i.e., they catalyze the GDP/GTP exchange process within the heterotrimeric G proteins that transduce the receptor signals. As summarized in an earlier review on the GTPase superfamily [36], the GPCR signaling cycle parallels those involving GTPase. Using the small Ras GTPase as an example, the role of a GPCR such as an opioid receptor basically is the same as the guanine nucleotide release protein GNRF in p21ras signaling (Fig. 9.1).

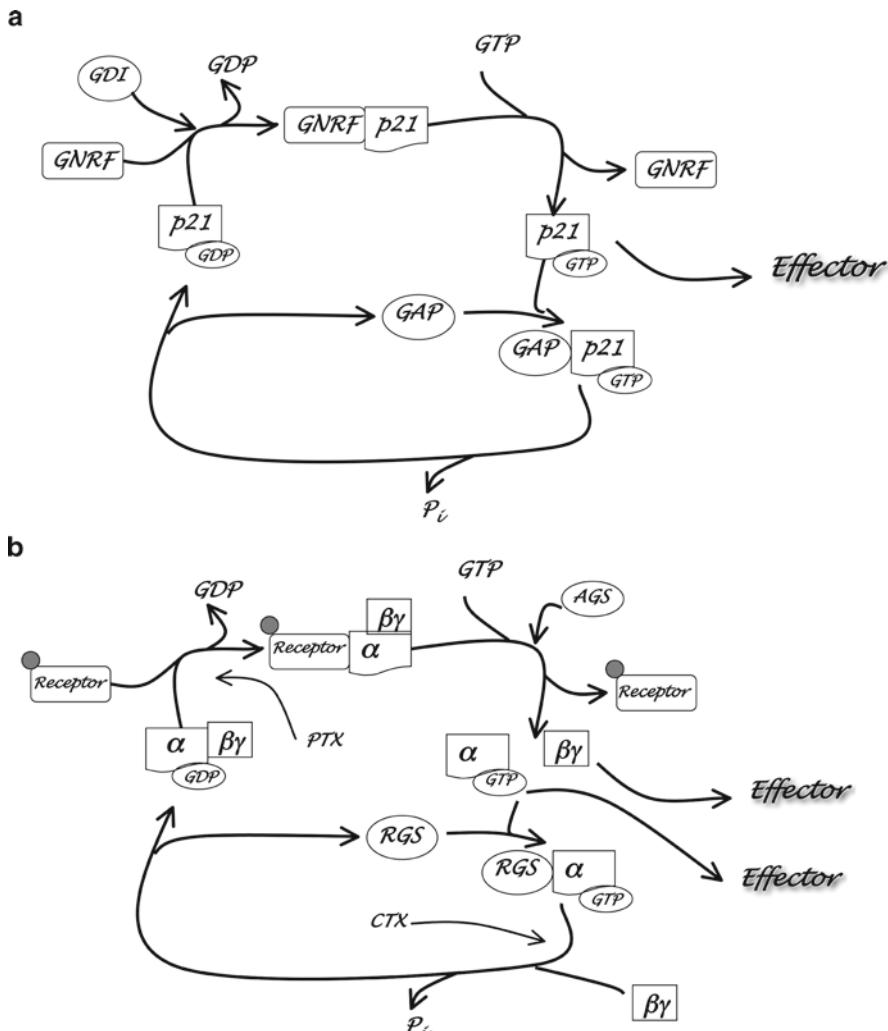


Fig. 9.1 Schematic representation of cellular proteins involved in (a) Ras and (b) GPCR activation and regulation

The interaction of the receptor with G proteins induces the release of bound GDP from the α subunit of the heterotrimeric G proteins, as in the case of GNRF interacting with p21. The dissociation of GDP allows for the binding of GTP, resulting in the dissociation of the heterotrimeric G proteins into two sets of signaling molecules, α - and $\beta\gamma$ subunits. Hydrolysis of the bound GTP to GDP by the endogenous GTPase within the $G\alpha$ -subunit results in the association of the various G protein subunits and returns the signaling cycle into its inactive state. Any proteins that enhance the GTPase activity (e.g., GTPase activating protein (GAP) in the case of p21 signaling, and regulators of G protein signaling (RGS) for GPCR) can reduce the potency and efficacy of the signals (Fig. 9.1).

Similarly, proteins, such as activator of G protein signaling (AGS), which regulate the GDP/GTP exchange within the heterotrimeric G protein, as in the case of guanine nucleotide dissociation inhibitor (GDI) in Ras signaling, could also affect GPCR activity. In addition, bacterial toxins, such as cholera toxin (CTX) or PTX, that ADP-ribosylate the $G\alpha$ -subunit could affect the signaling cycle also. CTX ADP-ribosylates the G_s α -subunit resulting in the inhibition of GTPase activity and subsequent potentiation of agonist signals. On the other hand, PTX, by mediating the ADP-ribosylation of G_i/G_o , inhibits the agonist-induced dissociation of GDP, resulting in the blunting of agonist signals.

In this general scheme of GPCR activation, any perturbation of the receptor from the inactive to active state, such as the binding of an agonist, triggers the GDP/GTP dissociation–association cycle within the heterotrimeric G proteins. By controlling the rate of GDP dissociation and GTP association, and/or the GTP hydrolysis rate, the duration and magnitude of the signals can be controlled.

The association of GTP onto the $G\alpha$ subunit of the heterotrimers results in the generation of two signaling molecules, $G\alpha$ and $\beta\gamma$ subunits, which have been known to activate different effector systems. Agonist activation of an opioid receptor results in the control of several effectors, such as inhibition of adenylyl cyclase [21, 37] and calcium channels activities [38–40], as well as stimulation of K^+ channels [41] and increases in intracellular calcium [42, 43]. In addition to the regulation of MAPKases cascades [44, 45] and the activation of phospholipase D [46], opioid receptor activation has resulted in Akt (PKB) [46] and phospholipase C activation [47–50]. Details of the receptor activating these effectors have been reviewed previously [51]. In the current chapter, we will briefly summarize the overall signaling process and examine recent models of opioid receptor signaling.

9.2 The Classical Mechanism of Opioid Receptor Signaling

9.2.1 Activation of Multiple Heterotrimeric G Proteins

As discussed earlier, since opioid receptors are members of the GPCR family, activation of an opioid receptor results in signal transduction via the heterotrimeric G proteins. The multiple effector systems that are regulated by the receptors could represent

different G proteins that are activated by the receptor, and subsequently, specific $\text{G}\alpha$ - and $\beta\gamma$ -subunits participate in the receptor signaling. The promiscuity of opioid receptors coupling to various heterotrimeric G proteins is well documented. Using either ^{32}P -azidoanilido GTP to label or CTX-mediated ADP-ribosylation to mark the agonist-induced dissociation of the $\text{G}\alpha$ subunits, we have shown that MOR, DOR, and KOR can activate all the Gi/Go with equal potency [52–57].

These G proteins can be co-immunoprecipitated with MOR from brain membrane [58]. In addition to the Gi/Go heterotrimers, in cells in which $\text{G}\zeta$ was over-expressed, opioid receptors activated these PTX-insensitive G proteins [59]. Opioid receptors also were reported to activate G12- and G16-proteins [60–63]. Even with such promiscuity, there appears to be selectivity in the G proteins involved in activation of specific second messenger systems. Studies with $\text{G}\alpha$ -specific antibodies suggest that $\text{G}\iota_2$ mediates DOR inhibition of adenylyl cyclase in NG10815 cells [64], whereas $\text{G}\alpha$ mediates MOR inhibition of enzyme activity in SHSY5Y cells and brain membrane [65].

Similar antibody studies indicate that $\text{G}\alpha$ mediates MOR and DOR inhibition of voltage-dependent Ca^{2+} channels [38, 66]. The ability of opioids to inhibit the Ca^{2+} channels in DRG neurons from $\text{G}\alpha \alpha$ -subunit knockout mice was impaired [67]. Although some studies suggest that an opioid receptor, such as DOR, activates one specific G protein ($\text{G}\iota\alpha 1$) more efficiently than the other ($\text{G}\iota\alpha$) [68], these studies and others appear to support the association of specific G proteins with specific regulation of effectors.

However, more than one type of G protein appears to be mediating the same opioid receptor signal. For example, when $\text{G}\zeta$ was co-expressed with either opioid receptor, the agonist-induced inhibition of adenylyl cyclase activity was PTX-resistant [69–71] and physical interaction between the receptor and $\text{G}\zeta$ was demonstrated by co-immunoprecipitation [72]. These studies suggest that, in addition to the Gi/Go proteins, $\text{G}\zeta$, the PTX-insensitive G proteins primarily expressed in neuronal tissues, can mediate opioid receptor-inhibition of adenylyl cyclase activity.

Another example of more than one G protein being involved in mediating the same opioid receptor signal is the agonist-mediated activation of phospholipase C PLC β . Using antisense oligodeoxynucleotides, $\text{G}\iota_2$ has been shown to mediate opioid-induced intracellular Ca^{2+} mobilization in ND8-47 neuroblastoma \times DRG hybrid cells [73]. However, co-injection of $\text{G}\alpha$ and receptor RNAs into *Xenopus* oocytes suggests that $\text{G}\iota\alpha_1$ is required for an opioid-induced Ca^{2+} -dependent chloride current [74]. Additionally, co-expression of MOR with $\text{G}\alpha 16$ in Cos-7 cells resulted in opioid agonist-induced Ca^{2+} mobilization via PLC β activation that was PTX-insensitive [75].

These divergent results could stem from the approaches used in defining the G protein involved in opioid receptor signaling. The use of antibodies or antisense oligonucleotides to blunt the responses may generate artifacts due to nonspecificity of the antibodies or instability of the oligonucleotides. The over-expression of the Gi/Go α -subunits to restore the responses also may lead to artifacts due to the relatively high level of $\text{G}\alpha$ -subunits being expressed, thereby eliminating any receptor-G protein selectivity.

This is best exemplified by the over-expression of various PTX-insensitive G α subunits to restore opioid receptor-inhibition of adenylyl cyclase after PTX treatment. By mutating the Cys residues serving as the PTX ADP-ribosylation sites within the various Gi/Go α subunits to either Gly or Ile, Clark et al. were able to rescue the PTX-blunting of the MOR-inhibition of adenylyl cyclase activity with either G α o , G α i , G α q_1 , or G α q_3 in C6 glioma cells [76, 77]. This is surprising since mutation of Cys to different amino acids has produced different efficacies in mutated G α -subunits [78].

In contrast, by controlling the expression levels of various Gi/Go α -subunits using the adenovirus-mediated transfer of PTX-insensitive G α -subunits, Zhang et al. demonstrated that only the PTX-insensitive mutant of G α q_2 was able to restore the DOR-mediated adenylyl cyclase inhibition in neuroblastoma N2A cells [79]. Similar observations were obtained with MOR expressed in N2A cells. Using receptor-G α fusion protein constructs, Moon et al. reported different levels of GTP γ S binding upon agonist stimulation between G α i and Go [68]. Therefore, there appears to be some G protein selectivity within the signal transduction processes.

The promiscuity in opioid receptor-G proteins interaction with G protein selectivity in effector activation can be reconciled by the involvement of both G α and G $\beta\gamma$ subunits in transducing the receptor signals. G $\beta\gamma$ -subunits mediate DOR-mobilized intracellular Ca $^{2+}$ via the PLC β pathway in NG1081-15 [80]. G $\beta\gamma$, rather than G α o , is responsible for the inhibition of Ca $^{2+}$ channels [81, 82]. G $\beta\gamma$ subunits also mediate the stimulation of the MAP kinase cascade by opioid receptors [83]. Since different G β subunits determine GPCR coupling to the same effector [84], multiple G proteins could participate in opioid receptor regulation of the same effector, depending on the G $\beta\gamma$ composition.

9.2.2 *Effectors Regulated by Opioid Receptors via the G α Subunits*

Among all effectors, adenylyl cyclase is generally accepted to be the one regulated by opioid receptors via the G α -subunits. Acutely, opioid agonist inhibits adenylyl cyclase activity. However, after prolonged agonist treatment, adenylyl cyclase activity increases above the control level when the agonist is removed. This is generally known as adenylyl cyclase superactivation (AC superactivation). The initial observation of an opioid agonist inhibiting adenylyl cyclase activity was reported by Collier and his co-workers [85, 86] and later defined in detail by Law et al. [87], with brain membrane homogenates.

Sharma et al. substantiated receptor-inhibition of adenylyl cyclase activity, with the cell line model of NG108-15 cells [37, 88]. The important aspect of adenylyl cyclase inhibition in opioid agonist in vivo action was demonstrated with the enhancement of morphine activity by over-expression of type 7 adenylyl cyclase in

mice [89] and the attenuation of MOR activities in mice lacking type 5 adenylyl cyclase [90].

As discussed earlier, either Gi/o or Gz can mediate opioid receptor-inhibition of adenylyl cyclase activity. With at least nine cloned isoforms of mammalian adenylyl cyclase exhibiting diverse sensitivities to G α or G $\beta\gamma$ subunits, Ca $^{2+}$ and protein kinases [91], it is not surprising that opioid-stimulation of adenylyl cyclase has been observed. Opioid agonists stimulated adenylyl cyclase in brain membranes [92], F-11 neuroblastoma-sensory neuron hybrid cells [93], olfactory bulbs [94], and spinal cord-ganglion explants [95]. These opioid stimulatory effects may be the result of G $\beta\gamma$ stimulating type 2, 4, and 7 adenylyl cyclases.

Stimulation of type 2 adenylyl cyclase requires the presence of GTP-bound G α_s [96, 97]. By providing activated G α_s , all three opioid receptors types were able to stimulate cAMP accumulation in transfected cells coexpressing type 2 adenylyl cyclase that was PTX-sensitive [69–71]. This stimulatory mechanism could account for the observed AC superactivation after chronic agonist treatment. However, AC superactivation has been shown to be adenylyl cyclase isozymes-selective, with type 1, 5, 6, and 8 exhibiting superactivation [98–100].

Type 2 and 8 adenylyl cyclase activities have been reported to be inhibited by various combinations of G $\beta\gamma$ [101, 102]. Reports have suggested direct interaction between opioid receptors and G α_s , thereby accounting for the AC superactivation [93, 103–105]. However, this cannot be the case. By using type 2 adenylyl cyclase activity as an index of G protein activation, MOR has been shown to couple to six members of the Gi/Go subfamily, but not to Gs [69]. Due to the complexity of the adenylyl cyclase types, the roles of the G α and G $\beta\gamma$ subunits will change, depending on the types of adenylyl cyclase present.

The complexity and versatility of the mammalian adenylyl cyclases allow opioid agonists to modulate the enzyme activity via mechanisms other than G α or G $\beta\gamma$. For example, Ca $^{2+}$ /calmodulin activated type 1 and 8 adenylyl cyclases [91]. Studies indicate that intracellular loop 3 of the opioid receptor contains a consensus calmodulin-binding motif and that agonist binding releases the bound calmodulin [106, 107]. This calmodulin binding and release, and the involvement of type 1 or 8 cyclase, could be the basis for opioid-induced intracellular cAMP level elevation in SK-N-SH cells that were shown to be dependent on Ca $^{2+}$ entry and calmodulin activation [108].

Other factors, such as covalent modification of the enzyme molecules or the G protein itself during chronic agonist treatment, also could account for the AC superactivation. Phosphorylation of G $\beta\gamma$, adenylyl cyclase and other molecules involved in receptor desensitization, such as β -arrestin and GRK2/3, during chronic morphine treatment appear to augment the association of these molecules and activation of the adenylyl cyclase [109–111]. Involvement of Raf-1 and other kinases in AC superactivation also has been reported [112, 113]. Depalmitoylation of G α_i was observed during chronic opioid treatment, resulting in the direct association of G α_i with adenylyl cyclase molecules preceding receptor activation [114]. All these observations and others provide alternative mechanisms, in addition to G α and G $\beta\gamma$, in the AC superactivation during chronic agonist treatment.

9.2.3 Effectors Activated by the $\beta\gamma$ Subunits of G Proteins

Voltage-gated calcium channels. The overall action of opioid drugs is to inhibit neurotransmitter release by inhibiting the voltage-gated Ca^{2+} channels and activating the G protein-coupled inward rectifying voltage-gated potassium channels (Kir). All three opioid receptors have been shown to inhibit different types of Ca^{2+} channels in various brain regions. For example, MOR and KOR inhibit N- and P/Q-type Ca^{2+} channels in the nucleus tractus solitarius of the rat [115, 116]. However, MOR, but not DOR or KOR, is responsible for the modulation of Ca^{2+} channel currents in mouse periaqueductal grey neurons [117]. When expressed in NG108-15 cells, cloned MOR is functionally coupled to the ω -conotoxin-sensitive N-type Ca^{2+} channels [118, 119]. On the other hand, cloned MOR and DOR inhibit voltage-activated L-type Ca^{2+} channels via Gi/Go proteins in GH3 pituitary cells [120, 121]. The differences in the Ca^{2+} types being regulated by the opioid receptors could stem from the multiple Ca^{2+} channel subunits that constitute the variety of voltage-gated Ca^{2+} channels, i.e., L-, N-, P/Q-, R-, and T-types. Thus far, it has been shown that MOR inhibits Ca^{2+} channels that consisted of α_{1A^-} , α_{1B^-} , α_{1D^-} or α_{1E} subunits [122–124]. Whether differences in Ca^{2+} channel composition will affect opioid receptor-mediated regulation remains to be elucidated.

Initially, the $\text{G}\alpha$ -subunits were considered to mediate the inhibition of Ca^{2+} channels by opioids. Using injected $\text{G}\alpha$ -subunits [38] and $\text{G}\alpha\alpha$ -specific antiserum [66], Hescheler et al. confirmed that $\text{G}\alpha\alpha$ mediates DOR-inhibition of Ca^{2+} channels. Now, it is accepted that $\text{G}\beta\gamma$ – rather than $\text{G}\alpha\alpha$ subunits mediate opioid agonist-inhibition of Ca^{2+} channels. Expression of $\text{G}\beta\gamma$ in rat sympathetic neurons mimicked GPCR-induced inhibition of Ca^{2+} currents [82]. Similar results were observed when $\text{G}\beta\gamma$ was coexpressed with Ca^{2+} channel subunits in a heterologous expression system [125]. The $\text{G}\beta\gamma$ binding domain on the Ca^{2+} channel has been mapped to the intracellular loop connecting domains I and II of the α_1 subunit [81]. This site contains the Q-X-X-E-R motif that is believed to form part of the $\text{G}\beta\gamma$ docking site. Intranuclear injections of different $\text{G}\beta$ subunits' cDNA into rat superior cervical ganglion neurons indicate that $\text{G}\beta_1$ and/or $\text{G}\beta_2$ subunits account for most of the voltage-dependent inhibition, while $\text{G}\beta_5$ produces minimal inhibition, and both $\text{G}\beta_3$ and $\text{G}\beta_4$ are ineffective in inhibiting N-type Ca^{2+} channels [126]. These data and others suggest that the exact composition of the G protein heterotrimers is important in determining the specificity of GPCR-induced inhibition of Ca^{2+} channels [127]. Although the $\text{G}\beta\gamma$ subunits are responsible for mediating the inhibition of Ca^{2+} channels, the $\text{G}\alpha\alpha$ subunits are indispensable in coupling the opioid receptors to the channels, as indicated with $\text{G}\alpha\alpha^{-/-}$ null mice studies. The ability of opioid agonists to inhibit Ca^{2+} channels in DRG neurons of these null mice was significantly impaired [67].

G protein-coupled inward rectifying K^+ channels. $\text{G}\beta\gamma$ subunits also regulate the G protein-dependent inward rectifying K^+ channels (Kir) that are involved in preventing neuronal excitation or action potentials propagation. Electrophysiological studies have shown that both MOR and DOR, but not KOR, can activate the Kir

channels via PTX-sensitive G proteins in the rat locus coeruleus [41]. However, in the intracellular recordings of substantia gelatinosa neuron preparations, all three opioid receptor types activate Kir currents [128, 129]. The ability of KOR to regulate the Kir channels was demonstrated by the coexpression of KOR and Kir3.1 in *Xenopus* oocytes, via the PTX-sensitive G proteins [130, 131]. These channels, which are located in the periaqueductal gray neurons, can be activated by MOR agonists and are shown to be involved in acute opioid analgesia [132, 133].

The cardioprotective effects of some of opioid agonists, such as TAN-67 for DOR, were shown to be mediated by activation of the Kir channels via Gi proteins [134]. The gating properties of these K⁺ channels were altered after chronic morphine treatment [135]. The identities of the Kir subunits involved in opioid functions were demonstrated clearly with Kir knockout mice. Kir3.2 and Kir3.3 mediate the acute inhibitory effects of opioids at locus coeruleus neurons [136]. Kir3.1 and Kir3.2 are likely to mediate the antinociceptive effects of morphine [137, 138].

Activation of Kir3 channels by opioid receptors appears to be mediated via the G $\beta\gamma$ subunits [139]. For a review of G $\beta\gamma$ regulation of Kir channels, please refer to Yamada et al. [140]. At least 12 distinct channel subunits are responsible for the complexity and diversity of inward rectifying K⁺ channels, with Kir3.1 being a major subunit. Using fusion proteins containing glutathione S-transferase and different N- and C-terminal deletion mutants of Kir3.1, two G $\beta\gamma$ binding sites have been identified [141, 142].

At the C-terminus of Kir3.1, the G $\beta\gamma$ binding domain is composed of two segments [142], one of which contains the N-X-X-E-R motif, similar to that observed in type 2 adenylyl cyclase shown to be critical for G $\beta\gamma$ interaction [143]. Interaction of the C-terminal domains with a small segment of the N-terminus of Kir3.1 resulted in a synergistic binding of G $\beta\gamma$.

Evidence suggests that different G β subunits have distinct efficacies in interacting with the N-terminal domain of Kir3.1 [144]. Since the Kir3 subunits have similar G $\beta\gamma$ interaction domains, any differences in opioid receptors to activate the Kir3 channels could result from the receptors interaction with G proteins having different G β subunits. However, this is unlikely since the specificity of G β subunit interaction with Kir disappears, as demonstrated by different combinations of G $\beta\gamma$ activating the Kir3.1 expressed in *Xenopus* oocytes [145]. Hence, other mechanisms, such as the PIP2 regulation of G $\beta\gamma$ -induced activation of Kir channels [146] or the scaffolding of signaling molecules as discussed in a later section, may participate in the opioid receptor regulation of these channels.

Activation of MAP kinase cascades. The MAP kinase pathways are comprised of three protein kinase cascades, i.e., the extracellular-signal regulated kinases (Erks), Jun N-terminal kinases (JNKs) and p38 kinases (review by Garrington and Johnson [147]). Activation of endogenous or heterologous expressed MOR, DOR, or KOR in various cell models has resulted in the activation of Erk1 and 2 [44, 45, 148–153], JNKs [154–156] and p38 [157–159].

Furthermore, opioid receptors have been reported to regulate brain Erk1/2 activity, as demonstrated by the in vitro opioid activation of Erk1/2 in the ventral tegmental area [160] and by the in vivo activation of Erk1/2 at distinct brain regions

[161–163]. In vivo chronic morphine studies suggest that Erk1/2 activity diminishes with treatment, but is dramatically increased during morphine withdrawal. Erk1/2 activation also appears to participate in the morphine rewarding effect in mice [164]. These studies implicate the role of Erk1/2 in the expression of chronic opioid effects. This was demonstrated by the ability of the Erk1/2 inhibitor PD98059 to attenuate opioid receptor desensitization, receptor phosphorylation, and internalization in cell models [165, 166]. However, persistent activation of Erk1/2 with glutamate and paclitaxel resulted in the blockade of agonist-induced DOR internalization [167]. There does not appear to be a correlation between Erk1/2 activation and AC superactivation activity after chronic agonist treatment [168]. Therefore, the exact role of Erk1/2 in chronic opioid effects remains to be determined.

Among the three kinases in MAP kinase pathways, Erks activation by opioids is the best understood. As expected, opioid activation of Erk1/2 occurs through the G $\beta\gamma$ subunit and in a Ras-dependent manner [83]. In jurkat cells stably expressing DOR, the opioid activation of Erk1/2 is Ras-independent [169]. The G protein most likely involved is the Go protein, as demonstrated by dominant negative form and RNAi studies [170].

However, there appears to be multiple mechanisms in opioid receptor activation of Erk1/2. These mechanisms can be divided into G protein-dependent and G protein-independent. The time course of G protein-dependent mechanisms is rapid, while that of G protein-independent mechanisms is slower. Coscia and his co-workers have suggested that, similar to the β_2 -adrenergic receptor, opioid receptor internalization is a prerequisite for Erk1/2 activation [171, 172]. In the case of the β -adrenergic receptor, receptor internalization is required because β -arrestin is involved in scaffolding the kinases participating in the Erk1/2 activation (review by Lefkowitz and Shenoy [173]). Similar β -arrestin-dependent activation of Erk1/2 and p38 by opioid receptors has been reported [157, 174, 175].

However, several laboratories have reported that opioid activation of these kinases does not require agonist-induced receptor internalization [176–179]. Coscia and his workers also suggested that opioid activation of Erk1/2 is mediated by a calmodulin-dependent transactivation of the epidermal growth factor receptor [180–182]. Others have not observed this transactivation, as reflected in phosphorylation of the tyrosine receptor kinases [183]. However, G protein-dependent activation of various subtypes of protein kinase C (PKC) in opioid regulation of Erk1/2 [169, 174, 184] and PKA in opioid activation of p38 [185] has been observed. Activation of Erk1/2 in astrocytes by DAMGO requires the activation of PKC ϵ via calmodulin, while activation of Erk1/2 in the same cells by U69,593 requires the activation of PI3 kinase and PKC ζ [184]. Since MOR can activate PKC α , PKC ϵ and PKC ζ isoforms [186], the reason for DAMGO selectively activating only one of these PKC isoforms is not obvious.

Activation of Erk1/2 has been linked to cell survival and proliferation. Opioid receptors have been shown to be involved in both apoptosis and cell survival [187–195]. Activation of MOR and KOR in embryonic stem cells, or MOR and DOR in adult hippocampal progenitor cells, promotes proliferation of these cells that is Erk1/2-dependent [77, 193, 196].

Thus, it follows that opioid receptor activation alters the activities of Erk1/2 cascades. Among the multiple kinases activated in Erk1/2 signaling cascades, opioid agonists have been reported to activate the phosphoinositide-3-phosphate (PI3)-dependent kinase, Akt (PKB), the p70 and p85 S6 kinases, and subsequent transcription factors such as Elk-1 and AP-1 complexes [149, 197–202]. In addition, the adaptor protein p52 Shc was tyrosine-phosphorylated upon DOR activation in Rat-1 fibroblast cells [203]. Collectively, the activation of Erk1/2, S6 kinase, PI3kinase, and Shc proteins provides a strong mitogenic signal for opioids to regulate cell growth. Activation of Erk1/2 and subsequent kinases could also be the basis for the observed cardioprotection effect of opioid pretreatment [159, 204, 205].

Activation of phospholipases and intracellular Ca²⁺ homeostasis. Direct activation of PLC β by Gq or G16 α -subunits is well documented. Gi/Go α subunits are not known to activate these phospholipases' activities [206]. Thus, the original observation that DOR activation results in mobilizing intracellular Ca²⁺ in NG108-15 cells was totally unexpected [43]. Since then, the ability of opioid agonists to stimulate IP3 production and mobilize intracellular Ca²⁺ has been demonstrated with human neuroblastoma SHSY5Y cells [42, 49, 207], human epithelial tumor cells [208], smooth muscle [209] and spinal cord [210].

Similar activation of phospholipase C, and thus an increase in intracellular Ca²⁺ transient, was observed with the heterologous expression of cloned opioid receptors in neuroblastoma cells [211], CHO cells [212], Ltk⁻ cells [70] and HEK293 cells [213]. PLC β has been implicated in opioid-mediated pain control in PLC inhibitors studies [214], antisense oligonucleotide studies [214, 215] and PLC β knockout mice studies [216, 217]. A PTX-sensitive PLC pathway appears to mediate the arrhythmogenic effect of KOR agonists in isolated rat heart [218].

In T cells, activation of DOR stimulates Ca²⁺ mobilization [219] and enhances IL-2 secretion [220]. Since an increase in PKC and Ca²⁺-dependent protein kinase activity usually follows an increase in intracellular Ca²⁺, activation of PLC leading to the stimulation of these kinases' activities has been suggested to be a mechanism for chronic opioid drug action. The activity of Ca²⁺/calmodulin-dependent protein kinase II (CaMK II) in the rat hippocampus is stimulated by morphine [221], and CaMK II was implicated in the phosphorylation and subsequent desensitization of MOR [222, 223] and DOR [224]. PKC was shown to translocate and participate in MOR down-regulation during chronic agonist treatment [186, 225]. PKC can mediate phosphorylation and endocytosis of DOR [226].

Phosphorylation of G α 1 and G α 2 by PKC was reported to be the basis for heterologous desensitization in Ca²⁺ mobilization [227]. The cross-talk between the PLC pathway and the adenylyl cyclase pathway [228, 229], and the increase in adenylyl cyclase activity during chronic agonist treatment, are related to PKC activities [230]. However, whether such PKC-mediated events have functional roles in chronic agonist responses is debatable, due to a feedback mechanism regulating PLC activities. PKC-mediated phosphorylation of PLC β_3 has been demonstrated to rapidly attenuate opioid-induced IP3 turnover in NG108-15 cells [231]. This feedback mechanism may limit the increase in PKC activities and the subsequent involvement of PLC β in the chronic actions of opioids.

The identities of the G protein subunits involved in PLC regulation have been debated. The involvement of Gi/Go in regulating the PLC activities was demonstrated by the ability of PTX to block these responses. Gi2 was identified to be responsible for PLC regulation, in studies using ND8-47 neuroblastoma × DRG hybrid cells with antisense [73].

When receptor and G α subunit mRNAs were co-injected into *Xenopus* oocytes and the chloride current measured, and in reconstitution studies with guinea pig cerebellum, G α 1 was identified to be involved in regulating the PLC activity [74, 232]. Since relatively high opioid agonist EC50 values were observed with PLC activation, and Gi/Go α subunits have a low affinity for PLC β , it has been accepted that G $\beta\gamma$ subunits mediate the regulation of PLC activity by opioids. The involvement of G $\beta\gamma$ subunits was demonstrated in the opioid-activated PLC β activity in both intestinal smooth muscle [209] and NG108-15 cells by the ability of the injected G $\beta\gamma$ -binding peptide (QEHA), but not the Gq-binding peptide (QLKK), to block the opioid response [80].

Opioid-induced intracellular Ca²⁺ increases may involve mechanisms other than G $\beta\gamma$ -activated PLC. In single-cell-fluorescence measurements using Ca²⁺ sensitive dye, the majority of the cells did not respond to the agonist addition. Only when chimera G proteins, such as Gq/Gi, or promiscuous G proteins, such as G α 16 were used, were robust responses to opioid agonists observed [61, 62, 75, 233].

However, the use of G protein chimeras or G α 16 resulted in responses that were PTX-insensitive. Moreover, this appears to depend on the cell line and whether the intracellular Ca²⁺ fluorescence was measured with suspended or attached cells. Furthermore, recent reports suggest that Gi/Go-coupled receptors, such as the opioid receptors, increase intracellular Ca²⁺ release only in the presence or after the pre-activation of Gq-coupled receptors [234–236]. Since G $\beta\gamma$ subunits have been implicated in opioid receptor action, the coincident signaling between the Gq-coupled receptor and the opioid receptor suggests that the binding of the G $\beta\gamma$ subunits at the N-terminal PH domain affects the interaction of Gq α -subunits with the C2 domain of the PLC β 3, thus potentiating the Gq α -subunit activity. This mechanism for the G $\beta\gamma$ potentiation of Gq α -subunit activities has been demonstrated with purified PLC β [206]. However, an increase in IP3 production in SHSY5Y cells did not parallel an intracellular Ca²⁺ increase [235]. The transient expression of the constitutive active Gq α -subunit does not substitute for an activated Gq-coupled receptor in opioid agonist-induced intracellular Ca²⁺ release (Law, unpublished observation).

Therefore, mechanisms other than co-activation of PLC may be involved in opioid-induced Ca²⁺ increases in some cell types. Ca²⁺ influx via the L-type Ca²⁺ channels was suggested to be the mechanism for MOR-mediated PLC activation in SHSY5Y cells [50]. DOR regulation of intracellular Ca²⁺ transient in human neuroblastoma SK-N-BE cells could be mediated by the ryanodine receptor and is PTX-insensitive [237]. These are the probable pathways involved. However, a more plausible explanation for the coincident signaling between Gi/Go- and Gq-coupled receptors is in the regulation of IP3-receptor activity. Phosphorylation of the IP3-receptor by PKC results in the ability of G β to activate Ca²⁺ release from the IP3-sensitive stores [238]. Thus, opioid agonists

could regulate the intracellular Ca^{2+} pools without directly activating PLC. Whether this is the mechanism remains to be demonstrated.

9.3 Classical Regulators of Opioid Receptor Signaling

9.3.1 Receptor Desensitization and Internalization

Since opioid receptors are members of the GPCR family and rhodopsin sub-family of receptors, mechanisms similar to those reported with other sub-family members, notably those reported with the β -adrenergic receptor, appear to apply to the regulation of opioid receptor signaling.

Lefkowitz and his co-workers have proposed and described a model for terminating the GPCR signaling in several review articles [173, 239, 240]. The model is summarized in Fig. 9.2. In this model, the binding of agonists results in the rapid phosphorylation of the receptor by protein kinases, including the G protein-coupled receptor kinases (GRKs), thereby promoting the association of the cellular protein β -arrestin. Association of β -arrestin with the receptor uncouples the receptor from the respective G protein, thus blunting the receptor signaling (receptor desensitization).

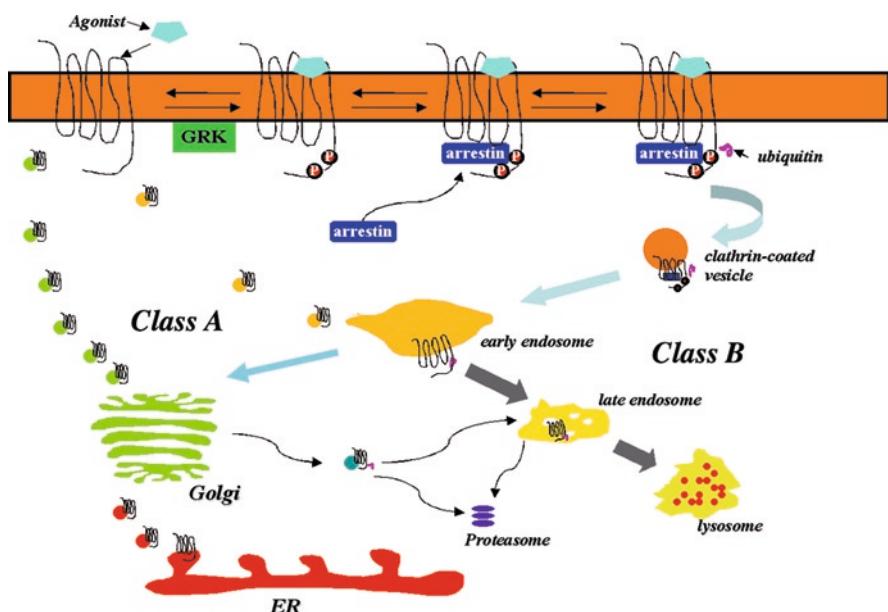


Fig. 9.2 Model for the regulation of GPCR activity

In addition, β -arrestin is involved in the agonist-induced, clathrin-coated vesicles-mediated receptor internalization. Internalized receptors, such as those classified as Class A receptors in Fig. 9.2, can resensitize and recycle back to the cell surface, where receptor signaling is continued. Other internalized receptors, such as those classified as Class B receptors in Fig. 9.2, recycle slowly to the cell surface, and their trafficking can be directed to other subcellular compartments, such as lysosomes, for degradation.

Eventually, with prolonged agonist exposure, there is a decrease or down-regulation of the overall cellular receptor content. β -arrestin itself serves as an adapter molecule in β_2 -adrenergic receptor signaling. In this case, a receptor-src kinase complex is formed through which the β_2 -adrenergic receptor activates the MAPkinase Erk1/2 [241]. The scaffolding of β -arrestin with the MAP kinase complexes also determines the eventual cellular location of the activated kinases, thereby provide another layer of receptor signaling processes.

Opioid receptor signaling is regulated by a mechanism similar to that of the β_2 -adrenergic receptor. Detailed discussion on the adaptational processes that occur during chronic agonist exposure can be found in subsequent chapters of this book. In general, several laboratories have reported rapid phosphorylation of MOR, DOR, and KOR [242–247]. The agonist-induced phosphorylation of DOR [247], MOR [242, 244] and KOR [248] suggest that the phosphorylation of the opioid receptor is at the carboxyl terminal Ser/Thr, and is mediated via GRKs and not by PKC.

Reports suggest that Tyr residues within MOR or KOR can be phosphorylated in the presence of an agonist and that phosphorylation of the receptors appears to be cellular background-dependent. Yu et al. reported that morphine can induce MOR phosphorylation in CHO cells [243], while Arden et al. and Zhang et al. reported that morphine could not induce MOR phosphorylation in HEK293 cells [245, 249].

The morphine-receptor complex is a poor GRK substrate, as demonstrated by the ability of over-expressed GRK-2 to phosphorylate MOR in HEK293 cells during morphine treatment [249]. The phosphorylation of the receptor has resulted in the subsequent recruitment of β -arrestin, causing agonist-induced receptor internalization and desensitization. The failure of morphine to induce receptor phosphorylation, and hence subsequent β -arrestin recruitment, has been the explanation for the inability of this agonist to induce MOR internalization. This hypothesis is supported by observations that morphine could not induce robust translocation of β -arrestin to the plasma membrane, and that over-expression of β -arrestin could rescue morphine's inability to induce MOR internalization [250, 251].

However, agonist-induced phosphorylation of other GPCRs results in an increase in the receptors' affinities for β -arrestin. Without phosphorylation, β -arrestin can interact with the agonist–receptor complexes. The recruitment of β -arrestin by non-phosphorylated opioid receptors was illustrated by studies in which the putative agonist-induced phosphorylation sites in DOR after Ser344, or in MOR after Ser363, residues were removed.

Agonists could induce endocytosis, which is a β -arrestin-dependent process [252, 253]. Opioid agonists could induce the translocation of the β -arrestin-GFP

fusion protein from the cytosol to the plasma membrane in truncated carboxyl mutants of MOR or in Ser/Thr phosphorylation mutants of DOR [253, 254]. The BIACORE studies reported similar arrestin-receptor interactions [255]. These arrestin–receptor interactions, which are independent of receptor phosphorylation, could explain the ability of morphine to induce MOR internalization in primary striatal neurons and not in hippocampal neurons [256, 257]. The ability of non-phosphorylated receptor-agonist complexes to interact with β -arrestin could also account for the observation of blunted morphine tolerance development in β -arrestin2 null mice [258, 259].

9.3.2 *Regulators of Receptor Signaling*

RGS. In the classical view of GPCR signaling, as outlined in Fig. 9.1, termination of the signals can be accelerated by an increase in GTPase activity within the $G\alpha$ subunits by proteins that exhibit GAP activities. In recent years, more than 25 proteins that exhibit negative regulatory roles in G protein signaling, i.e., RGS, have been discovered. Early studies on RGS proteins indicated that they could act on multiple equivalents of GTP-bound $G\alpha_i/o$ or $G\alpha_q$, to increase the rate of GTP hydrolysis by these G proteins [260, 261]. They all share a 130 a.a. RGS domain that allows them to interact with $G\alpha$ subunits and accelerate termination of the signaling. They can be grouped into five distinct subfamilies according to their structures and genetic similarities: Rz, R4, R7, R12, and RA [261]. In most cases, effects of individual RGS on $G\alpha$ GTPase activities can be observed in solution, in the absence of any other proteins. However, reconstitution of RGS and G proteins, together with GPCR, in phospholipids vesicles resulted in an increase in both the RGS affinity for the G proteins and the degree to which it accelerated GTP hydrolysis [262, 263].

These data and others suggest that individual GPCRs independently can regulate RGS- $G\alpha$ GTPase activities. Some of the RGS proteins, such as those in the R7 subfamily (RGS6, RGS7, RGS9, RGS11), have multiple domains that can facilitate interaction with GPCRs. The R7 subfamily contains a GGL or $G\gamma$ -like domain that forms a stable complex with the G protein $G\beta 5$, and also can bind to other proteins [264–266]. It also contains the Disheveled/EGL-10/Plextrin homology (DEP) domain that serves as an interface for other proteins. The DEP domain allows the RGSs to target their G proteins and specific GPCR signaling systems via DEP domain-associated syntaxin-like proteins called R7BP and R9AP [267–269]. The scaffolding of RGS with proteins such as R7BP, sphinophilin or 14-3-3 could regulate the cellular locations and RGS association with signaling proteins such as GPCR effectors, resulting in the alteration of GPCR signals by RGS [267].

RGS can regulate both the *in vitro* and *in vivo* activities of opioid receptors. RGS2 and RGS3 increase, while RGS4, RGS9-2, and RGS16 decrease, MOR activities [270, 271]. In addition, over-expression of the RGS domain can modulate *in vitro* MOR activities [272]. Similar to their selectivity toward various GPCRs,

RGS also appear to exhibit selectivity toward the opioid receptors. RGS-Rz members selectively inactivated $\text{G}\alpha_z$, regulating MOR, but not DOR, activities; and RGS-Rz members co-immunoprecipitated with MOR, but not MOR in PAG [273–275]. This selectivity is supported by RGS4, RGS9-2, RGS14, RGSZ1, and Z2 physically associating with MOR in PAG; while RGS2, RGS7, RGS10, and RGS11 could not [276]. However, direct pull-down assays with GST fusion proteins indicate that the carboxyl tail and intracellular loop 3 domains of DOR, in addition to carboxyl tail domain of MOR, can interact with RGS4 [277]. Thus, RGS proteins also should regulate DOR activities. As for the *in vivo* regulation of opioid receptor activities, an increase in RGS4 level in the spinal cord corresponded to the development of hyperalgesia [278].

The knockout of RGS9-2 in mice resulted in an increase in sensitivity to morphine and to physical dependence and addiction to the drug, and a decrease in tolerance development [279]. The results of this knockout mice study agree with those observed using a knockdown of RGS9 with the antisense oligonucleotide approach. This approach resulted in an increase in morphine analgesia and a decrease in analgesic tolerance [270]. Since the mRNA and protein levels of these RGS were altered during acute and chronic morphine treatment [279–281], the direct modulation of opioid receptor signaling by RGS could contribute to analgesic tolerance development. In the case where RGS9-2 levels can be elevated within 2 h of morphine injection [279], post-transcriptional regulation of the RGS levels may contribute to the acute tolerance development generally observed. Although the exact mechanism is unknown, such post-transcriptional regulation could involve the sumoylation of the RGS proteins, such as that in RGSZ2, resulting in the increase in $\text{G}\alpha_i$ and $\text{G}\alpha_z$ interaction [282], or involve the correct cellular targeting and stability of RGS proteins by their interacting partners, such as R7BP's effect on RGS9-2 [283, 284].

AGS. An alternative mechanism by which GPCR signaling can be regulated is by a novel class of proteins that can directly regulate the activation state of G proteins. Using the pheromone response pathway in *Saccharomyces cerevisiae* to screen the mammalian expression library, and using growth as the readouts, ten cDNAs were shown to affect growth in a $\text{G}\beta\gamma$ -dependent manner, and therefore were classified as receptor-dependent activators of G protein signaling (AGS).

These proteins can be sub-divided into three classes: Group I, consisting of AGS1, whose activities can be antagonized with RGS4; group II, consisting of AGS3–AGS6, the activities of which depend on the $\text{G}\alpha$ subunits (active with $\text{G}\alpha_i$ and $\text{G}\alpha_s$, but not with $\text{G}\alpha\alpha$ or $\text{G}16\alpha$); and group III, consisting of AGS2 and AGS7–AGS10, the activities of which are independent of the $\text{G}\alpha$ present [285]. With the exception of those in Group II that possess the G protein regulatory motif GPR, these AGS proteins have no generally conserved structural features. AGS1, a Ras-related protein also known as RASD1, interacts with $\text{G}\alpha\alpha$ and increases $\text{GTP}\gamma\text{S}$ binding to purified G proteins; while AGS2 and AGS7–AGS10 interact with the $\text{G}\beta\gamma$ -subunits. These AGS proteins could act by (1) serving as a guanine nucleotide exchange factor, thereby promoting G protein activation; (2) interfering with subunit interaction during nucleotide exchange and hydrolysis; (3) promoting subunit

dissociation, independent of nucleotide exchange; and (4) forming stable complexes with G α replacing the roles of G $\beta\gamma$ subunits. These AGS proteins also could regulate G protein functions by serving as scaffolds and chaperones for G proteins.

Among all the AGS, AGS3 has been shown to be enriched in neurons and to regulate G protein signaling in the nucleus accumbens (NAc) [286]. An increase in AGS3 level was observed in the prefrontal cortex and in the core region of NAc after three weeks of withdrawal from repeated cocaine treatment. The cocaine sensitization-like phenotype could be induced by elevating AGS3 content in these brain regions. These observations may be due to AGS3 stabilization of the G α_3 -GDP complex prior to the reassociation of the free G α_3 and $\beta\gamma$ subunits [287]. Similarly, AGS3 can potentiate the MOR-mediated activation of PKA in NAc, via the $\beta\gamma$ -directed activation of adenylyl cyclase type II and IV [288]. Interestingly, in rats self-administering heroin, AGS3 antisense in the NAc core, but not the shell, eliminated the reinstatement of heroin-seeking behavior in these animals, paralleling the AGS3 antisense actions on MOR-mediated PKA activation. These studies indicate that the ability of AGS to regulate opioid receptor activities could have broad functional impact.

9.4 Nonclassical and Novel Mechanisms in Opioid Receptor Signaling

In detailing the heterotrimeric G proteins involved in opioid receptor signaling, various biochemical approaches have demonstrated the ability of the receptor to interact with multiple G proteins. The paradoxical findings are that, depending on the cell models, MOR can mediate the same functions via either G i_3 , G α , or G i_2 [79, 272, 289, 290]. These differences may be due to RGS or AGS proteins in the system that modulates the receptor activity. However, there is accumulating evidence to indicate that the location, the proteins that scaffold together, and the ligand-induced signal selectivity alter the GPCR signals. Opioid receptor signaling is no exception. In the following sections, we will examine the various mechanisms that could affect opioid receptor signaling.

9.4.1 Cellular Location of the Receptors

The partition of GPCRs into microdomains, such as lipid rafts, has been shown to have dramatic effects on their signaling [291]. Opioid receptor signaling has been reported to depend on the receptor location within the lipid rafts [292–295]. Extraction of membrane cholesterol with methyl- β -cyclodextrin resulted in receptor redistribution and potentiation of KOR activity, but blunted of MOR activity [292].

The divergence in opioid receptor signaling within microdomains may be due to cellular proteins recruited to the receptor vicinity. Evidence supporting the formation of opioid receptor signaling complexes will be discussed in detail in a later section. Cellular proteins recruited to the receptor vicinity or that function as protein scaffolds also could regulate GPCR activities [296, 297].

An excellent example is the *Drosophila* InaD gene that codes for a protein with five PDZ domains [298]. InaD associates through these PDZ domains with a light-activated Ca^{2+} channel (TRP), PLC β and PKC. The organization of these effectors by InaD allows for the efficient activation of TRP by PLC β , in response to the stimulation of rhodopsin and Gq α and the inactivation of TRP by phosphorylation by PKC. The scaffolding of signaling proteins and GPCRs via interaction with PDZ domains has been well documented. For example, the β_1 -adrenergic receptor associates, via its carboxyl tail, with the post-synaptic density protein-95 (PSD-95) and the membrane-associated guanylate kinase-like protein inverted-2 (MAGI-2) [299, 300].

The β_2 -adrenergic receptor associates with the PDZ domain-containing protein Na^+/H^+ exchanger regulatory factor/ezrin binding protein 50 (NHERF/EBP50) that may control the recycling of the receptor to the cell surface, in addition to controlling agonist-induced intracellular Na^+/H^+ exchanges [301, 302]. Similar interactions and functions of NHERF/EBP50 have been reported with KOR [303, 304]. In addition to static interaction with domains on scaffolding proteins to form initial signaling complexes, activation of GPCRs can recruit cellular proteins that alter the signaling amplitude and duration. An excellent example is the recruitment of β -arrestin molecules after agonist-induced phosphorylation of GPCR.

Initially, β -arrestin molecules were considered to be proteins involved in receptor desensitization [305, 306]. β -arrestin has since been demonstrated to associate with proteins involved in receptor endocytosis [307], such as AP2 [308, 309], ARF6 [310], *N*-ethylmaleimide-sensitive factor (NSF) [311], ARNO [310], Mdm2 [312], Src [241, 313], c-Jun *N*-terminal kinase 3 (JNK3), apoptosis stimulating kinase 1 (ASK1) [314, 315], and Erk1/2 [316, 317]. Since some of these proteins, such as Src and Erk1/2, have been reported to regulate opioid receptor phosphorylation and trafficking [165, 167], the organization of opioid receptors, G proteins and other cellular proteins in microdomains could determine which G proteins are used in receptor signaling [318].

9.4.2 Scaffolding of Cellular Proteins with Receptors and Formation of Signaling Complexes

Scaffolding with cellular proteins. Since the early studies with target inactivation analyses, it has been apparent that opioid receptor size far exceeds the combined molecular weights of the opioid receptor and the heterotrimeric G proteins [319]. The physical association of a ~25 KDa protein, labeled by ^{125}I - β -endorphin, binding to an opioid receptor has been demonstrated [320]. Using either fusion protein pull-down or a yeast two-hybrid system with specific receptor domains, a variety of

cellular proteins, such as sorting nexin 1, GPCR-associated sorting protein (GASP), filament A, protein kinase C-interacting protein (PKC1), phospholipase D2, and GEC, have been shown to form complexes with opioid receptors [321–326].

These receptor–protein interactions can involve consensus motifs other than the PDZ interaction domains found in the receptor sequences. A highly conserved ARF binding motif NP(X)_{2–3}Y is present within the seventh transmembrane domain of the opioid receptor [327]. The interaction of rhodopsin with Rho and ARF via this sequence may be the basis for phospholipase D activation [123]. Activation of phospholipase D by opioid agonists has been reported [46] and is ARF dependent [322]. In the case of opioid receptors, the direct interaction of phospholipase D₂ with the receptor appears to participate in this enzyme’s activation, since yeast two-hybrid assays using the carboxyl tail domain as the bait have demonstrated association with the phospholipase [322].

A consensus sequence for calmodulin binding also was reported to locate at the third intracellular loop. Agonist activation of the receptor results in the dissociation of the calmodulin-receptor complex [106, 107]. The functional significance of the receptor-calmodulin interaction is magnified by the presence of a single nucleotide polymorphism at this interaction domain that can alter basal G protein coupling and calmodulin binding [328].

Although a direct interaction between a specific sequence of receptors was not provided, the isolation of the phosphatidylethanolamine binding protein (PBP) from the morphine affinity column, and the ability of heterologous expression of PBP to enhance opioid receptor and G protein coupling, suggest the possible association of the PBP with opioid receptors [329].

The direct association of opioid receptors with signaling proteins can affect receptor activities. Modulation of receptor activity by protein association is commonly observed with GPCRs. The ability of the angiotensin II AT₁ receptor to regulate Jak/STAT signaling has been demonstrated to be dependent on the direct interaction of the carboxyl terminus of this receptor [330–333]. Association of Jak2 with the AT₁ receptor promotes the phosphorylation of STAT1, the translocation of the complex into nucleus, and the subsequent activating transcription. Similar to the interaction with proteins via PDZ or non-PDZ domains, the direct interaction of Jak2 requires a specific sequence identified in the AT₁ carboxyl tail domain.

Co-immunoprecipitation studies suggest direct interaction of signaling proteins such as RGS with opioid receptors [276, 334]. Endogenous RGS can influence the efficacies and potencies of opioid agonists in C6 glioma cells and also the in vivo function of morphine [270, 272, 273, 275, 279, 335]. Again, in lieu of inactivated receptors recruiting RGS [86], β₅ subunits associating with RGS to affect MOR activities [336], and RGS interacting with known scaffolding proteins such as 14-3-3 and spinophilin [267], it is probable that opioid receptor-RGS interaction results in a larger complex formation that alters the final outcome of the agonist-receptor activation.

This alteration of the outcome of receptor activation can be demonstrated by the direct interaction between MOR and a PKC interacting protein, PKCI. Using a yeast two-hybrid screen with the carboxyl domain of MOR, Guang et al. identified

PKCI as one of the proteins that interacts with the domain, resulting in the reduction of acute agonist inhibition of adenylyl cyclase and blunting the desensitization responses [321]. Interestingly, mice lacking the PKCI gene exhibited greater antinociceptive responses to morphine and a greater extent of tolerance development. Clearly, the receptor activities were altered by direct interaction with PKCI.

The association of opioid receptors with cellular proteins, which regulates their trafficking, also is a mechanism by which receptor activities can be regulated. Removal of the cell surface receptor and the recycling and resensitization of the internalized receptor greatly affect receptor signaling duration and magnitude. The absolute requirement for and association with β -arrestin in receptor endocytosis processes has been demonstrated [249, 251]. The association of β -arrestin requires multiple receptor domains [255] and β -arrestin can serve as a platform for recruitment of additional proteins into the receptor vicinity.

As we will discuss in a later section, the interaction of opioid receptors with β -arrestin also determines the agonist-selective signaling process. Nevertheless, the association of β -arrestin and other proteins with the receptor determines the ability of the agonist to induce receptor internalization. Over-expression of β -arrestin or GRK2 resulted in morphine-induced MOR internalization, which is not normally observed [249, 251]. Therefore, β -arrestin within these protein scaffolds determines the opioid receptor trafficking, as illustrated by morphine's ability to induce MOR internalization in the dendrites, but not the cell bodies, of nucleus accumbens neurons [256].

Other proteins also can regulate the trafficking of opioid receptors. Using a yeast two-hybrid system, Whistler et al. reported specific interactions between GASP and DOR in DOR's lysosomal trafficking [324]. However, GASP does not appear to be selective for DOR. In a later study, Simonin et al. demonstrated the association of GASP with MOR and KOR, among other GPCRs [337]. Probably, GASP is involved in the eventual targeting of internalized receptors to lysosomes for degradation. This function of GASP was best illustrated by its involvement in CB1 down-regulation [338].

Other proteins serve as chaperones for opioid receptors, and thus regulate the eventual targeting at the cell surface. One such protein is GEC-1, also known as GABA_A receptor-associated protein-like and Apg8L [325]. Using a yeast two-hybrid screen with the KOR carboxyl domain, Chen et al. identified GEC1 as a protein that selectively interacts with KOR, and not with MOR or DOR. GEC1 expression enhances the level of mature KOR over immature KOR, thus facilitating the trafficking of KOR from the ER/Golgi to the plasma membrane. GEC1 tightly controls the plasma membrane expression of KOR. We also have identified a protein with a similar function as GEC-1 in MOR trafficking to the membrane.

By partially purifying the MOR receptor complex and using mass spectrometry for proteomic analyses of the complex, we have identified ribophroin 1 (RPN1), a member of the oligosaccharide transferase family that is responsible for N-glycosylation of newly synthesized proteins, as being a critical mediator of the transport of MOR from the endoplasmic reticulum to the cell membrane [339]. By controlling the expression of RPN1 by either over-expression or siRNA, the plasma membrane level of MOR can be altered. Again, RPN1 controls the maturation of MOR, similar to GEC1 controlling KOR maturation.

In addition, in the same proteomic studies, we have identified GRIN1 (G protein-regulated inducer of neurite outgrowth 1) also to associate with the third intracellular loop of MOR serving as a tether between the receptor and the G protein α -subunit [340]. Such action of GRIN1 enables the receptor to be retained within the lipid rafts upon agonist-stimulation and potentiated the agonist effect. Thus, by the association with the proteins involved in receptor maturation and cell surface location, the activity of opioid receptors can be controlled.

Activity controlled by the oligomerization of the receptor. An emerging concept in GPCR signaling is that these proteins are able to oligomerize and alter the signaling processes of the receptors. GPCRs can either homo- or hetero-oligomerize. A clear picture of GPCR existing as dimers was presented in atomic force spectroscopy studies of the disc membrane [341, 342]. The oligomerization of the GPCR not only could alter the ligand selectivity or function, it could also regulate the trafficking of the respective components of the oligomers, as in the case of the cell surface expression of the GABA_B receptor [343, 344].

Opioid receptors are no exception. The oligomerization of opioid receptors can be demonstrated using either co-immunoprecipitation or BRET or FRET studies [345–348]. Intriguingly, the heterodimerization of opioid receptors with each other or with other receptors has resulted in the appearance of unique phenotypes [349–352]. The heterodimerization of DOR and KOR results in a decrease in the affinities of receptor-selective ligands [350, 353]. DOR and KOR also can heterodimerize with β_2 -adrenergic or substance P receptors, resulting in an alteration of these receptors' functionalities [349, 354].

Furthermore, the heterodimerization of MOR and DOR appears to affect both the *in vitro* and *in vivo* activities of MOR-selective agonists [355, 356] and to alter the signaling pathways of the receptors. The activation of ERK1/2 by these receptors individually can be attenuated by PKC inhibitors, but not in cells expressing both MOR and DOR [174].

The β -arrestin-dependent activation of ERK1/2 in cells expressing both MOR and DOR was demonstrated by the use of siRNA knockdown of β -arrestin, and the eventual cytoplasmic location of the phospho-ERK1/2. The putative heterodimerization of MOR and DOR resulting in alteration in phenotypes could be the basis for previously reported observations in which co-administration of DOR selective antagonists, such as naltrindole, TIPP[ψ] or DOR antisense oligonucleotides, resulted in attenuation of morphine tolerance and dependence development [357–359], and the potentiation of morphine activities by DOR selective antagonists in wild type, but not DOR-null, mice [355]. Receptor oligomerization resulting in synergism in receptor activities has been implicated in earlier studies reported by Vaught and co-workers in which Leu⁵-enkephalin potentiated morphine effects [360, 361].

9.4.3 Agonist-Dependent Signaling, or Biased Agonist Signaling

Classic concepts of quantitative pharmacology have been challenged by numerous observations in which GPCR agonists exhibited functional selectivity. An excellent

example of functional selectivity was reported with the serotonin 5HT₂ receptors, which transduce their signals via the Gq-coupled pathways, resulting in an increase in IP3 production via PLC activation and an increase in arachidonic acid release via PLA2 activation [362, 363]. Berg et al. reported that the relative efficacy of a series of 5-HT ligands in activating 5-HT2A or 5-HT2C differed depending on whether PLC or PLA2 activities were measured [364]. Additional functional selectivity has been reported with β₂-adrenergic receptors, Vassopressin 2 receptors, and D2L and D1 dopamine receptors [365]. In most of these cases, the ligands' functional selectivity was derived from the activation of different receptor subtypes. Thus, it is probable that the observed selectivity is caused by partial agonism in one receptor subtype and full agonism in another receptor subtype.

However, functional selectivity can be observed with a single receptor. Using mass spectrometry to identify and quantitate residues phosphorylated in the presence of agonists, Trester-Zeditz et al. demonstrated agonist bias in multiple residues within the carboxyl tail and third intracellular loop of β₂-adrenergic receptors [366]. Biased agonism can be demonstrated with different ligands in the same receptor.

A perfectly biased ligand for angiotensin II type 1 receptor, Sar¹, Ile⁴, and Ile⁸ AngII (SII), was shown to stimulate β-arrestin-dependent activation of ERK1/2 in the absence of detectable G protein-dependent activities [367, 368]. Such G protein-independent signaling of AT1 receptors could be the basis for the *in vivo* activities of mutant AT1 receptors in transgenic mice to produce cardiac hypertrophy and bradycardia [369]. Similar biased agonism was reported with the type 1 parathyroid hormone receptors. Truncated or modified parathyroid hormone-related peptides (PTHrP), PTHrP(2–36) and Bpa¹-PTHrP(1–36) exhibited Gα-mediated activation of adenylyl cyclase activity, but lost both Gq-mediated PLC activation and β-arrestin recruitment [370]. In addition, ligands that were described as inverse agonists for Gα-coupled signals of PTH receptors, such as D-Trp¹² and Tyr³⁴PTH(7–34), were shown to stimulate β-arrestin signaling in the absence of Gα or Gq signals [371]. Thus, these studies and others point to ligand-selective signaling of GPCR.

Opioid receptors also appear to be ligand-selective in their signaling. As summarized earlier, the putative hetero-oligomerization of MOR and DOR can direct the pathway-selective activation of ERK1/2 [174]. Agonist-selective activation of opioid receptors was demonstrated by the weak activity of morphine to induce MOR phosphorylation and internalization, in contrast to ligands such as etorphine or DAMGO [245, 372].

Mutation of the MOR intracellular loop 3, a putative G protein-interacting domain, resulted in variable attenuation of the potencies of different MOR agonists [373]. Recently, a novel compound, herkinorin, was shown to produce *in vivo* antinociceptive effects with minimal tolerance development, possibly due to its inability to recruit β-arrestin [374]. These results suggest probable receptor conformational differences between the morphine- and etorphine-receptor complexes. Differences in the ability of PKA to phosphorylate morphine- and DAMGO-receptor complexes suggest probable differences in agonist-receptor conformation [375].

Biased agonist signaling of opioid receptors can be demonstrated further with a single signaling pathway. One of our recent observations indicated that agonists such as morphine, etorphine, fentanyl and methadone can select for the signal pathways to elicit the same effect. Similar to other GPCRs, MOR activation of Erk1/2 can be mediated via a G protein- and PKC-dependent, or via the β -arrestin-dependent, pathway. However, in contrast to other GPCRs, all these agonists' activation of Erk1/2 can be blocked by PTX pretreatment and are mediated by the same G protein, G_i_2 . Divergence occurs after activation of the heterotrimeric G proteins.

In contrast to other earlier observations that PKC-mediated Erk1/2 activation preceded β -arrestin-mediated activation [174], morphine- and etorphine-mediated Erk1/2 activation exhibit similar kinetics; and these agonist activities can be blocked by PKC inhibitors or by depleting β -arrestin in the cellular content, respectively (Fig. 9.3).

Differences in the pathway selected by these agonists also are reflected in the eventual cellular location of the activated Erk1/2. For morphine, the activated Erk1/2 remains in the cytosol, as indicated by the phosphorylation of one of the Erk1/2 substrates, p90RSK, and the subsequent nucleus translocation of p90RSK and phosphorylation of the transcriptional factor CREB. In the case of etorphine activation, the activated Erk1/2 translocates into the nucleus, as reflected by the phosphorylation and activation of transcriptional factor Elk-1, followed by an increase in the transcript levels of GRK2 and β -arrestin2, two transcripts that could be controlled by Elk-1 activity.

Among the agonists tested, fentanyl is similar to etorphine, while methadone is similar to morphine, in their pathway selectivity. Further, agonist selectivity in ERK1/2 activation can be demonstrated in cell models that express MOR endogenously, such as human neuroblastoma SHSY5Y cells, or in primary hippocampal neurons. Although the exact mechanism is unknown, the demonstration of agonist-selectivity introduces another layer of complexity in the regulation of opioid

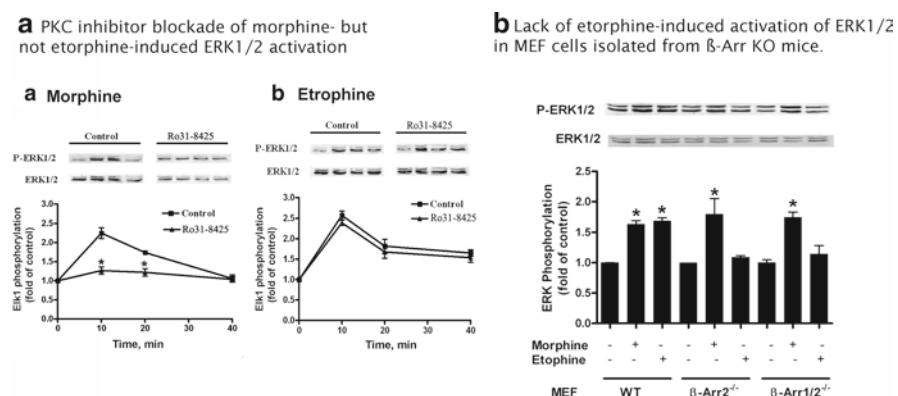


Fig. 9.3 Morphine and etorphine activate ERK1/2 via different pathways. (a) PKC inhibitor blockade of morphine but not etorphine ERK1/2 activation mice. (b) Lack of etorphine activation of ERK1/2 in MEF cells isolated from β -Arr KO

receptor signaling. This can be demonstrated by the pools of microRNAs (miRs) that are regulated differentially by the agonists. Using a miR array, and the primary hippocampal neuron cultures or isolated hippocampi from mice treated either with morphine or with fentanyl, we could demonstrate that these two agonists differentially regulate the miRs, one of which is miR-190, that has significant effects on subsequent targets such as NeuroD, a transcription factor involved in neural differentiation and maturation [376]. Hence the agonist-dependent pathway selective signaling of opioid receptors could lead to differential responses in neuron maturation.

Perspectives. The original model in which GPCR serves as a GTP/GDP exchanger remains unchallenged. However, the linear relationship between receptor activation and cellular responses appears to be dissolving. This is due to the continuous evolution of the events that follow the activation and dissociation of the heterotrimeric G proteins. There is no question that opioid receptors, similar to other GPCRs, exist as oligomeric units with other cellular proteins, and possibly with each other.

Experiments with the yeast two-hybrid, protein pull-down assays clearly indicate that multiple proteins can interact with the receptors, thereby modulating their functions. Some of these proteins, such as β -arrestin, RGS or AGS, actually can modulate the amplitude and duration of the signals, and serve as scaffolds for other cellular proteins. Thus, they could be critical in the eventual blunting or prolonging of receptor signals. As will be discussed in detail in a later chapter of this book, the hetero-oligomerization of opioid receptors may alter receptor selectivity toward signal molecules. Evidence supports location-dependent signaling. Our and others' recent observations that agonist bias exists in opioid receptor signaling processes add another layer of complexity.

One could imagine that, depending on the agonist used to activate the opioid receptor, eventual cellular responses could be different, even though the same effector is being activated by the various agonists. By manipulating the interacting partners or scaffolding proteins, the overall signaling of the receptor could be different. In one way, this signaling complexity could be an alternative explanation for the observed diverse pharmacological responses exhibited by opioid agonists, without evoking the concept of multiple spliced variants of the same receptor gene.

One could imagine that, within a simple response such as Erk1/2 activation, different opioid agonists exhibit pathway selectivity, and the cross-talk between signaling pathways and the various kinases that are activated can modulate and result in different overall responses to various agonists. Whether various agonists would elicit different receptor conformations or different post-translational modifications, such as phosphorylation, ubiquitination or nitrosylation, or affect the eventual receptor location remains to be investigated. Transition of the receptor between membrane domains or locations could greatly affect the overall response. An excellent example is the "compartment-selective" morphine-induced MOR internalization in nucleus accumben neurons [257].

Whether the receptor is located at the soma or the dendrites determines morphine's ability to internalize the receptor. In this respect, receptor location and cellular contents determine the eventual responses to the activation receptor. The generalization that

the opioid receptor signaling mechanism is based on a single cellular background or selected group of agonists might not be valid anymore. The identification of the signaling complexes, or “receptosomes,” participating in opioid receptor signaling will greatly facilitate the eventual understanding of signal regulation. The identification of the receptosomes’ components could also be critical in future drug development. The ligand’s selectivity toward various receptors must be considered, and pathway selectivity due to recruitment of receptosomes’ components or to the translocation of the receptor-ligand complex to specific domains must be evaluated.

If indeed the chronic actions of the drugs are pathway-dependent, then designing drugs that select for specific pathways, as in the case of morphine- and etorphine-induced Erk1/2 activation, is possible. Both agonists activate Erk1/2, but the cellular locations of the Erk1/2 activated by these two agonists are different. In one sense, this opioid agonist pathway selectivity is reminiscence of the activities exhibited by the AT1 receptor-inverse agonists that can activate β -arrestin-dependent, but not G protein-dependent, signaling [365, 366]. Hence, if the signaling pathways involved in the chronic effects of the drugs could be identified, then it might be possible to design drugs that would not activate these pathways, but still activate the pathways involved in the acute action of the drugs. If such drugs could be identified, then an ideal opioid agonist could be developed in pain management without the association of tolerance and dependence development.

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Chapter 10

Mu Opioid Receptor Mediated Analgesia and Reward

Howard L. Fields

Abstract Agonists acting at the mu-opioid receptor (MOP-R) have two important functions related to motivational systems: (i) to suppress pain and (ii) to promote reward seeking. Their most important clinical application is for pain relief. In fact, MOP-R agonists are the most powerful analgesic agents currently available. The unparalleled therapeutic efficacy of MOP-R agonists undoubtedly results from the fact that they have several pain-inhibitory actions. They directly inhibit pain transmitting neurons in the periphery and central nervous system. They also act on descending pain modulatory circuits that control spinal cord pain transmission. In addition, MOP-R agonists produce powerful positive motivational effects. These effects are mediated by a circuit that includes dopaminergic neurons in the midbrain ventral tegmental area and their connections to limbic forebrain regions including sub-cortical areas such as the nucleus accumbens and amygdala. Because MOP-R agonists produce robust positive reinforcement, their repeated use can lead to addiction, which limits their use as analgesics. In this chapter, I will outline the actions of MOP-R agonists on the neural systems that transmit and modulate pain and at the central nervous system sites that underlie analgesia and reward.

Keywords Pain • Addiction • Reward • Motivation • Medulla • Spinal cord • Midbrain • Synapse

10.1 Targets for the Analgesic Action of MOP-R Agonists

When administered systemically, MOP-R agonists simultaneously target receptors at multiple sites in the central and peripheral nervous system. MOP-R targets relevant to analgesia can be grouped into three distinct classes: peripheral nociceptors,

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central nervous system pain transmission pathways and pain modulatory pathways. When acting on pain modulating targets, direct activation of MOP-R agonists elicits the release of endogenous opioid peptides that bind MOP-R at downstream brain sites. Activation of MOP-R receptors at multiple CNS sites and the resulting secondary effects on opioid receptors by endogenous opioids combine to enhance the analgesic power of MOP-R agonists and likely account for their uniquely potent analgesic effect.

10.2 Pain Transmission Targets

10.2.1 Pathways for Nociception

Pain perception depends upon the activation of specialized peripheral neurons called primary afferent nociceptors (PANs). Different PANs are tuned to respond to a variety of intense, potentially tissue damaging, thermal, mechanical and chemical stimuli. PANs arise from unipolar cell bodies in the dorsal root ganglion. Their axon bifurcates to send a peripheral process out to innervate somatic and visceral tissues and a central process into the spinal cord where it terminates in the gray matter of the superficial dorsal horn. Some PANs directly contact second-order dorsal horn neurons that project to the contralateral brainstem and thalamus (Fig. 10.1, left side).

Other PANs synapse upon dorsal horn interneurons, which then contact the projection cells. Nociceptive projection cells terminate in the brainstem and thalamus. In the brainstem, the projection cells target the reticular formation in the medulla and pons, and the dorsolateral region of the pons and midbrain. The thalamus receives both direct inputs from nociceptive dorsal horn neurons and indirect connections from the spinal cord via the brainstem. There are several distinct thalamic regions that receive nociceptive input and each thalamic region has a different cortical projection target. Cortical areas receiving nociceptive input include the primary and secondary somatosensory cortex, the anterior cingulate and insular cortices. Human functional imaging studies have demonstrated that activity in each of these areas correlates with perceived pain intensity and/or unpleasantness [1, 2].

10.3 MOP-R Mediated Actions in Peripheral Tissues

The MOP-R is synthesized by PANs and it is expressed on both their peripheral and central terminals [3]. In PANs, MOP-R agonists inhibit voltage activated calcium currents as well as cation currents that are activated in the presence of inflammatory mediators [4]. Consistent with these anti-nociceptive actions on PANs, MOP-R agonists reduce the excitability of peripheral nociceptor terminals and inhibit the

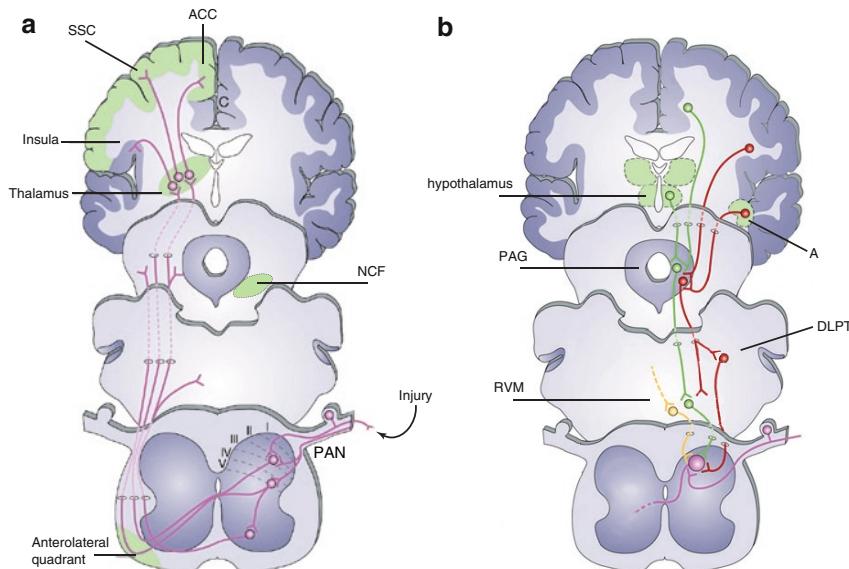


Fig. 10.1 Pain transmission and modulation pathways. **(a)** Transmission pathway. Intense, tissue damaging stimuli activate primary afferent nociceptors (PAN) with cell bodies in the dorsal root ganglion (DRG). The central terminals of the PAN contact second-order neurons in the dorsal horn of the spinal cord gray matter. The axons of pain projection neurons cross the midline and ascend to supraspinal targets, including the brainstem nucleus cuneiformis (NCF), and the thalamus. Different thalamic regions relay the nociceptive message to several cortical regions including the insula, somatosensory cortex (SSC), and anterior cingulate cortex (ACC). **(b)** Descending pain-modulating pathway. This pathway has critical links in the midbrain periaqueductal gray (PAG) and rostral ventromedial medulla (RVM). Regions of the frontal lobe including the insula and medial prefrontal cortex and amygdala (A), project directly and via the hypothalamus (H) to the PAG. The PAG in turn controls spinal nociceptive neurons through relays in the RVM and the dorsolateral pontine tegmentum (DLPT). The RVM contains both serotonergic and non-serotonergic projection neurons; the DLPT provides noradrenergic innervation of the dorsal horn Adapted from [35]

peripheral release of vasoactive peptides from these terminals [3]. Importantly, in addition to behavioral studies demonstrating an analgesic action of MOP-Rs in the peripheral tissues of rodents [5–7], there is a growing body of evidence that MOP-R agonists acting in the periphery can produce clinically significant pain relief [3, 5].

The peripheral anti-nociceptive action of MOP-R agonists is greatly increased in inflamed tissues [8]. This is due in part to stimulation by cytokines of MOP-R synthesis in the dorsal root ganglia and their transport to peripheral terminals. In addition, immune cells, guided to the inflamed region by cell adhesion molecules, are stimulated by local cytokines to secrete endogenous opioid peptides, including β -endorphin and enkephalin that bind to the MOP-R [3, 9].

Clearly, MOP-R agonists can produce a significant analgesic effect through direct actions on peripheral receptors (presumably those expressed on the peripheral terminals of PANs). However, it is difficult to resolve the issue of how much

this peripheral target contributes to the analgesic effect of systemically administered MOP-R agonists. Rodent studies have suggested that the peripheral contribution can be quite significant. For example, Labuz et al. [10] using a chemically induced abdominal pain model, found that a peripherally restricted antagonist, naloxone methiodide, reduced the analgesic effect of interpleural (i.p.) morphine by about 60%, whereas the same dose of morphine was fully reversed by a centrally penetrant antagonist.

Furthermore, using the tail flick reflex, Kolesnikov et al. [6] showed that intra-dermal morphine can produce a MOP-R mediated analgesic effect that is highly localized and that interacts synergistically with morphine given concomitantly directly into the central nervous system. The significance of synergy between central and peripheral MOP-R receptor actions is supported by work of King et al. [11] who found that morphine can be transported out of the brain and into the blood stream by a saturable P-glycoprotein transport system. When the function of this transport system is reduced, the analgesic efficacy of systemically administered morphine is enhanced (presumably due to higher brain levels of the drug). In contrast, this same manipulation significantly reduces the analgesic efficacy of centrally administered morphine. This result indicates that a peripheral action of morphine is required for its full analgesic potency.

In summary, MOP-R receptors on the peripheral terminals of primary afferents can mediate a clinically significant analgesic effect when an agonist is locally applied in relatively high concentration. This local analgesic effect is particularly robust in the presence of inflammation. Animal studies also suggest that the optimal analgesic effect of systemically administered MOP-R agonists depends upon a synergistic action of peripheral and central nervous system MOP-Rs. On the other hand, it is unclear to what extent peripheral MOP-Rs contribute to the analgesic effect of systemically administered opioid agonists in most clinical situations.

10.4 MOP-R Actions on Pain Transmission in the Spinal Cord Dorsal Horn

Rodent studies show that MOP-R agonists exert a powerful analgesic effect when applied directly to the spinal cord [12, 13]. This effect has been replicated in humans and has lead to the widespread clinical use of intrathecal and epidural spinal opioids for the management of acute and chronic pain [14, 15]. The value of this approach is that clinically effective analgesia can be produced while minimizing unwanted side effects of MOP-Rs including sedation, respiratory depression and nausea.

The central terminals of PANs terminate densely in the superficial laminae of the dorsal horn, especially lamina II (the substantia gelatinosa) (Fig. 10.2). Lamina II also contains local interneurons that relay the nociceptive message from the PAN to the neurons that give rise to ascending pain pathways [16]. The density of MOP-R is greatest in lamina II and local microinfusion of MOP-R opioids into

lamina II inhibits dorsal horn nociceptive neurons [17]. The mechanism of this analgesic effect depends upon both presynaptic and postsynaptic MOP-R agonist actions in superficial dorsal horn. As with their peripheral terminals, the central terminals of PANs contain MOP-R [18]. The fast excitatory neurotransmitter released by PANs is glutamate and MOP-R agonists presynaptically inhibit excitatory glutamatergic input to lamina II interneurons [19, 20]. A subset of PANs also contains substance P, a peptide that has been implicated in nociceptive transmission [21–23].

MOP-R agonists inhibit the release of substance P from PANs and this likely contributes to their analgesic action [24, 25]. This presynaptic inhibitory effect is most likely the result of direct inhibition of an N-type calcium channel by a guanine nucleotide regulatory (G) protein [26]. In addition to this presynaptic effect, MOP-R agonists strongly hyperpolarize lamina II neurons (Fig. 10.2) [19, 27], including those with axonal arbors to the pain projection neurons in lamina I and deep dorsal horn [28]. These interneurons relay excitatory input from PANs to lamina I and lamina V projection cells [29, 30]. Similar to other neurons directly inhibited by MOP-Rs, the hyperpolarization in lamina II neurons is due to activation of a G protein coupled inwardly rectifying potassium conductance [19, 31].

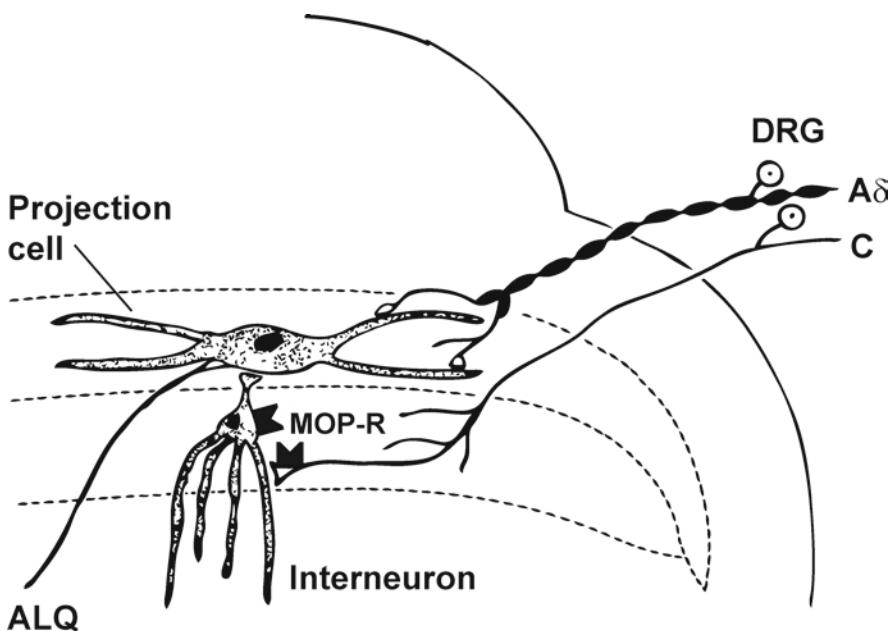


Fig. 10.2 Location of MOP receptors in superficial dorsal horn. MOP-Rs (MOP) are present on the central terminals of primary afferent nociceptors and on interneurons in lamina II of the dorsal horn. Both actions block the transmission of the nociceptive message to pain projection neurons located in lamina I. The lamina I neurons project to supraspinal sites through the contralateral anterolateral quadrant (ALQ) of the spinal cord. The primary afferent cell body is located in the DRG. Adapted from Fig. 3.8 in Fields, H.L. (1987) Pain, McGraw-Hill, New York

In summary, the spinal actions of MOP-R agonists are focused on lamina II of the dorsal horn. At this site they inhibit the release of the excitatory neurotransmitter glutamate and neuropeptides including substance P from PANs onto dorsal horn nociceptive neurons. They also directly hyperpolarize excitatory dorsal horn interneurons that relay the nociceptive message to pain projection neurons. The combination of these two actions contributes to the robust analgesic action of MOP-R agonists directly applied to the spinal cord.

10.5 MOP-R Actions on Supraspinal Pain Transmission Pathways

MOP-R actions on supraspinal pain transmission relay nuclei are not fully understood. The anatomical distribution of MOP-R suggests several possible analgesic targets. MOP-R binding is dense in several thalamic nuclei that receive nociceptive inputs, including the ventroposterior, parafascicular and mediodorsal [32], however, MOP-R agonists microinjected into these afferent relay nuclei do not produce powerful analgesia. They do, however, produce an analgesic effect when microinjected into the nucleus submedius in the rat, which contains a dense concentration of MOP-R agonists, receives input from superficial dorsal horn and projects to frontal cortical areas [33].

Although there is widespread MOP-R binding in the cerebral cortex, the relevance of cortical receptors to analgesia remains largely unexplored. One cortical area implicated in MOP-R analgesia is the anterior insula, which is activated by nociceptive stimuli in humans and is sensitive to the analgesic action of locally injected morphine [34]. In summary, while MOP-R actions at the spinal cord clearly include blocking transmission of the nociceptive message, the analgesic effect of MOP-Rs at supraspinal relays of the pain sensory pathway is currently unclear. An important question is whether the MOP-R actions at these sites relates to direct inhibition of pain transmission neurons or to activation of the pain modulating circuits discussed below.

10.6 Pain Modulatory Circuits

In addition to peripheral and central pain transmission neurons, MOP-R agonists produce analgesia by acting on a pain modulation circuit (Fig. 10.1) that includes regions of the frontal cortex, hypothalamus, amygdala, midbrain periaqueductal gray (PAG), dorsolateral pontine tegmentum (DLPT) and rostral ventromedial medulla (RVM) [35, 36]. This circuit controls pain through a direct pathway from the medulla to dorsal horn laminae that contain the cells of origin of ascending pain transmission pathways. MOP-R and endogenous ligands for MOP-R are present in the constituent nuclei of the pain modulating pathway

including: insular cortex, amygdala, hypothalamus, PAG, the DLPT, RVM and spinal cord dorsal horn [37, 38].

Morphine and MOP-R selective agonists, produce analgesia when microinjected into any of these sites [39]. Furthermore, lesions, reversible inactivation or micro-injection of opioid antagonists into these sites reduces the analgesic effect of systemically administered opioids. This opioid mediated modulatory network is reliably recruited to suppress responses to noxious stimuli under conditions of threat or, in humans, by the anticipation of pain relief, such as occurs when a placebo analgesic is administered. In addition, the anticipation of reward is associated with an analgesic effect and MOP-R agonists produce analgesia when microinjected into regions associated with reward such as the ventral tegmental area and nucleus accumbens (NAc) (*vide infra*).

10.6.1 The Midbrain Periaqueductal Grey and Stimulation-produced Analgesia

The discovery of stimulation-produced analgesia (SPA) was a critical step leading to the elucidation of descending pain-modulating circuitry [40]. Electrical stimulation of the PAG dramatically inhibits responses to noxious stimuli while leaving responses to other sensory stimuli unchanged. The most sensitive region for stimulation is the ventrolateral PAG. In addition, SPA can be elicited from the laterally adjacent nucleus cuneiformis [36, 41]. SPA from either the PAG or nucleus cuneiformis is blocked by the opioid antagonist naloxone [42]. Importantly, an SPA-like effect is produced in humans by electrical stimulation of the PAG region [43, 44].

10.6.2 Connectivity of the PAG

Major inputs to the PAG arise from the frontal cortex, hypothalamus and amygdala (Fig. 10.1, right side) [45]. Cortical projections to the PAG are derived from the anterior cingulate and anterior insular cortex. Human functional imaging indicates that there is also a major pain modulatory input to PAG from the dorsolateral pre-frontal cortex [46]. The amygdala is another key source of PAG afferents. In particular, the basolateral nucleus of the amygdala (BLA), which receives input from both the hippocampus and broad areas of the frontal neocortex [47]. The BLA then projects to the central nucleus of the amygdala, which in turn projects densely to the PAG [48]. Both the BLA and PAG have dense concentrations of MOP-R [37, 49]. Furthermore, the analgesic effect of opioid agonists injected into the BLA is blocked by lidocaine inactivation of, or opioid antagonist injection into, the PAG [50, 51].

Importantly, the central nucleus of the amygdala receives nociceptive input directly from the spinal cord [52, 53], and indirectly via the parabrachial nucleus [54]. This nociceptive pathway may be modulated indirectly by opioids acting in the BLA.

The BLA may also contribute to pain modulation through its connections to the NAc, located in the ventromedial striatum. Although primarily thought of as part of the circuitry for reward (*vide infra*), opioids injected into the NAc can produce an analgesic effect [55]. The NAc also receives a major projection from the medial prefrontal cortex and although it does not project directly to the PAG, it does project to the lateral hypothalamus [56]. The hypothalamus has moderate to dense MOP-R binding [32, 49] and provides a massive input to the PAG. Furthermore, electrical stimulation or opioid microinjection in the hypothalamus produces analgesia [57–59].

Brainstem inputs to the PAG arise from the adjacent nucleus cuneiformis, the pontomedullary reticular formation, the locus coeruleus and other brainstem catecholaminergic nuclei, all of which have moderate to dense MOP-R binding [60, 61]. The PAG is reciprocally connected with the RVM (see below). Finally, the PAG and adjacent nucleus cuneiformis receive a significant projection from the dorsal horn, including spinal lamina I nociceptive neurons [62, 63].

10.6.3 MOP-R Synaptic Actions in the PAG

The PAG was one of the first sites to be identified as a critical site for the analgesic effect of centrally administered MOP-R agonists [33, 39]. In rats, the most sensitive part of the PAG for this effect is the ventrolateral region. A subpopulation of PAG neurons in this region is hyperpolarized by MOP-R agonists through activation of a G-protein coupled inwardly-rectifying potassium conductance [64]. In this same region, MOP-R agonists presynaptically inhibit release of GABA [65]. In several CNS sites, the presynaptic inhibitory effects of MOP-R agonists are mediated by direct G-protein inhibition of voltage gated calcium channels [66]. However, in the PAG, the inhibition is prevented by drugs that block voltage-gated potassium channels and by drugs that inhibit arachidonic acid metabolism [67].

While it is unclear to what extent the analgesia elicited by MOP-R agonists in the PAG depends on presynaptic inhibition of GABA release or postsynaptic inhibition of GABAergic neurons, it is likely that inhibition of GABAergic inputs to antinociceptive PAG output neurons (i.e., disinhibition) contributes significantly to the analgesic effect of PAG MOP-R agonists. In support of this idea, microinjecting the GABA-A receptor agonist Muscimol into the PAG, can reverse the analgesic effect of PAG morphine [68].

10.6.4 The Rostral Ventromedial Medulla

The RVM includes the midline nucleus raphe magnus and the adjacent reticular formation ventral to the nucleus reticularis gigantocellularis. Electrical stimulation

of, or microinjection of excitatory amino acids into the RVM produces analgesia and inhibits spinal dorsal horn neuronal responses to noxious stimulation [22].

The PAG and nucleus cuneiformis are the major source of inputs to the RVM. The RVM also receives input from serotonergic neurons in the dorsal raphe [69], from noradrenergic neurons in the A5 and A7 cell groups of the dorsolateral pons [70], from the medial preoptic region of the hypothalamus [71] and from limbic and prelimbic cortex, including the anterior insula [72]. The RVM does not receive direct inputs from the spinal cord but has a major input from the adjacent medullary nucleus reticularis gigantocellularis, which receives a large direct projection from nociceptive spinal cord neurons.

The pain-modulating action of the PAG upon the spinal cord is relayed through the RVM. Inactivation of the RVM abolishes the analgesia produced by stimulation of the PAG [22]. Furthermore, the connection between the PAG and RVM appears to amplify the analgesic action of MOP-R agonists. Thus the analgesic effect of the MOP-R selective peptide [D-Ala₂,MePhe₄,Gly(ol)₅]-enkephalin (DAMGO) shows robust synergy when concomitantly microinjected into the PAG and RVM [73].

10.6.5 Physiology of Pain-Modulating Neurons in the RVM

Electrical stimulation of the RVM can produce either inhibition or facilitation of spinal pain transmission [74]. This suggests that there are parallel inhibitory and facilitatory output pathways from RVM to spinal cord. In fact, there are two pain relevant types of spinally projecting RVM neuron: those that discharge beginning just prior to the occurrence of withdrawal from noxious heat (“on-cells”), and those that pause firing just prior to a withdrawal reflex (“off-cells”) (Fig. 10.3) [22, 35, 75]. On- and off-cells project specifically to laminae I, II and V of the dorsal horn [76]. MOP-R agonists given systemically or into the PAG inhibit on-cells and activate off-cells (Fig. 10.3, right side). Since inactivation of the RVM blocks MOP-R analgesia, activation of off-cells is necessary and sufficient for MOP-Rs to produce behavioral analgesia [77]. In contrast, selective activation of RVM on-cells *facilitates* nociception [78–82]. On- and off-cells are also found in the PAG and the adjacent DLPT [83], which suggests that a common neural mechanism for pain modulation exists at medullary, pontine and midbrain levels.

10.6.5.1 Direct Opioid Actions in the RVM

Similar to the PAG, MOP-R agonists directly hyperpolarize a subpopulation of neurons in the PAG and RVM. This hyperpolarization is mediated by G protein-coupled inwardly rectifying potassium channels (Fig. 10.4b) [64, 84]. Local iontophoretic application of morphine directly inhibits RVM on-cells, but has no direct effect on off-cells [85]. Thus, the RVM cells that are directly hyperpolarized by

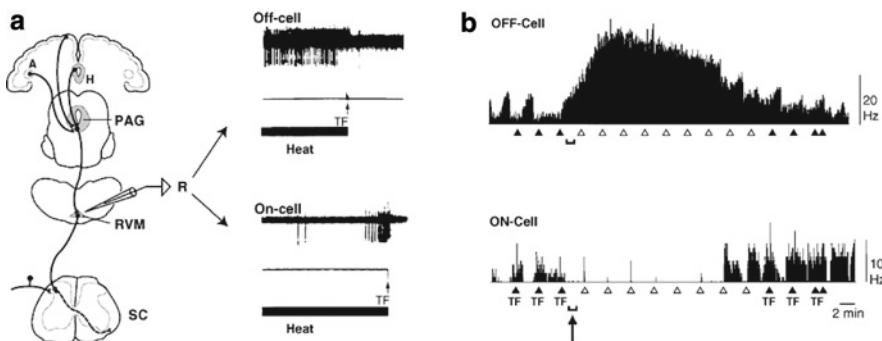


Fig. 10.3 (a) Left: Microelectrode placement in the RVM for single unit extracellular recording. Right: top, a single oscilloscope sweep showing the off-cell pause occurring just prior to the tail-flick reflex (TF, middle trace shows force transducer output with movement) in response to noxious heat (lower trace). Below; typical on-cell firing pattern, which is a burst beginning before the tail-flick. (b) Focal application of the MOP-R agonist DAMGO into the RVM inhibits on-cells and activates off-cells. Adapted from [75]

MOP-R agonists in vitro are the pain-facilitating on-cells. Because inactivation of the RVM does not produce analgesia, this direct opioid inhibition of on-cells is not by itself sufficient to account for behavioral antinociception produced by systemically administered MOR agonists under normal conditions [86]. However, direct opioid inhibition of RVM on cells may contribute significantly to relief of hyperalgesic states such as inflammation or opioid dependence that are characterized by increased on-cell activity [87, 88]. MOP-R agonists activate off-cells indirectly, by inhibiting the release of GABA from presynaptic terminals (Fig. 10.4a) [84]. As mentioned above, this action is sufficient to produce analgesia.

10.6.6 RVM Neutral-Cells and the Contribution of Serotonin to the Descending Modulation of Nociceptive Transmission

Because a significant number (about 20%) of RVM neurons are serotonergic [89] and the RVM is the source of dorsal horn serotonin, serotonin was thought to be critical for the analgesic action of MOP-R agonists. However, the role of serotonin is not at all clear. Significantly, in the adult rat, all serotonergic neurons recorded *in vivo* have been neither on- nor off-cells [90, 91]. RVM serotonergic neurons *in vivo* (in adult rats) are not affected by MOP-R agonists or by electrical stimulation of the PAG [92, 93].

Despite the lack of direct evidence tying RVM serotonergic neuron firing to pain modulation, there is nevertheless an extensive literature supporting a role for 5HT in descending modulation of pain transmission [33, 94]. Electrical stimulation of the RVM evokes the release of 5HT in the spinal cord and the analgesia produced

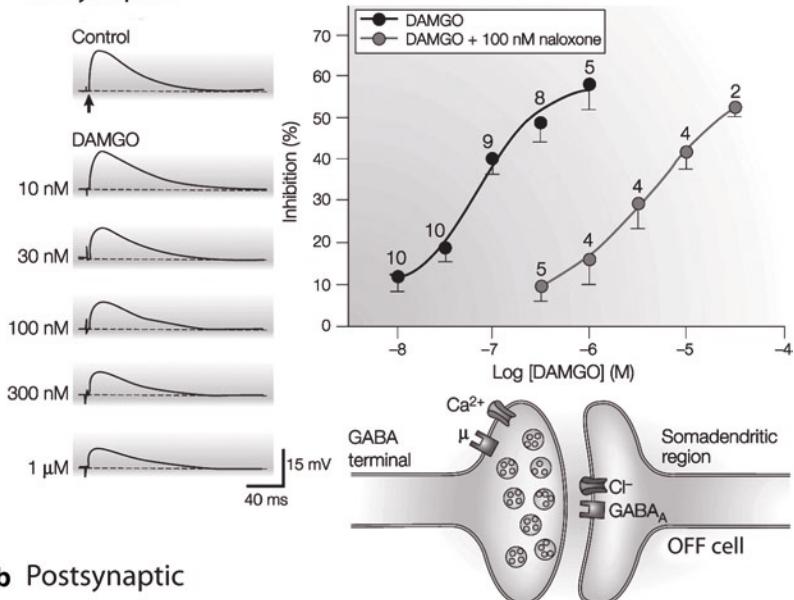
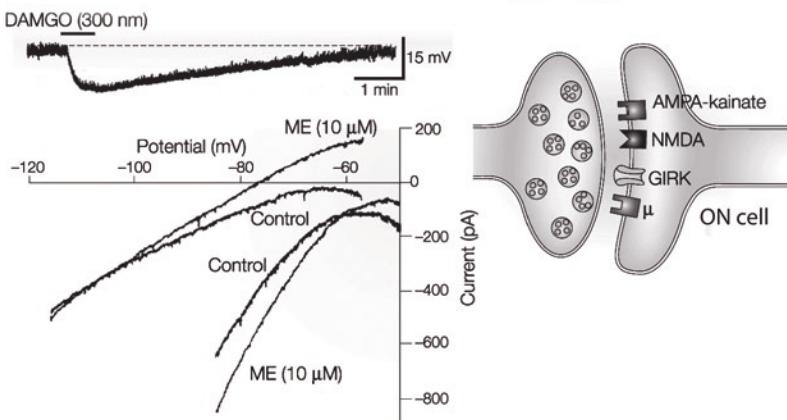
a Presynaptic**b Postsynaptic**

Fig. 10.4 Synaptic actions of MOP-R agonists in the RVM. **(a)** Pre-synaptic actions. Dose-related and naloxone reversible inhibition of electrically evoked GABAergic inhibitory post-synaptic potential. *Inset cartoon* illustrates the MOP-R inhibition of a calcium channel on the GABA terminal. The GABA-A receptor on the somadendritic region of the o off cell opens a chloride channel. **(b)** Post-synaptic action. In this case, the MOP-R selective agonist DAMGO acts on the soma-dendritic region of the on-cell to hyperpolarize it through activation of a G-coupled inwardly rectifying potassium conductance. From [35]

by this stimulation is reduced by non-selective 5HT antagonists given intrathecally (for review, see Le Bars [94]). Iontophoresis of 5HT inhibits the response of dorsal horn neurons to noxious stimulation [95, 96] and 5HT applied directly to the spinal cord inhibits nociceptive transmission [97]. Furthermore, the analgesic action of

systemically administered opiates can be at least transiently reduced by depletion of 5HT or by neurotoxic destruction of spinal 5HT terminals. (See Le Bars [94] for review). Mason and colleagues have proposed that serotonergic RVM neurons have a permissive action on pain modulation at the level of the spinal cord [98–100].

10.6.7 The Dorsolateral Pontine Tegmentum (DLPT) and Brainstem Noradrenergic Neurons

Noradrenergic neurons of the DLPT (the locus coeruleus, and the A5 and A7 noradrenergic cell groups) are the major source of noradrenergic projections to the spinal cord and they provide a critical link to the spinal cord for brainstem pain modulating sites (Fig. 10.1, right side, DLPT) [101]. Stimulation of the DLPT produces analgesia and inhibition of dorsal horn neurons mediated by spinal α_2 adrenergic receptors [33, 102].

The PAG projects directly to the locus coeruleus and the A7 region [103], and RVM neurons containing substance P or enkephalin also project to A7 [104, 105]. Focal application of substance P into the A7 region produces analgesia. Electrical stimulation of the RVM evokes the release of norepinephrine at the spinal cord [106]. Furthermore, the inhibition of spinal withdrawal reflexes by RVM activation is attenuated by spinal application of noradrenergic antagonists [107–109]. In addition, the inhibition of nociceptive dorsal horn neurons by PAG stimulation is blocked by local iontophoresis of α_2 -adrenergic receptor antagonists [102].

10.7 Endogenous Opioid Peptides in MOP-R Circuitry

That endogenous opioid peptides contribute to pain modulation was established by showing that SPA from the PAG in animals and humans is reduced by the opioid antagonist naloxone. Naloxone also worsens postoperative pain in patients who have not received exogenous opioid therapy, thus establishing the relevance of endogenous opioids to common clinical situations. (See Fields [110] for review.) The endogenous ligand for the MOP-R that mediates these analgesic effects has not been established [111].

The enkephalins appear to be released in proximity to MOP-R. Both leu-enkephalin and met-enkephalin have significant affinity for MOP-R, but also act at the delta opioid receptor (DOP-R). Like met-enkephalin, β -endorphin (BE) has approximately equal affinity for DOP-R and MOP-R and much lower affinity for the kappa-opioid receptor (KOP-R). BE-containing neurons in the ventromedial hypothalamus project to the PAG. This BE projection contributes to stimulation-produced and stress-induced analgesia in rats [112]. In contrast to the limited distribution of BE terminal fields, met- and leu-enkephalin cells and terminal fields are widely distributed throughout the neuraxis. All of the CNS structures involved in the opioid mediated pain modulation circuit have

significant levels of both enkephalins [111]. This includes the amygdala, hypothalamus, PAG, DLPT, RVM and superficial dorsal horn.

The very short peptides, endomorphin-1 (Tyr-Pro-Trp-Phe-NH₂) and endomorphin-2 (Tyr-Pro-Phe-Phe-NH₂) have high selectivity for MOP-R [113]. The endomorphins have over 1,000-fold selectivity for MOP-R vs. KOP-R and DOP-R. They do not share the Tyr-Gly-Gly-Phe amino acid sequence common to the N-terminal of the other known endogenous opioid peptides. The endomorphins inhibit PAN evoked responses of dorsal horn neurons [114] and have analgesic sites of action and potency similar to morphine [115, 116]. Endomorphin-2 is concentrated in many, but not all regions that express MOP-R, including the hypothalamus, amygdala, PAG, locus coeruleus and dorsal horn [117]. There is co-localization of endomorphin-2 with substance P in PANs [118]. Moreover, in primary afferent terminals, endomorphin-2 is co-localized in and likely released from the same dense core vesicles as substance P [119].

Endogenous opioids play a critical role in linking the component nuclei of the pain-modulating circuit. For example, the analgesic effect of a MOP-R agonist microinjected into the basolateral amygdala is reversed by a MOP-R antagonist in the PAG [120]. Similarly, the antinociceptive effects of PAG morphine can be blocked by naloxone or a selective MOP-R antagonist microinjected into the RVM [121, 122]. Moreover, an enkephalinase inhibitor injected into the RVM produces analgesia [123].

Endogenous opioids also contribute to RVM inhibition of pain transmission at the spinal cord. MOP-R agonists injected into either the PAG or the RVM produce analgesia via inhibition of spinal pain transmission neurons [124, 125]. This inhibition is exerted in part via inhibitory enkephalinergic interneurons in the spinal cord dorsal horn since the vast majority of opioid terminals in the dorsal horn derive from local interneurons (Fig. 10.2) [126]. As discussed above, spinal application of opioids produces analgesia and opioid iontophoresis inhibits dorsal horn nociceptive neurons [127]. Furthermore, direct application of naloxone to the spinal cord reduces the analgesic effect of electrical stimulation of RVM [128, 129] or supraspinal opioid administration [130] and, conversely, blocking enkephalin-degrading enzymes at the spinal cord level produces behavioral antinociception [131] and inhibits nociceptive dorsal horn neurons [132]. Finally, the inhibition of dorsal horn neurons by activation of the PAG is blocked by local iontophoresis of MOP-R selective antagonists [133]. In summary, the pain inhibiting action of the descending modulatory system is mediated in part through the release of endogenous opioids, likely enkephalin, acting on local MOP-Rs.

10.8 Physiological Function of Pain-Modulating Networks

When rodents are threatened, for example when exposed to an inescapable noxious stimulus, a predator, or sensory cues associated with pain there is an analgesic effect that involves opioid mediated modulating circuits. For example, in rats forepaw electric shock analgesia is completely blocked by naloxone and by RVM lesions

[134, 135]. Animals can also learn to activate this circuit in contexts associated with pain [136] or in response to a light or tone that has been paired with foot shock (conditioned fear). The analgesia that accompanies this conditioned fear paradigm is blocked by lesions of the basolateral and central amygdala, the RVM and the DLF [137–139]. It is also blocked by naloxone administered systemically, into the PAG [137, 140] or RVM [141].

10.8.1 Evidence That an Endogenous MOP-R Mediated Pain-Modulatory System Exists in Humans

The opioid mediated pain modulatory system is highly conserved in a variety of mammalian species, including rodents, carnivores, primates and marsupials. The distribution of neurotransmitters, including opioid peptides, in this pathway is also conserved across species, including humans [142, 143]. The earliest evidence that endogenous opioids play a physiologically significant role in pain modulation came from studies using naloxone. If endogenous opioids can produce analgesia in humans, it should be possible to identify situations in which naloxone worsens pain. Using a human clinical pain model, Levine et al. [144] were the first to demonstrate that, compared with placebo, naloxone significantly worsens postoperative pain. Subsequent studies have confirmed that naloxone produces hyperalgesia using both clinical and experimental pain models [145, 146]. Furthermore, Levine et al. [147], and later, Benedetti and colleagues [148, 149] demonstrated that placebo analgesia can be blocked by systemic naloxone.

Although these studies implicate endogenous opioids, they do not tie placebo analgesia to the modulating circuitry described above. The first evidence linking the two was provided by Petrovic et al. [150], who used positron emission tomography to study human CNS activity in subjects given the MOP-R agonist fentanyl or a saline placebo and then subjected to a painful stimulus. They found that many of the same brain areas were activated by fentanyl and the saline placebo. These areas included the anterior cingulate cortex and a region in the caudal pons/rostral medulla.

Human imaging studies using the MOP-R selective radioligand [¹¹C]carfentanil have provided direct evidence that MOP-Rs in the classical pain modulating circuitry are activated in individuals reporting placebo analgesia [151]. In these studies, positron emission tomography was used to localize the radiolabeled MOP-R ligand and a reduction of receptor binding was taken as evidence for the release of an endogenous MOP-R ligand. Evidence for opioid release (reduced binding) was found in the amygdala, PAG and nucleus cuneiformis in the placebo condition. Together with the naloxone studies, these results confirm the physiological significance of endogenous MOP-R ligands in human analgesia.

In summary, MOP-R analgesia depends upon a set of widely distributed neural targets that include PANs, ascending pain projection neurons and a top-down pain modulatory circuit that includes cortical, amygdala, diencephalic and brainstem components.

In the modulatory pathway, the component nuclei are linked by endogenous opioids. MOP-R agonists have a synergistic analgesic effect when acting concomitantly at two separate target locations; a fact that contributes to their singular potency and selectivity for pain control.

10.9 MOP-R Agonists in Reward and Motivation

MOP-R agonists can produce powerful rewarding effects. This property has led to widespread recreational use of MOP-R agonist drugs like morphine and heroin and, in some individuals to addiction. Although addiction is rare when opioids are used appropriately to treat pain, the risk of this outcome has led to caution in the long term use of opioids for chronic pain [152]. Animal studies of opioid reward have mostly used morphine. Morphine produces reward largely, if not exclusively, through its action on the MOP-R [153, 154]. Furthermore, endogenous ligands for MOP-R contribute to the reinforcing actions of non-opioid drugs including ethanol, nicotine and psychostimulants like cocaine and amphetamine [155, 156]. These drugs are significantly less rewarding in mice with experimentally inactivated MOP-R [156, 157].

10.10 Mesolimbic Dopamine Projections in MOP-R Reward

There are a variety of methods to measure drug reward [158, 159]. The two most commonly used are conditioned place preference (CPP) and drug self-administration. In the CPP paradigm animals are given the drug and then immediately placed in one chamber of a two- or three-chambered apparatus. On alternate days, they are placed in the opposite chamber after vehicle administration. After conditioning with drug and vehicle, the animal is allowed to roam freely in the apparatus on the test day. If the drug is rewarding, the animal will spend an increased amount of time in the drug-paired chamber. Morphine and other MOP-R agonists are self-administered and produce CPP.

Studies of the neural circuitry underlying opioid reward have focused on the mesolimbic dopamine pathway, in particular, the dopaminergic neurons of the VTA and their limbic forebrain targets (see [160] for a review of the connectivity of the VTA). VTA neurons receive inputs from GABAergic neurons in the ventral pallidum and from glutamatergic and cholinergic neurons of the dorsal pontomesencephalic tegmentum. Both of these VTA inputs have been implicated in opioid reward [158]. The VTA is also reciprocally connected to the amygdala, lateral hypothalamus, NAc and the medial prefrontal cortex. MOP-R agonists produce CPP and are self-administered into the lateral hypothalamus and NAc [155, 156, 159, 161]. Furthermore, opioid antagonists microinjected into the NAc reduce the rewarding effect of systemic opioids (see Chap. 4 in [158]). Microinjection of

morphine directly into the VTA produces a robust CPP [155, 156, 162]. In addition, rodents will learn to press a lever to deliver morphine [163] or the MOP-R selective peptide, endomorphin [164] directly into the VTA. These studies demonstrate that VTA neurons contribute to circuits that are critical for the rewarding actions of MOP-R agonists. Importantly, microinjection of opioid antagonists into the VTA blocks the CPP produced by systemic morphine [165].

About 60% of VTA neurons are dopaminergic; the rest are GABAergic and glutamatergic [160]. Each VTA projection target receives input from a distinct group of VTA neurons which consists of different proportions of dopaminergic and non-dopaminergic neurons. Although VTA injected morphine can produce reward through a non-dopaminergic mechanism [166], there is strong evidence that dopaminergic VTA neurons are capable of mediating reward. Thus, systemic or intra-VTA MOP-R agonists raise dopamine levels in the NAc [160, 167] and both systemic morphine CPP and morphine self-administration are reduced by dopamine receptor antagonists [156, 168]. Furthermore, acquisition of CPP produced by systemic or VTA morphine is blocked by dopamine antagonist microinjection into the NAc [166, 169]. In summary, VTA dopaminergic neurons that project to the NAc are a critical element of opioid reward circuitry that includes other brainstem, diencephalic, cortical and subcortical forebrain regions (e.g., see [158, 160, 170] for reviews).

10.10.1 Synaptic Actions of MOP-R Agonists in the VTA

As in pain transmission and modulating circuits, the synaptic actions of MOP-R agonists are predominantly inhibitory; either pre-synaptic inhibition of neurotransmitter release or postsynaptic hyperpolarization of neurons through activation of an inwardly rectifying potassium channel. MOP-R agonists excite VTA dopaminergic neurons and *in vitro* studies indicate that this effect is produced indirectly through inhibition of VTA GABAergic neurons that inhibit DA neurons [171]. Although MOP-R agonists act directly on a variety of VTA cell types, including a subset of dopamine neurons, it is clear that there is a subclass of non-dopaminergic VTA neurons that is hyperpolarized by MOP-R agonists through activating a potassium conductance [172]. MOP receptor agonists also directly inhibit release of the inhibitory neurotransmitter GABA on to VTA DA neurons [173]. These observations lead Johnson and North to propose that MOP-R agonists inhibit VTA GABAergic neurons with local connections to dopaminergic neurons. While there are clearly non-dopaminergic mechanisms of VTA reward [36], disinhibition of DA VTA neurons projecting to the NAc is widely accepted as a key synaptic mechanism for the rewarding and motivational effects of MOP-Rs acting in the VTA [174].

In summary, MOP-R agonists are highly rewarding themselves and also contribute to the rewarding effect of nicotine, ethanol and psychostimulant drugs. While there are several circuits capable of mediating the rewarding actions of MOP-R agonists, the evidence implicating VTA dopamine neurons that project to the ventral striatum

is most complete. MOP-R opioids can indirectly activate these VTA neurons by inhibiting their GABAergic inputs.

10.11 MOP-R Agonist Enhancement of Consumption of Palatable Foods

Opioid antagonists reduce the pleasantness ratings of sweet and fatty foods in human subjects [175, 176]. In rodents, morphine increases and naloxone selectively reduces the consumption of palatable foods but not overall caloric intake [177]. Furthermore, even in mildly thirsty rats, MOP-R agonists selectively increase drinking of sucrose and ethanol solutions but not water [178]. While the central sites for the enhancement of palatability have not been fully elucidated, they overlap with the areas discussed above that contribute to the directly rewarding effects of MOP-R agonists. Sites involved in palatability include the NAc and ventral pallidum, the VTA, the nucleus of the solitary tract, the amygdala and the dorsomedial and lateral hypothalamus [177, 179]. As with the pain modulatory system, serial endogenous MOP-R agonist links play a critical role in this motivational pathway. Thus enhanced consumption of palatable foods elicited by MOP-R agonists in the VTA can be antagonized by opioid antagonist microinjection into the ventral pallidum or VTA [180, 181].

As with morphine CPP, the enhanced feeding produced by MOP-R agonists in the VTA is blocked by dopamine receptor antagonists microinjected into the NAc. Furthermore, the increased consumption produced by inactivation of the NAc is correlated with an increase in immediate early gene expression in orexin-containing neurons of the lateral hypothalamus and is mimicked by orexin microinjected into this region. These orexin-containing neurons project to and excite VTA dopamine neurons. Accordingly, increased feeding produced by MOP-R agonists in the NAc is associated with increased dopamine release in the shell region of the NAc [182, 183] and is blocked by inactivation of the lateral hypothalamus or VTA or by dopamine antagonists or naloxone microinjected into the NAc. In summary, MOP-R agonists play an important role in enhancing the palatability of food and promoting consumption in animals that are not initially hungry. The circuit mediating this effect in rodents overlaps with the central nervous system sites where direct microinjection of MOP-R agonists produces reward.

10.12 Summary

The most striking biological functions of the MOP-R are manifested behaviorally in the realm of motivation. Pain and reward are the two major forces that shape behavior and MOP-Rs affect both dramatically. By shutting down pain transmission MOP-Rs reduce the motivation to escape and can permit the individual to endure pain in order to survive a threat or pursue a reward. On the other hand,

MOP-R agonists enhance the motivation to consume palatable foods and can produce reward directly. Most of known direct actions of MOP-Rs are inhibitory; either direct hyperpolarization of neurons through activation of a G-protein activated inwardly rectifying potassium conductance or inhibition of neurotransmitter release from nerve terminals. One common motif is that MOP-R agonists can produce neuronal excitation indirectly through postsynaptic inhibition of GABAergic neurons or inhibition of GABA release from their terminals. Another common feature of both analgesia and reward circuitry is that activation of MOP-Rs in one brain region leads to release of an endogenous MOP-R agonist at a remote downstream site. Furthermore, when MOP-R agonists are given systemically, they act synergistically at multiple sites to amplify their biological action. This organizational principle contributes to their powerful medicinal and recreational effects.

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Chapter 11

Kappa Opioid Receptor Function

Julia C. Lemos and Charles Chavkin

Abstract This chapter describes recent developments in the analysis of kappa opioid receptor (KOR) function at a molecular, cellular and systems level within the central and peripheral nervous system. A combination of neuroanatomical, electrophysiological and behavioral techniques have been used to probe the functional consequences of KOR activation in the brain and spinal cord in both normal and pathological conditions. As a result of a number of studies conducted over the last three decades it is apparent that kappa opioid receptors will emerge as an important therapeutic target for both psychiatric and neurological pathologies. The following chapter reviews where the field has been, but importantly discusses gaps in our knowledge and highlights important new directions for the future.

Keywords Kappa opioid receptor • Dynorphin • Epilepsy • Stress • Glutamate • Dopamine • Norbinaltorphimine • Hippocampus • Mesocortical limbic pathway

11.1 Overview

As described in the other chapters in this book, the central and peripheral nervous systems express three main classes of opioid peptide receptor, mu (MOPr or MOR), kappa (KOPr or KOR), and delta (DOPr or DOR), as well as several possible receptor subtypes. Activation of these receptors by selective endogenous and exogenous ligands has been shown to produce striking differences in behavioral output. For example, activation of MOR results in a feeling of euphoria [1], whereas activation of KOR results in dysphoria [2, 3]. To understand the mechanisms underlying this dichotomy, one must first understand how these receptors influence cellular and neurosystems functioning. Other chapters in this book focus on the functional analysis of MOR and DOR activation. This chapter focuses on the role of KOR activation in modulating cellular and systems functioning and how these events result in behavioral consequences.

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KOR has a wide, yet distinct distribution throughout the CNS [4], and it is positioned to exert a large influence over many different behaviors. Recently, there have been significant advances in our understanding the role of KOR in controlling cognition and emotion in addition to insights into its involvement in neurological diseases such as epilepsy and neuropathic pain. These pathologies share the common feature of disruption of normal synaptic neurotransmission and disruption of the induction of neuroplasticity. While this is not a particularly novel idea in the field of epilepsy, an emerging scheme in the field of psychiatric disorders is that diseases such as addiction and depression also stem from disruption in normal synaptic physiology and aberrant neuroplasticity that ultimately lead to maladaptive learning.

Similarly exposure to intense behavioral stressors, which can facilitate drug abuse and depression, are also thought to disrupt normal neuronal adaptations (for review see [5, 6]). There is compelling evidence to suggest that dynorphin, the endogenous ligand for KOR [7], is released in response to stressor exposure as well as during pathological hyperexcitability typical of seizure, neural injury, and CNS inflammation. However, the specific relationships between stress exposure, dynorphin release, and subsequent changes in synaptic functioning remain unclear.

The central question of this chapter is: How does the dynorphin/KOR system function in normal and pathological neurological and psychiatric states? This chapter will review a substantial body of literature that posits a role for the dynorphin–KOR system in modulating synaptic strength and controlling neuroplasticity at several different synapses in brain regions associated with cognition and emotion.

KOR activation has been shown to directly change cell excitability in specific cell types. The majority of primary literature reviewed uses electrophysiological techniques including whole cell patch clamp, in current and voltage clamp configurations as well as field potential recordings from neurons in *in vitro* brain slice preparations and cell-culture systems. Activation of KOR has been found to reduce both synaptic strength and cellular excitability by inhibiting calcium channel activation, enhancing potassium conductances, and mobilizing intracellular calcium. Interestingly, MOR activation is also inhibitory in nature, however, the differences in behavioral consequences between MOR and KOR likely result from differences in regional, cell type and subcellular distribution of the two opioid receptors.

Recent evidence suggests that KOR activation does not only change cellular functioning acutely via the aforementioned mechanisms, but can activate a number of protein kinases downstream of KOR that also influence long-term synaptic plasticity and regulate transcription events known to influence learning and memory processes [8, 9].

Throughout the chapter, there will be several points where discrepancies or controversies in the current literature will be discussed in order to underscore areas in where further study is needed. Furthermore, some of leading models of how KOR is involved in pathological conditions will be presented to emphasize the potential for KOR selective drugs as therapeutic tools.

11.2 Tools

The development of *in vitro* slice preparations and dissociated cell culture systems used to study synaptic transmission and cellular excitability have proven advantageous for several reasons. The ease of molecular manipulations in these systems allows for detailed elucidation of the mechanisms underlying changes in synaptic strength or cellular excitability. Manipulations can range from including inhibitors of downstream signaling molecules in the recording pipette, bath application of ion channel blockers thought to be coupled to KOR, transfection of mutated channels or receptors into culture systems or exclusion of necessary proteins from transfected culture systems. *In vitro* studies using isolated synaptosomes have also provided important mechanistic insights [10, 11].

Synaptosomes are isolated synapses from neurons that, following homogenization and centrifugation, remove most of the cell, yet preserve the molecular machinery necessary for neurotransmitter release and uptake. A principal site of KOR action is on pre-synaptic terminals, and thus studies using synaptosomes are useful and complementary to conventional electrophysiology. Most *in vitro* electrophysiology experiments are done using a within cell design where agonist is exogenously bath applied and then either reversed by drug-free buffer wash or by subsequent antagonist application. Alternatively, the effects of the agonist are blocked via pre-treatment of antagonist or addition of an inhibitor of a downstream signaling event. Although *in vivo* analysis of drug effects allows better correlations between cellular actions and behaviors, the use of defined drug and inhibitor concentrations during experiments done *in vitro* allows more confidence in the interpretation of their sites and mechanisms of action.

The most commonly used exogenous KOR agonist in *in vitro* electrophysiology preparations is (5a,7a,8b)-(-)-N-methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro(4,5)-deca-8-yl] benzeneacetamide (U69,593) (typically at 100 nM to 10 μ M concentrations), however, trans-(\pm)-4,3-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)cyclohexyl]-bezene-acetamide methane sulfonate (U50,488H) is also used [12, 13]. Two of the endogenous dynorphin-opioids derived from the preprodynorphin precursor polypeptide dynorphin A (1–17) and dynorphin B are also used and have the structural features required for KOR selectivity [14].

In a few instances, researchers have demonstrated the ability of stimulated or activity-dependent release of endogenous dynorphins to influence synaptic strength and/or cell excitability in a KOR-dependent manner [15–17]. Demonstration of a physiological role for KOR also hinges on the use of selective antagonists. Typically, the KOR selective antagonist norbinaltorphimine (norBNI) is used to block the effects of exogenously applied or stimulated release of endogenous KOR ligands. However, recently additional KOR antagonists have been developed including (3R)-7-hydroxy-N-(2-methylpropyl)-1,2,3,4-tetrahydro-3-isoquinoline-carboximide (JDtic), 5'-acetamindinoethylindatridole (ANTI) and 5'-guanidinonaltrindole (GNTI). In some instances, researchers will use the nonselective opioid antagonist naloxone to reverse the effects of bath applied KOR agonists; however naloxone has about tenfold higher affinity for MOR than KOR [18].

The development of KOR knockout mice ($\text{KOR}^{-/-}$) as well as prodynorphin knockout mice ($\text{Dyn}^{-/-}$) has been useful in confirming effects seen with KOR agonists and antagonists (for review see [3, 19]). In some cases, $\text{KOR}^{-/-}$ or $\text{Dyn}^{-/-}$ may be more useful, for example, in longer studies in which stable inactivation is required. In studies such as these, the disparity between the half-life of the agonist and duration of the experiment could confound the results. Moreover, the use of genetic knockouts allows researchers to gain information about developmental time course and possible compensatory changes that result from KOR or prodynorphin deletion. This is arguably important in understanding potential genetic causes of pathology in humans. For example, a functional polymorphism in the prodynorphin gene promoter has been linked to temporal lobe epilepsy in humans [20]. However, possible compensatory changes following gene deletion must be considered when interpreting the results.

There is significant pharmacological evidence along with some physiological and behavioral evidence demonstrating three KOR subtypes (KOR1, KOR2, KOR3) [21–24]. Similar data suggests two MOR and DOR subtypes (MOR1,2 and DOR1,2, respectively). While this pharmacological evidence is compelling, presently there have only been three homologous opioid receptor genes that have been cloned. Therefore, the correlation between pharmacologically unique subtypes and *in vivo* biological activity of encoded proteins remains unclear [25]. Pharmacologic heterogeneity may arise from alternative splicing mechanisms of the known KOR gene or variance in the ability of KOR1, 2, or 3 to form functional heterodimers. It has been demonstrated that KOR can form functional heterodimers with DOR in *in vitro* gene expression systems [24]. It has been suggested that *in vivo* differences in responses may also result from different KOR subtypes. The behavioral ramifications of activation of the different KOR subtypes remains an active area of research, but will be mentioned only briefly in the remainder of the chapter.

Through use of electrophysiology, specific questions about modulation of synaptic physiology can be asked via implementation of intracellular recordings in a whole-cell voltage clamp or current clamp configuration. Typically, the effects of KOR activation on evoked (via stimulation of afferent fibers) excitatory or inhibitory post-synaptic currents (voltage clamp) (EPSC or IPSC, respectively) or potentials (current clamp) (EPSP or IPSP, respectively) are measured. Particularly, when interested in the distinction between pre- and post-synaptic effects, a paired-pulse paradigm (two electrical pulses delivered typically 50 ms apart) is employed, and the paired pulse ratio (PPR) or the ratio of the amplitude of the second PSC over the first PSC is measured. Although the exact mechanisms remain unclear, the paired pulse ratio is thought to be inversely proportional to the pre-synaptic release probability. Often times, calcium independent TTX-insensitive miniature PSCs will also be recorded to accompany evoked data. Changes in mPSC frequency, particularly in the absence of mPSC amplitude change, also suggests a pre-synaptic site of action of modulation. In studies, researchers will sometimes report coefficient of variation (defined as standard deviation/mean amplitude of a response assuming a Poisson distribution), which is a measure that is inversely related to probability of transmitter release independent of amplitude.

Furthermore, use of strontium (Sr^{2+}) produces asynchronous transmitter release that allows one to count individual quanta. Field excitatory post-synaptic potentials (fEPSPs) as well as population spiking are also commonly used, particularly in laminar structured brain regions like the hippocampus [17, 26]. Furthermore, the effects of KOR on the induction and maintenance of long-term potentiation at excitatory synapses, particularly in the hippocampus, have been examined using both chemical and electrical (tetanic) stimulation [17, 26–28].

In both slice preparations and dissociated cell culture systems, the effects of KOR on intrinsic characteristics of the cell including resting membrane potential (RMP), input resistance and spike width and duration and spontaneous firing have been studied [16, 29]. Also, in voltage clamp configuration the effect of KOR activation on isolated calcium and potassium currents using current pulses or ramp protocols and pharmacology have been examined [30–34].

In recent years, use of slice electrophysiology has advanced such that animals are first exposed to acute or chronic drug treatment or a certain behavioral manipulation prior to slice preparation. In this way, the slice preparation captures stable changes in synaptic functioning following specific pharmacologic or behavioral manipulations over a specific time course. This method has been employed extensively in the study of the effects of drugs of abuse on synaptic plasticity [5, 35] and to some extent has been used in the study of KOR on synaptic function [36, 37].

Functional neuroanatomy and ultrastructural analysis by electron microscopy [15, 38, 39] has been key to our understanding of the effects of KOR on synaptic functioning and communication between brain regions. Different neuroanatomical assays that have been effectively used include classic immunohistochemistry [40], radioligand binding of [^3H] U69,593 following stimulation or behavioral manipulation [41], use of phospho-receptor specific antibodies [42, 43], and retrograde tract tracing [29, 44]. In addition, sites of action have also been identified using *in vitro* radioligand displacement assays [41].

Finally, another complementary tool to conventional electrophysiology is the use of *in vivo* and *in vitro* neurochemical detection using microdialysis or fast scan cyclic voltammetry. Through different mechanisms, these two techniques can indirectly measure the release of neurotransmitters other than excitatory amino acids or GABA, specifically these neurochemical studies often look at the release of monoamines as measured by the relative changes in extrasynaptic spillover. Monoamines such as dopamine have been shown to have dramatic effects on fast synaptic transmission and plasticity at excitatory synapses (for review, see [45]). It is in this context that these studies are discussed below, particularly since there is evidence that KOR is present on monoaminergic pre-synaptic terminals [38, 46].

11.3 Behavioral Studies

Behavioral studies, mostly in rodent models, have been particularly useful in guiding physiological work done using *in vitro* systems. Examining the effect of KOR agonist/antagonism or gene deletion (use of $\text{KOR}^{-/-}$ or $\text{Dyn}^{-/-}$) in behavioral assays

where certain brain regions are known to be involved has been instrumental in isolating regions of interest for electrophysiological studies. Moreover, the use of region specific microinjections of KOR agonists or antagonists has been particularly important in understanding the role of different brain regions in mediating all or select components of complex behavioral outputs. Initially, interest in modulation of synaptic transmission by the dynorphin-KOR system focused on its role in neurological conditions including neuropathic pain and epilepsy.

However, recent studies of the dynorphin-KOR system have broadened to include examination of its role in cognition and emotion. Pfeiffer et al. [1986] reported that in male human subjects, KOR agonists produced feelings of dysphoria, aversion and anxiety. Some subjects report racing thoughts and feelings of body distortion as well, while others report pseudohallucinations, loss of self-control and inability to focus attention, and these responses were blocked by naloxone.

Salvinorin A (Sal A), the active component of the hallucinogen salvia divinorum, is a potent and selective KOR agonist, and cognitive-emotional alterations have been reported following human recreational use of salvia divinorum (for review, see [47]). An important theme to appreciate is that in all these different behavioral phenomena, dynorphin is released following a state of cellular hyperexcitability.

11.3.1 Dynorphin-KOR System and Nociception

Pain perception or nociception involves forebrain regions as well as regions in the midbrain, brainstem and spinal cord, each of which contributes a different component to the overall sensory phenomenon. The role for opioids in analgesia has been extensively studied. In particular, an effort has been made to study mechanisms of opioid receptor tolerance and dependence in hopes of improving pain therapeutics. While morphine, a MOR agonist, is often used to treat acute pain, it is not as efficacious in treating neuropathic pain [48, 49]. Several behavioral studies using KOR agonists/antagonists as well as knockout animals have demonstrated a potential role for the dynorphin-KOR system in analgesia of neuropathic pain [3]. Dynorphin immunoreactivity is widespread in several regions that are associated with nociception and several forms of peripheral inflammation result in upregulation of dynorphin [49]. It has been shown that KOR gene deletion increases sensitivity to chemical visceral pain [50]. Mice subjected to partial sciatic nerve ligation (pSNL), a model of neuropathic pain demonstrate an increased phospho-KOR immunoreactivity mostly in GABAergic neurons and astrocytes [43, 48].

Interestingly, KOR^{-/-} and Dyn^{-/-} mice with pSNL demonstrate different behavioral phenotypes: KOR^{-/-} mice demonstrate enhanced hyperalgesia (increased pain sensitivity) and increased allodynia (behavioral sensitivity to non-noxious stimuli) following pSNL, whereas Dyn^{-/-} demonstrate hypoalgesia and decreased allodynia [48]. Schepers et al. [51] similarly found that inflammation-induced hyperalgesia by intraplantar complete Freund's adjuvant (CFA) was significantly

exacerbated in KOR^{-/-} and also leads to mechanical hyperalgesia in sites remote from the site of inflammation. It is thought that in a neuropathic pain state, dynorphin is secreted chronically, leading to desensitization and internalization of KOR. One interpretation of these data is that KOR^{-/-} mice mimic animals that have desensitized receptors, while Dyn^{-/-} mice do not show dynorphin-dependent receptor desensitization [48].

Chronic pain can pre-dispose or potentiate psychopathological states. For example, chronic inflammatory pain induced by formalin injection was shown to potentiate morphine preference in the conditioned place preference assay (see below) in a norBNI-sensitive manner [52]. In addition to neuropathic pain, there is also the phenomenon of stress-induced analgesia, in which prior stress exposure produces analgesia to noxious stimuli (for review, see [53]). In mice, certain forms of stress-induced analgesia appear to be norBNI sensitive and not apparent in Dyn^{-/-} mice [42, 54]. These behavioral studies, as well as others, underscore the need to understand KOR functioning in regions associated with nociception. Electrophysiological studies have been conducted focusing on the role of KOR in modulation of synaptic transmission and direct effects on cellular excitability in regions associated with nociception including different segments of spinal cord (e.g., dorsal root ganglia), nucleus raphe magnus (NRM) and rostral ventralmedial medulla (RVM).

11.3.2 *Dynorphin-KOR System and Epilepsy*

There is a body of evidence to suggest that dynorphin peptide and message expression is upregulated in both epileptic humans and animals models of epilepsy [55–59]. Normally, the bulk of dynorphin immunoreactivity in the hippocampus is confined to the granule cell layer of the dentate gyrus and mossy fiber terminal region in the hilus and CA3 regions [40, 57]. In contrast, hippocampi taken from epileptic humans show expanded dynorphin immunoreactivity in the inner molecular layer of the dentate gyrus, suggesting that dynorphin is now expressed in sprouted recurrent collaterals that may underlie some of the hyperexcitability evident in this syndrome [57, 58]. In a rodent pilocarpine-induced model of epilepsy, prior administration of U50,488 attenuated pilocarpine induced seizure activity by increasing seizure latency, decreasing seizure duration, decreasing mossy fiber sprouting and increasing hilar neuronal survival [55]. There are also significant increases in dynorphin expression in the hippocampus following amygdala kindled seizures [60, 61]. Along the same line, Dyn^{-/-} animals have a lower threshold for onset of seizure, which can be rescued by pretreatment with U50,488 in a GNTI-sensitive (KOR-dependent) manner [56]. This study is particularly interesting since there have been reports that polymorphisms in the prodynorphin gene promoter are associated with temporal lobe epilepsy [20, 62]. These studies suggest that in instances of pathological hyperexcitability dynorphin might be upregulated as a compensatory mechanism.

11.3.3 *Dynorphin-KOR System and Stress, Depression, and Drug Abuse*

It is now thought that the dysphoric elements of stress contribute to the development of anxiety states, clinical depression and drug abuse. Furthermore, there is recent evidence to suggest that the dysphoric components of stress are encoded by the dynorphin-KOR system [63]. In preclinical studies using rodent models, various stressors including forced swim stress, foot shock and social defeat stress (SDS) are typically employed to produce anxiety, learned helplessness and depression-like behavior. The effects of stress potentiation of drug preference, seeking and reinstatement can be studied by combining these behavioral stressors with assays such as drug self-administration, intracranial self-stimulation and conditioned place preference and aversion.

Restraint (or immobilization) stress as well as forced swim stress (FSS) (for review of FSS, see [64]) and induction of learned helplessness increases both dynorphin A and B immunoreactivity in the dentate gyrus and CA3 region of the hippocampus as well as nucleus accumbens (NAcc) core and shell [65], two regions that have extensively been implicated in affective disorders (for review see [66]). Similarly, repeated stress exposure increases norBNI-sensitive phospho-KOR immunoreactivity in these regions; an effect not evident in Dyn^{-/-} mice [63].

Treatment with U69,593 or Sal A increases immobility in the FSS assay, indicating a KOR dependent increase in pro-depressive behavior [67, 68]. Microinjection of norBNI into either of these regions increases escape behavior (i.e., a marker of antidepressant behavior) in the learned helpless paradigm [65]. Systemic treatment with norBNI, GNTI, ANTI or the novel KOR antagonist MCL-144B prior to FSS exposure decreases immobility [68–70]. It has been demonstrated that stress decreases BDNF expression which in turn, predisposes the individual to depressive mood [71]. Acute pre-treatment (24 h prior) with high doses of norBNI i.c.v. has been shown to increase BDNF mRNA expression in the areas of hippocampus and the amygdala [70].

Along the same vein, KOR antagonists norBNI and JDtic decrease anxiety-like behavior in other behavioral assays developed to test anxiety including elevated plus maze (EPM), open field test (OF), and fear-potentiated startle (FPS) [72]. Exposure to FSS produces a norBNI-sensitive conditioned place aversion (for review of CPP/CPA see [73]) to the treatment paired context (side) that is not apparent in Dyn^{-/-} mice [54]. This effect is mimicked by U50,488 treatment [74, 75]. Keep in mind that the CPP/CPA assays are measures of contextual learning as well as affect. Similar to FSS studies, pretreatment with norBNI or prodynorphin gene disruption attenuates stress responses and submissive posturing associated with SDS [76]. Microinjection of U50,488 specifically into NAcc produces a similar CPA to that seen with systemic U50,488 treatment in a dose-dependent fashion [77]. Taken together, it seems that stress induces dynorphin release and that subsequent activation of KOR elicits pro-depressive and pro-anxiety-like behaviors as well as contextual aversion.

Early studies have shown that KOR activation by U69,593 depresses cocaine-induced locomotor sensitization in rats, a common behavioral output for measuring cocaine sensitivity [78]. However, it is unclear if this reduction is due to KOR effects on locomotion or actual valence of a euphoric stimulus. Stress exposure, either by FSS or SDS, potentiates preference to the cocaine paired context, and this is again attenuated by pretreatment by norBNI or prodynorphin gene disruption [54, 76]. In another study using cocaine CPP, swim stress-induced reinstatement of extinguished place preference to the cocaine-paired side was blocked by pretreatment of the KOR antagonist arodyn [79]. Moreover, stress-induced reinstatement (intermittent footshock) to lever pressing for cocaine in the absence of drug delivery is attenuated by pretreatment with JDtic in a dose-dependent fashion. Similarly, alcohol consumption is reduced in mice lacking preprodynorphin [80].

In an interesting study by Todtenkopf et al. [81], the researchers found using an intracranial self-stimulation assay with electrodes implanted in dopaminergic medial forebrain bundle, that systemic pretreatment with U69,593 resulted in a rightward shift in the response \times frequency function. In other words, rats required higher stimulus intensities (i.e., greater amount of dopamine delivery) in order to press a certain number of times compared to vehicle treated rats. ANTI was shown to block this effect. While this has clear implications for the effects of KOR on dopamine itself (see Sect. 11.7), from a behavioral standpoint this suggests that KOR activation controls the amount of effort required for a given rewarding stimulus. Hence, stress-induced activation of KOR produces a dysphoric state that can lead to depressive-like behavior and an increase in the preference for rewarding, euphoric stimuli.

11.3.4 Dynorphin-KOR System and Learning

Stressors can have effects on cognitive functioning in addition to producing a negative affect. Specifically, mild or transient stress can enhance learning, while chronic or acute severe stress can impair learning (for review, see [82]). Jiang et al. [83] has shown using radioimmunoassay, that dynorphin A immunoreactivity is also increased in the hippocampus and frontal cortex of aged rats. Moreover, the authors demonstrated a significant negative correlation between the level of spatial learning and the level of dynorphin A immunoreactivity in the hippocampal formation. In humans, the brains of Alzheimer's disease patients have significant increases in dynorphin expression compared to age matched controls [84].

In more recent studies, using standard behavioral models of learning, intra-hippocampal injection of U50,488 impaired contextual freezing (contextual fear conditioning) and increased swim path length in the Morris water maze assay, both of which indicate that acute KOR activation in the hippocampus produces learning deficits [85]. In a novel object recognition task, FSS exposure produced deficits in object recognition. Systemic U50,488 treatment mimicked the effects of FSS, suppressing novel object recognition [86]. In a context CS-US blocking paradigm

(for review, see [87]), intra-accumbal infusion of U50,488 facilitated blocking [88], suggesting that KOR activation in the accumbens not only produces aversion, but facilitates attending to the most relevant associations.

As all of these behavioral studies suggest, KOR activation in areas such hippocampus and NAcc appear to be important for mediating behaviors associated with affect and cognition. As such, much of the slice work done in the last decade has focused on KOR modulation of glutamatergic synaptic transmission in isolated hippocampus and cortico-striatal preparations (Table 11.1).

11.4 Hippocampus

KOR inhibition of glutamatergic excitatory synaptic transmission has been extensively studied in sub-regions of the hippocampus and has been extended to several other brain regions of interest in the past few years. The hippocampus is an example where KOR and MOR activation have opposing effects (inhibition and excitation respectively) based on cellular localization. MOR activation has a predominantly excitatory role in the hippocampus resulting from disinhibition of GABAergic interneurons [89].

The effects of KOR activation are discussed below. Interest in the hippocampus is threefold: First, the hippocampus has been widely studied as a nexus for seizure activity associated with various forms of epilepsy. Second, the hippocampus has been extensively studied for its role in explicit learning and long-term potentiation. Third, changes in hippocampal excitability have been widely implicated in models of depression. Demonstration of the actions of endogenous dynorphin release in the hippocampus was some of the first evidence of peptidergic neurotransmission in the CNS.

As demonstrated by Jan and Jan [90] using the sympathetic ganglia of the frog, release of small molecules (i.e., acetylcholine) and peptides (leutinizing hormone releasing hormone) from nerve terminals require different strengths and durations of electrical stimulations and have different time courses and binding properties at the synapse. KOR modulation of excitatory synaptic transmission and cellular excitability was demonstrated primarily at three synapses within the hippocampus: the perforant pathway to molecular layer, the mossy fiber pathway to CA3 pyramidal cell layer and recurrent collateral contact of mossy fiber pathways to dentate granule cells (DGCs). Schaffer collateral synapses onto CA1 pyramidal cells have been examined, however, both histological and electrophysiological studies have shown a lack of KOR modulation at this particular synapse [91].

Wagner et al. [41] employed a radioligand competition assay using [³H]U69,593 to demonstrate stimulated release of dynorphin (i.e., reduction in [³H]U69,593 is indicative of an increase in endogenous dynorphin release) in guinea pig hippocampal slices in a calcium-dependent and TTX-sensitive manner. Stimulation was done through high frequency hilar stimulation (HFHS), perforant pathway stimulation (50-Hz stimulus trains of 300 μA pulses of 0.3 ms durations for 1 s every 10 s)

Table 11.1 Summary of KOR modulation at different effectors

Effector	Consequence of KOR activation	Cell/brain region
Synaptic release		
Glutamate	↓	Dentate gyrus, CA3, nucleus accumbens, dorsal raphe, locus coeruleus, RVM, NRM,
GABA	↓	CA1, globus pallidus, nucleus accumbens, ventral tegmental area
Dopamine	↓	Nucleus accumbens, prefrontal cortex, VTA
Serotonin	↓	Dorsal raphe, nucleus accumbens, hippocampus
Norepinephrine	↓	Hippocampus
Ion channels		
N-type CaV	↓	Dorsal root ganglia, globus pallidus
P/Q-type CaV	↓	Anterior pituitary
L-type CaV	↓	Globus pallidus, anterior pituitary
NMDA	↓	CA3
HCN	↑	Nucleus raphe magnus
Kv 1.1 (K_A)	↑	Dentate gyrus, CA3, supraoptic nucleus
Kv 1.7 (K_M)	↑	CA1, CA3
GIRK	↑	Xenopus oocytes, spinal trigeminal nucleus, VTA
Kinases		
ERK 1/2	↑	COS-7 cells, striatum
p38 MAPK	↑	COS-7 cells, hippocampus, striatum, cortex, spinal cord

or through chemical stimulation using application of veratridine. Identification of the specific opioid released was done using dynorphin antisera. As expected, pre-incubation of dynorphin antisera blocked the stimulation induced reduction of [³H] U69,593 binding.

Exogenous application of U69,593 to guinea-pig hippocampal slices depresses EPSP and EPSC amplitude and population spike responses in recordings from DGCs [17, 92]. Using the same antidromic HFHS stimulation protocols described above, endogenous release of dynorphin also inhibits perforant path (PP) evoked EPSCs again in intracellular recordings made from DGCs, which is blocked by naloxone, norBNI and pretreatment with dynorphin antisera [17]. Accordingly, ultrastructural analysis has shown dynorphin localization in dense core vesicles in dendrites and some unmyelinated axons typical of collaterals of DGCs [15].

In a set of follow-up studies, Simmons et al. [94] demonstrated that the inhibition of excitatory transmission from perforant pathway synapses was due to a pre-synaptic site of action using anatomical and functional assays [93]. Antidromic release of dynorphin during hilar stimulation suggests that dynorphin acts as a retrograde transmitter locally and is able bind to KOR receptors on PP axon terminals through volume transmission. Perforant path stimulation reliably evoked LTP. However, if induction was preceded by HFHS, the LTP was blocked. Again, norBNI or

dynorphin antisera pretreatment blocked the effects of HFHS on LTP induction [17, 27]. In a pilocarpine model of epilepsy in rat, though spontaneous excitatory currents were increased and stimulation threshold decreased, KOR inhibition was increased again suggesting that in a state of pathologic hyperexcitability, dynorphin release acts as a natural anti-convulsant [94].

Mossy fiber stimulation also caused release of dynorphin from axon terminals synapsing onto CA3 pyramidal cells resulting in depression of fEPSPs and eEPSCs [28, 95]. Stimulated release of dynorphin as well as exogenously applied U69,593 increased PPR, again indicating a predominantly pre-synaptic site of action. Interestingly, in the presence norBNI, subthreshold stimulation was able to induce LTP, and pre-synaptic inhibition by dynorphin was more pronounced in cells that expressed LTP [28]. These data suggest that at the mossy fiber-CA3 pyramidal cell synapse, endogenous dynorphin may regulate the threshold of LTP induction. It should be noted that at the time these experiments were carried out, little was known about different types of LTP and LTD that occur at different synapses (e.g., early LTP, late LTP, cannabinoid-dependent LTD).

To better understand the conditions in which KOR influences plasticity, closer attention must be paid to which types of LTP KOR modulates in the future. KOR modulation of population spiking and glutamatergic release probability is likely due to KOR1 activation. Caudle et al. [21] demonstrated that KOR2 inhibited NMDA mediated currents evoked from Schaffer collateral/commissural stimulation. GR 86,696 was identified as a KOR2 selective agonist and KOR1 selective antagonist based on its ability of inhibit NMDA-mediated currents in a naloxone-sensitive fashion and reverse the effects of U69,593 on population spiking when co-applied [22]. It is possible that KOR1 and KOR2 may modulate different types or phases of LTP based on their different physiological effects.

In addition to regulating excitatory synaptic activity pre-synaptically, KOR activation also results in an augmentation of M-current (Kv 1.7) directly in CA3 pyramidal neurons [96]. While KOR may not influence post-synaptic glutamate receptor efficacy or distribution at this synapse, it is possible that depression at this synapse is a combination of direct effects on CA3 cell excitability as well as pre-synaptic inhibition of glutamatergic synapses.

Finally, a third site of action for KOR is the recurrent collateral mossy fiber synapse onto DGCs that is independent of the perforant pathway. Similar to the other pathways discussed, KOR activation by dynorphin or U69,593 inhibits EPSCs and population spike responses at this synapse as well [97]. A caveat to this finding is that hilar excitatory influences are not evident without pretreatment of the $GABA_A$ blocker bicuculline. However, a transient disinhibition could result in unmasking this hilar excitatory influence in a long-term fashion. As stated above, there does not seem to be a large KOR influence on the Schaffer collateral to CA1 pyramidal cell excitatory synapse. Application of U50,488 had no effect on LTP induction or maintenance at this synapse, yet did inhibit population spiking [98]. Madamba et al. [32] demonstrated that dynorphin application augments M-current in CA1 neurons similarly to CA3 neurons, which could account for decreased spiking in this region.

There is also evidence that KOR is located on GABAergic interneurons proximal to the CA1 pyramidal neuron, suggesting a disinhibition of CA1 neurons [91, 99]. In a different slice preparation containing globus pallidus neurons, it was demonstrated that dynorphin A application depressed calcium-dependent IPSCs via pre-synaptic inhibition, hyperpolarized and increased input resistance in a subset of neurons [100]. A similar mechanism may also be true in CA1. Further studies on this region of hippocampus must be conducted to resolve these disparities. However, the take home message of these studies is that at several synapses with the hippocampus, dynorphin release is activity-dependent and subsequent KOR activation results in inhibition of the excitation. Activity-dependent release of dynorphin may have a physiological role during Hebbian-type learning as modeled by LTP, pathological hyperexcitability characteristic of seizure states, or hyperexcitability that results from stress exposure.

The behavioral ramifications of dynorphin release in hippocampus remain unclear. In hippocampal formations from epileptic patients, dynorphin immunoreactivity is increased compared controls [58], however, the functional actions of dynorphin are disrupted [101]. Repeated U50,488 exposure *in vivo* produced tolerance to exogenously applied U69,593 or stimulated release of endogenous dynorphin on DGC population spikes in a NMDA-independent fashion [36]. In a separate study using the same methodology, U69,593 tolerance in hippocampal slices was correlated with increased KOR phosphorylation [102]. These data suggest that chronic activation of KOR ultimately results in desensitization and may produce chronic pathologic hyperexcitability.

Both the mechanisms underlying endogenous dynorphin release and KOR mediated inhibition of glutamatergic release have also been studied. At every synapse studied, dynorphin release appears to be calcium-dependent. Axonal release of dynorphin at recurrent collaterals within the hilar-dentate gyrus region as well as at the MF-CA3 pyramidal synapse was found to be mediated by N-type CaV [26, 97]. In contrast, dendritic release of dynorphin is dependent on both N-type and L-type CaV channels [26]. Through blockade of dynorphin release, L-type channel block facilitated LTP induction at the PP-DGC synapse [26].

Consistent with these findings, Castillo et al. [103] found that inhibition of internal calcium flux through application of EGTA-AM blocked release of dynorphin following tetanic stimulation, however, did not affect the inhibitory action of KOR activation. Despite evidence for KOR coupling to CaV channels through G $\beta\gamma$, blockade of N-type, P-type, or L-type channels did not disrupt KOR mediated fEPSP inhibition when U69,593 or dynorphin A was exogenously applied at either the PP-DGC or MF-CA3 synapse [26, 103]. Further study is required to clarify the mechanism of release at these synapses. It is likely that KOR activation has similar effects on excitability in several limbic brain regions. In a very recent study it was shown that KOR activation decreases synaptic transmission and inhibits LTP in the basolateral amygdala (BLA) [104]. There has also been more extensive work done in the striatum discussed below. It is important to remember that while the cellular events following KOR activation may be similar in different brain regions the behavioral ramifications of KOR activation in different brain regions are likely very different.

So how does KOR activation result in pre-synaptic inhibition of transmitter release? In hippocampal slice whole-cell voltage clamp recordings of CA3 pyramidal neurons, application of potassium channel blockers 4-aminopyridine (4-AP), mast cell degranulating peptide (MCDP) or dendrotoxin, which selectively blocks Shaker type Kv channels (Kv 1) markedly increased EPSC amplitude by prolonging glutamatergic release and blocked U69,593 inhibition of EPSCs. Consistent with other studies, cadmium, a blocker of CaV, inhibited EPSC amplitude, but did not attenuate U69,593 EPSC inhibition [95]. Low doses of tetraethylammonium chloride (TEA) (1 mM), administration of K_{ATP} blocker glibenclamide or administration of BaCl₂, a blocker of G-protein coupled inward rectifying K⁺ (GIRK, Kir3) channels did not affect KOR inhibition of EPSCs [95]. Taken together, at the MF-CA3 synapse, KOR is coupled to Shaker-type potassium channels ($I_{K(A)}$) pre-synaptically.

This type of coupling would be consistent with other studies showing that MOR can activate voltage sensitive potassium channels [105, 106]. These channels are known to influence repolarization following an action potential and therefore, can indirectly influence calcium influx. Studies of KOR coupling to calcium conductances and potassium conductances as well as effects of intracellular calcium stores have been done in several different types of culture systems as well as other *in vitro* brain slice preparations. Some of these studies are reviewed in the following section.

Figure 11.1 summarizes the majority of studies just discussed. Within the hippocampus, acute increases in excitation may result in the release of dynorphin and subsequent KOR activation that in turn inhibits glutamatergic synaptic transmission pre-synaptically as a negative feedback mechanism used to halt the excitation. However, chronic increases in excitability within the hippocampus may result in chronic secretion of dynorphin and importantly KOR desensitization and internalization that leads to long-term hyperexcitability.

11.5 KOR and Ion Channel Coupling

11.5.1 KOR and Ca²⁺

In the hippocampus, inhibition of excitatory neurotransmitter release seems to be dependent on KOR positively coupling to a potassium channel, rather than negatively coupling to a calcium channel. However, in other regions of the CNS there is strong evidence to indicate that KOR reduces calcium conductance, particularly high threshold calcium channels. In the neurohypophysis (the anterior pituitary), KOR activation reduced Ca²⁺ currents and hormone secretion by mechanisms that were blocked by inhibitors of L-type, N-type and P/Q-type channels [107]. In forebrain, cortical synaptosomes and in GABAergic accumbal synapses, pre-synaptic inhibition by KOR was also dependent on negative coupling to N-type

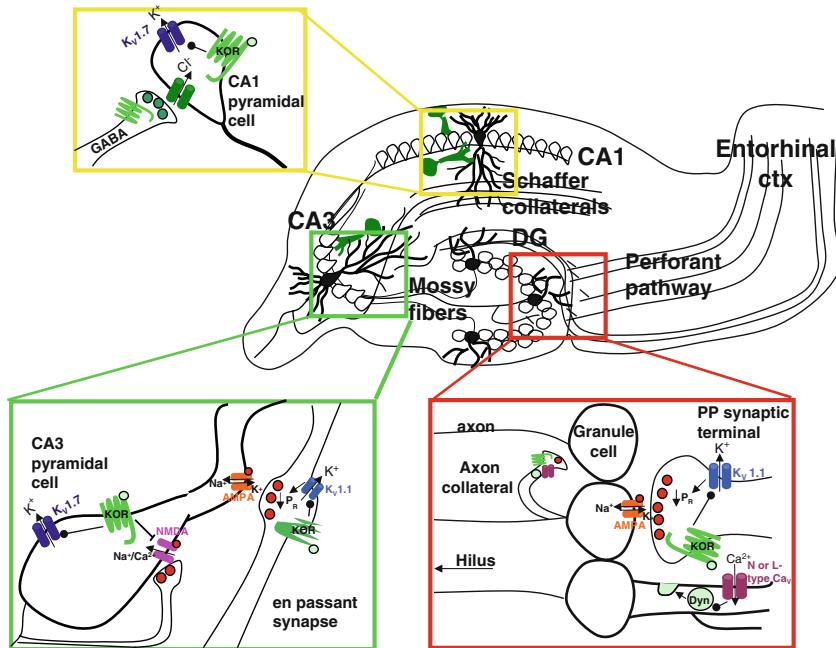


Fig. 11.1 Summary of KOR modulation in the hippocampus. Cartoon summary of sites of KOR modulation within the hippocampus, mechanisms of dynorphin release and of KOR actions. KOR modulates synaptic transmission and cell excitability in the dentate gyrus (red box), CA3 (green box) and CA1 (yellow box). KOR (bright green squiggle), dynorphin (light green circles), glutamate (red circles), AMPA-R (orange cylinders), NMDA-R (pink cylinders), GABA (dark green circles), GABAergic interneurons (dark green small cells). Kv1.1 (blue cylinders), Kv 1.7 (navy blue cylinders), L-type or N-type CaV (purple cylinders). A line with a ball at the end indicates that KOR activates or enhances the function of the effector while a line with a horizontal line at the end indicates that KOR inhibits the function of the effector

CaV channels in ref [11]. Similarly, both dynorphin and U50,488 inhibited high-voltage activated Ca^{2+} channels in small-to-medium (i.e., non-GABAergic) globus pallidus neurons. This was sensitive to both conotoxin (N-type) and nimodipine (L-type) pre-treatment [108]. KOR coupling to CaV channels in the dorsal root ganglia has been studied in greatest detail where KOR predominantly coupled to N-type channels and consequently inhibits Ca^{2+} currents in DRG neurons directly in a pertussis-toxin sensitive $\text{G}_{\alpha\text{o}}$ dependent manner [31, 109–111]. In primary neurons from slices of NRM, U69,593 enhances the amplitude of the hyperpolarization activated non-selective cation channel (I_{H}). The KOR mediated augmentation of I_{H} is dependent on heparin-sensitive IP_3 mediated release of intracellular Ca^{2+} [33]. In Neuro2 cells stably expressing KOR, calcium imaging studies revealed that activation of KOR following U50,488 application mobilized intracellular stores of calcium.

11.5.2 KOR and K^+

KOR couples to both voltage-gated (K_v) and inwardly rectifying (K_{ir}) potassium channels. For example, KOR can inhibit release pre-synaptically by enhancing a K_v conductance and also reduce cell excitability by enhancing K_{ir} conductance. G-protein gated inwardly rectifying potassium channels or GIRKs ($Kir3$) are activated by the $G\beta\gamma$ protein subunits following GPCR activation and produce a small outward current at normal resting potentials above E_{Kir} , but at hyperpolarized potentials produces a large inward current. They are typically defined by both their current–voltage relationship and sensitivity to barium. Many studies had been conducted on MOR coupling to GIRKs prior to investigation of KOR coupling to GIRK. In acutely dissociated CA1/subiculum neurons, MOR activation by DAMGO augmented K_{ir} conductance and a K_v conductance that was $CsCl_2$ -sensitive, but $BaCl_2$ insensitive in two different populations of nonpyramidal neurons respectively [106]. Several studies have examined actions of MOR in the noradrenergic locus coeruleus (LC) nucleus (for review see [112]). For example, in mice lacking $Kir3.2/3$, LC neurons had a significantly more depolarized RMPs and [Met]5-enkephalin induced hyperpolarization was reduced [113].

Similar studies have been conducted examining KOR coupling to GIRKs. In approximately one third of primary neurons recorded from slices of the spinal trigeminal nucleus of the *pars caudalis*, U69,593 and dynorphin A produced a hyperpolarization that was sensitive to manipulations of extracellular K^+ ($[K^+]_o$) [30]. Co-expression of KOR with GIRK subtypes (1, 2, or 3) in *Xenopus* oocytes has demonstrated the ability of KOR activation following either U50,488 or U69,593 application, to augment GIRK conductances using a ramp protocol in whole-cell voltage clamp mode [34, 114, 115].

Importantly, Ippolito et al. [116] found that dynorphin-dependent regulation of $Kir3.1$ by KOR-induced tyrosine phosphorylation occurs during both acute and chronic inflammatory pain and also during behavioral stressors, both of which increase release of dynorphin. KOR dependent tyrosine phosphorylation of GIRK may be the possible mechanism for the actions of U69,593. Similarly to studies done in hippocampus, KOR modulation of GIRK also desensitizes. Recent studies have shown that reduction in GIRK augmentation following prolonged KOR activation is a result of desensitization and internalization of KOR. This is mediated by an interaction of G-protein receptor kinase 3/5 and β -arrestin 2 with the Ser369 phosphorylation site on the C-terminal region of KOR [117–119]. Interestingly, desensitization of KOR mediated functional responses can persist for several days. Restoration of functional sensitivity required production and incorporation of new KOR receptors into the cell membrane rather a dephosphorylation event [119].

Along with a homologous mechanism of desensitization, a recent study by Clayton et al. [120] demonstrated that KOR activation can cause heterologous desensitization by phosphorylating a tyrosine residue located on the N-terminal tail of $Kir3.1$ (GIRK 3). In Att-20 cells, following treatment with 10 μM U50,488, somatostatin-mediated increases in GIRK currents were desensitized. The authors

went on to show that this heterologous desensitization was dependent on KOR induced p38 MAP kinase activation (see KOR and downstream signaling) that in turn activation Src kinase to phosphorylate the tyrosine residue. This study has interesting implications given the vast number of receptors shown to couple to GIRKs including 5-HT_{1A}, GABA_B and D₂ receptors. Strong activation of KOR may cause both homologous desensitization due to receptor internalization, but additionally may lead to heterologous desensitization of several other GPCRs at the level of the channel.

While the majority of studies have focused on opioid receptor coupling to GIRK, as stated above there is evidence that KOR can also couple to other Kv channels (e.g., Shaker, M channel). In addition to the work done in the hippocampus, KOR augments I_A as well as another voltage sensitive K⁺ current in vasopressin-containing magnocellular neurons of the supraoptic nucleus (see KOR and other neuropeptides) [121]. While both MOR and KOR can couple to GIRK or Kir3, they do diverge in their coupling to another Kir, K_{ATP}. MOR does positively couple to K_{ATP} presumably by mobilizing ATP, however, KOR has not yet been demonstrated to couple to K_{ATP} [122, 123].

Thus, KOR can clearly couple to different calcium and potassium channels to suppress or enhance conductance respectively as well as modulating intracellular calcium stores through downstream signaling mechanisms. What should be apparent from these last two sections is the diversity of KOR coupling and how disparate this coupling might be depending on the synapse or cell type of interest. These data suggest that KOR activation acutely reduces excitability by directly reducing calcium conductance, directly activating voltage-sensitive potassium channels, indirectly activating calcium-dependent potassium channels, and may also initiate calcium-dependent downstream signaling cascades.

11.6 KOR and Downstream Signaling

As discussed above, the first studies on KOR modulation of synaptic strength demonstrated that the dynorphin-KOR system regulates glutamatergic synaptic transmission and plasticity primarily through inhibition of pre-synaptic release in synapses within the hippocampus. This section concerns signaling molecules downstream of KOR that may be responsible for changes in synaptic strength, plasticity and behavior. Specifically, it has been shown that KOR activation leads to subsequent activation of several mitogen-activated protein kinases (MAPKs) [124]. In addition to acute effects, dynorphin-KOR modulation of long-term synaptic plasticity and learning-based behavioral outputs likely requires long-term changes in *de novo* gene transcription as well as up- or down-regulation of membrane protein expression.

Members of the MAPK family have been shown to influence both long-term and short-term synaptic plasticity, specifically extracellular-signal related kinase (ERK) and p38 MAPK (for full review see [8, 9]). Briefly, ERK can couple to dendritic

Kv channels, which in turn fine tunes membrane excitability. In classic models of LTP, *N*-methyl-D-aspartate (NMDA) channels act as coincidence detectors of membrane depolarization and binding of glutamate. Kv channels can regulate the depolarization sensed by the NMDA channel. Moreover, ERK regulates AMPAR insertion into the membrane and promotes dendritic spine growth and stabilization in CA1 pyramidal cells [8, 9]. ERK also phosphorylates the learning related transcription factors CREB and elk which go on to regulate the expression of several learning/memory related genes. ERK signaling has been widely implicated as being important for both spatial learning and fear conditioning [9].

Recent studies have suggested a distinct role of p38 MAPK in regulating neuroplasticity. Bolshakov et al. [125] found ERK and p38 MAPK can influence synaptic plasticity via parallel pathways such that p38 MAPK specifically regulates a form of mGluR-dependent hippocampal LTD at the CA3-CA1 synapse. Interestingly, the authors demonstrated that p38 MAPK induction influenced pre-synaptic function via regulation of a retrograde messenger. ERK and p38 MAPK have also been shown to having opposing effects on AMPA-R surface expression and function in the same cell [126, 127] suggesting divergent pathways for regulating plasticity. Additionally, p38 MAPK has been shown to phosphorylate sodium channels (specifically NaV 1.8) in the dorsal root ganglia, increasing current density in these neurons [128]. Therefore, p38 MAPK may cause a synaptic depression in some regions while increasing cell excitability in others.

The dynorphin-KOR system has been shown to alter both ERK and p38 MAPK activation. In both COS-7 and immortalized astrocytic cultures, norBNI-sensitive KOR activation causes an increase in ERK1/2 activation that is dependent on G $\beta\gamma$ G-protein subunits [129, 130]. MOR and KOR activation are both able to increase ERK1/2 activation, but through different isoforms of PKC (PKC ϵ and PKC ζ /PI3K for MOR and KOR, respectively). Interestingly, blockers of L-type calcium channels (e.g., nifedipine) or a blocker of release from intracellular Ca $^{2+}$ stores (e.g., dantrolene) both attenuated the activation of ERK1/2 induced by U69,593 [129].

In both striatal astrocyte and neuronal cultures, U50,488 application activated both p38 MAPK and ERK1/2 [131]. As described above, KOR forms a complex with β -arrestin 2 that is dependent on GRK3 phosphorylation of the Ser369 residue of the C-terminal tail of KOR. While U50,488 induced p38 MAPK activity was dependent on GRK3, β -arrestin 2 and Ser369, the early phase of increased ERK1/2 activity was not dependent on this complex.

Similar to the study by Bolshakov et al. [125] and Zhu et al. [126], it is possible that KOR activation of ERK1/2 and p38 MAPK may occur in the same cell or synaptic formation, yet go on to influence different forms of plasticity through different signaling complexes. KOR mediated astrocytic proliferation in the spinal cord following pSNL is dependent on KOR increases in p38 MAPK activity [43]. Changes in or up-regulation of astrocytic function as a result of p38 MAPK induction could have dramatic impact on synaptic strength. Astrocytes have been shown to influence glutamatergic synaptic function both by generation of Ca $^{2+}$ waves and glutamatergic uptake [132]. This KOR mediated increase in astrocytic proliferation could contribute to changes in cell excitability associated with neuropathic pain states.

Very little is known about the role of p38 MAPK in regulating learned behaviors. Classical eye-blink conditioning, a form of associative learning that is dependent on the cerebellum, is prevented by infusion of the selective p38 MAPK inhibitor, SB203580, into the cerebellar vermis [133]. Intra-accumbal injections of amphetamine evoked a conditioned place preference that blocked by intra-accumbal injection either ERK or p38 MAPK inhibitor in a dose-dependent fashion [134]. This study is interesting in that it was the first to demonstrate a potential role for p38 MAPK in a form of contextual associative learning that was dependent on a forebrain region.

Bruchas et al. [42] recently demonstrated that swim stress increases phospho-p38 MAPK expression in the hippocampus, striatum and cortex. This increase in phospho-p38 MAPK expression was blocked by norBNI, mediated by KOR activation, and dependent on β -arrestin 2 and GRK3 expression. Activation of KOR also increased activation of the transcription factor Zif268 (Egr1) in a p38 MAPK dependent fashion. Inhibition of p38 MAPK by infusion of SB203580 attenuated U50,488 induced CPA and decreased immobility in the FSS assay, but did not attenuate stress-induced analgesia. This study suggests that KOR activation of p38 MAPK in key brain regions plays a critical role in associative learning of context with aversive stimuli in specific behavioral assays. Certainly further study is needed on the role of p38 MAPK in regulating synaptic plasticity in different cell types and within different brain regions, both in and of itself and in relation to KOR. Moreover, the relationship between KOR-p38 MAPK effects on synaptic plasticity and learning related behavioral outputs remains to be elucidated. Interestingly, in a follow-up study, Bruchas and colleagues found that repeated swim stress also increased ERK1/2 activation in a norBNI sensitive manner, however, this activation was GRK3 independent [135]. These two findings illustrate how one receptor can have a multitude of downstream signaling cascades that are activated by similar behavioral stimuli, but likely have profoundly different effects on long term cellular excitability.

11.7 KOR and the Corticostriatal Pathway: Focus on the Nucleus Accumbens (NAcc)

The last three sections have dealt with the majority of studies on KOR modulation at the level of the synapse, post-synaptic cell and downstream signaling molecules. The following sections demonstrate how this knowledge has been extended to various other regions and circuits in the brain. Neuroplasticity as modeled by LTP and LTD have been most extensively studied in the hippocampus. However, there is compelling evidence that both short-term and long-term synaptic plasticity also occurs in several other brain regions.

The nucleus accumbens (NAcc), comprised mostly of GABAergic medium spiny neurons, receives glutamatergic cortical inputs and midbrain dopaminergic inputs. The nucleus accumbens is involved in several goal-directed behaviors,

including those which are reinforced by drugs of abuse [136]. Dynorphin is expressed in medium spiny neurons (MSNs) of the dorsal striatum and the NAcc. In the dorsal striatum, dynorphin is expressed exclusively in D1 receptor expressing neurons that project directly back to the substantia nigra, making up the “direct” pathway [137, 138]. Similarly, NAcc MSNs send projections back to the ventral tegmental area (VTA), the majority of these neurons express D1 receptors as well [139, 140]. In both the ventral and dorsal striatum, MSNs have axon collaterals that also can release dynorphin. It has been shown that drugs of abuse selectively increase dynorphin expression in a D1 receptor dependent fashion [141].

As mentioned above, intra-accumbal injection of U50,488 is able to significantly alter such goal-directed learning based behaviors. Electrophysiology, ultrastructural analysis, and neurochemical detection studies including microdialysis have been employed to assess the effects of the dynorphin-KOR system on synaptic function and neuroplasticity in NAcc. Figure 11.2 summarizes studies examining

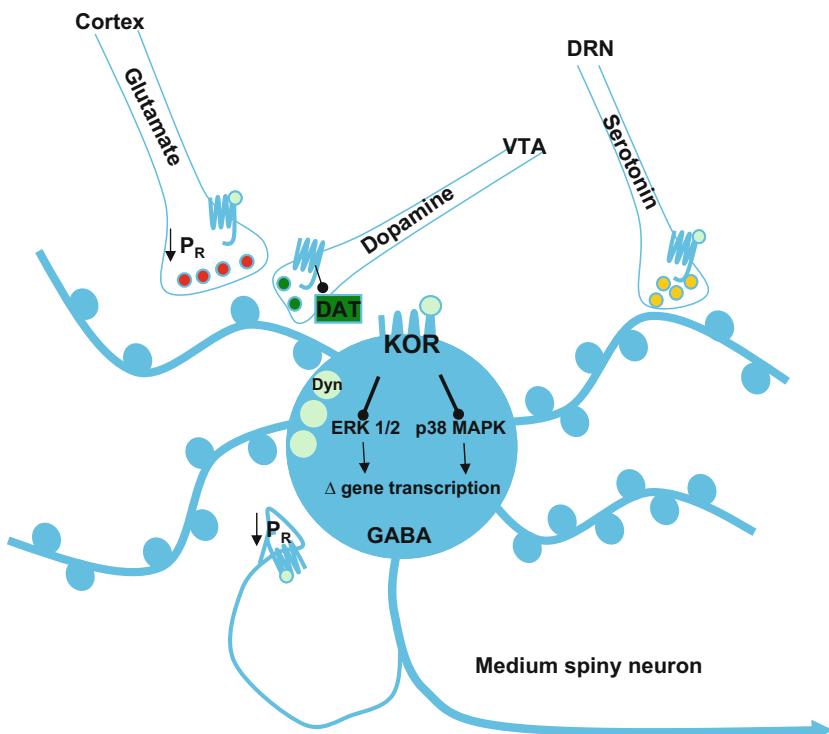


Fig. 11.2 Summary of KOR modulation and downstream signaling in NAcc. Cartoon summary of the sites of KOR modulation in a medium spiny neuron of the NAcc and downstream effectors of KOR activation. Following release of dynorphin (light green circles), KOR (bright green squiggle) can inhibit the release of glutamate (red terminal), dopamine (green terminal), GABA (blue axon collateral), and serotonin (orange terminal). It has been shown that KOR increases DAT function (dark green box) to inhibit dopamine release. Post-synaptically KOR increases (line with ball at the end) ERK 1/2 and p38 MAPK activity through parallel pathways

the neuroanatomical and functional localization of KOR and its downstream effectors in the nucleus accumbens.

Researchers have found that KOR can modulate both glutamatergic and GABAergic synaptic transmission [142, 143] as well as dopaminergic release [144–147] within NAcc. Keep in mind that modulation of dopaminergic release can ultimately influence fast excitatory neurotransmission. Ultrastructural analysis has shown that dopaminergic terminals synapse onto the sides of post-synaptic spines receiving glutamatergic stimulation from cortex, and there have been several studies demonstrating dopaminergic modulation of LTP/LTD [45]. However, despite fairly convincing evidence that all these KOR effects are occurring in NAcc, very little is known regarding how KOR modulation at different synapses within the accumbens affect behaviors.

Ultrastructural studies of KOR immunoreactivity within the accumbens by Svingos et al. [39] showed that KOR is localized to axon terminals, the majority of which belonged to small unmyelinated axons characteristic of dopaminergic or acetylcholinergic positive profiles that make contacts onto dendritic spines receiving convergent input from large asymmetric excitatory synapses. A smaller portion of KOR positive axon terminals either opposed dynorphin positive terminals or contained dynorphin. There was also a small percentage (21%) of KOR positive axon terminals that were large and formed asymmetrical synapses characteristic of excitatory synapses. A very small percentage (<4%) of KOR positive terminals formed large symmetric inhibitory synapses onto unlabeled dendritic shafts. A small portion of KOR positive structures were either cell bodies characteristic of MSNs (10%) or were astrocytes (10%). A follow-up study confirmed approximately half of the pre-synaptic structures positive for KOR also expressed the dopamine uptake transporter (DAT) [38]. These studies posit that KOR can modulate release of glutamate, acetylcholine, and dopamine.

Much like the hippocampus, through use of both field recordings and patch-clamp techniques, exogenous application of U69,593 depresses excitatory potentials (fEPSPs) and currents (evoked EPSCs) exclusively through pre-synaptic modulation, both of which are reversed by norBNI application [142]. While this is not surprising, the ultrastructure studies suggest that at least in a subpopulation of MSNs, KOR can act both pre- and post-synaptically. A follow-up study demonstrated that U69,593 can inhibit both glutamatergic EPSC(P)s and GABAergic IPSCs (albeit a moderate ~30% inhibition) both pre-synaptically, but through different mechanisms [143]. U69,593-induced increases in coefficient of variation (CV) were significantly correlated with decreases in IPSC amplitude and U69,593 also significantly decreased sIPSC frequency without altering amplitude. Pretreatment with 4-AP did not block KOR-dependent synaptic depression at either glutamatergic or GABAergic synapses. The researchers found that KOR modulation of GABAergic inhibition was dependent on N-type calcium channels, yet at glutamatergic synapses pretreatment with selective N-type or P/Q-type channel blockers did not attenuate the U69,593 effect.

Psychostimulants increase extracellular dopamine by inhibiting dopamine uptake (inhibition of DAT). Through activation of D1-like receptors, dopamine increases

CREB activation, which in turn up-regulates dynorphin gene expression [66, 148]. Normally, the actions of dynorphin-KOR in NAcc may act as a “brake” at different synapses, much like in the hippocampus. However, indirect up-regulation of dynorphin following psychostimulant exposure may desensitize and internalize KOR (see Sect. 11.5) such that the “brake” is effectively removed and the system is in a hyperexcited state [66]. Xia et al. [37] found that KOR mediated depression of fEPSPs was absent when animals were acutely exposed to amphetamine prior to slice preparation. This study is the first to posit a direct change in KOR modulation of glutamatergic release following drug exposure.

Several studies have focused on KOR inhibition of dopamine release. For these studies, *in vivo* microdialysis and *in vitro* voltammetry techniques, rather than electrophysiology, are often employed. MOR and KOR appear to have opposite effects on dopamine release. While MOR activation increases dopamine release via disinhibition of GABAergic neurons in or projecting to the VTA, both systemic administration and intra-accumbal administration of U50,488 or Sal A decrease extracellular dopamine [67, 145, 146, 149–152].

Repeated cocaine treatment causes persistent locomotor sensitization, increases VTA firing rate, dopamine levels in dialysates, and decreases DAT uptake, even after cessation of cocaine treatment [153, 154]. Co-administration of U69,593 with cocaine reduced the increases in dopamine [147]. Consistent with these findings, KOR^{-/-} mice exhibit significant increases in basal levels of accumbal dopamine [144]. As stated above, approximately half of KOR immunoreactive axon terminals also express DAT. In addition to directly inhibiting dopamine release, KOR activation may directly affect DAT function to influence dopamine release.

In vivo microdialysis is also used to measure the extraction fraction, an indirect measure of dopamine uptake or clearance and hence DAT activity. Repeated U69,593 exposure decreases extraction fraction and radioactive DAT binding, however does not effect total DAT protein levels [147]. This finding suggests that KOR disrupts DAT efficacy, but not necessarily expression. Interestingly, samples of dialysate from the accumbens of KOR^{-/-} mice demonstrate an increase in extraction fraction, which may be due to a compensatory up-regulation of DAT [144]. In direct contrast to KOR^{-/-} mice, basal DA levels are decreased in NAcc of Dyn^{-/-}, while the extraction fraction is unaltered [155]. Cocaine evoked DA release as well as psychomotor effects were decreased in Dyn^{-/-} compared to wild-type animals. U69,593 induced decrease in DA release was enhanced in Dyn^{-/-} mice suggesting that in these animals the KOR receptor is functionally up-regulated [155]. From the electrophysiological and microdialysis studies done in the accumbens, it seems that KOR is able to act at glutamate, GABA, and dopamine containing synapses. However, more work must be done to understand how different loci of modulation function together to produce NAcc-mediated behaviors.

Along with studies demonstrating KOR modulation of the dopamine system at release sites, the effects of KOR on excitatory synaptic transmission driving dopaminergic neurons of the VTA that project to NAcc, have recently garnered some

attention. In the same study demonstrating CPA following intra-accumbal injection of U50,488, the researchers also found that intra-VTA injection of U50,488 similarly produced CPA in a dose-dependent manner [77]. Moreover, intra-VTA injection of dynorphin A increases feeding behavior in sated rats [156]. Ultrastructural analysis of dynorphin immunoreactivity in the VTA revealed only sparse dynorphin immunoreactivity localized to symmetric inhibitory-type synapses (presumably originating from NAcc), though there was some localization at large asymmetric synapses as well [157].

However, intra-VTA injections of U50,488 had no effect on levels of accumbal dopamine in rats [149]. In recent retrograde tract tracing studies, it now seems likely that KOR is expressed at sites poised to modulate cell excitability and synaptic activity in subsets VTA dopaminergic neurons. In electrophysiologically classified subpopulations of VTA neurons (principal and tertiary), KOR activation directly inhibits firing through coupling to GIRK [158]. Depending on the target location of dopaminergic VTA projecting neurons, KOR activation has disparate effects on cell excitability (U69,593 induced hyperpolarization) and GABA_A and GABA_B mediated currents [29, 44]. In these studies there were some disparities between recordings done in rats [44] compared with those done in mice [29].

Consistent with microdialysis studies done in rats [149], KOR mediated activation of GIRK currents and subsequent membrane hyperpolarization was only apparent in dopaminergic neurons (i.e., tyrosine hydroxylase positive cells) that project to the prefrontal cortex and not the nucleus accumbens in recordings made from the VTA of rats [44]. However, in VTA recordings from mice, KOR activation produces a larger membrane hyperpolarization and GABA_B slow IPSC augmentation in dopamine cells projecting to NAcc compared to those projecting the BLA. Yet inhibition of fast GABA_A-mediated IPSCs was greater in dopamine neurons projecting to the BLA [29]. Consistent with findings in the accumbens that KOR is, at least part, localized to dopaminergic pre-synaptic terminals, U69,593 also inhibits D₂ mediated slow IPSCs that occur following “burst-like” stimulation [29, 159]. (Note: This electrophysiological phenomenon has been extensively characterized by Williams and colleagues and is one proposed mechanism for inhibiting phasic dopamine release.)

Again, these studies show what KOR activation can do, not what happens during discrete behaviors. These studies raise several issues. First, they suggest that KOR can both depress cellular excitability while producing a disinhibition synaptically in the same cell type when U69,593 is exogenously applied. Second, these data suggest a difference in the kinetics of KOR modulation dependent on target location. What is clear from this set of studies is that along with dynorphin-KOR modulation of cellular signaling in the corticostriatal pathway, there is similar modulation occurring directly in the VTA that may ultimately affect both striatal synaptic strength, but also change synaptic transmission in other projection sites such as the prefrontal cortex and amygdala. The disparity between the studies done by Margolis et al. (2006) in rats and Ford et al. [29] in mice may reflect a species difference. However, further work must be conducted to resolve this difference.

11.8 KOR and Monoamines

The majority of studies on dynorphin–KOR interactions with monoamine systems have focused on dopamine. In fact, very little is known about how KOR modulates other monoaminergic systems to affect behavior. However, there is some evidence that KOR can also modulate both serotonergic and noradrenergic systems by modulating synaptic transmission in serotonergic (dorsal raphe) and noradrenergic (LC) nuclei [160–162] and also at release sites [46, 163]. In recordings made from putative serotonergic or noradrenergic neurons of the dorsal raphe (DR) or locus coeruleus (LC) respectively, application of the KOR agonist CI-977 (enadoline) [161, 162] or U50,488 [160] depressed EPSPs. Ultrastructural analysis examining dynorphin immunoreactivity in the LC is consistent with the electrophysiological data. Dynorphin is mostly (89% of profiles) localized to axon terminals of asymmetrical excitatory synapses with some dendritic labeling [164].

Surprisingly, Reyes et al. [164] found that of the dynorphin positive axon terminals, only 28% contacted TH positive post-synaptic processes. In a follow-up study examining KOR localization in the LC, Reyes et al. [165] found that KOR was primarily localized to the nuclear core of the LC as well as peri-coerulear region where noradrenergic dendrites extend and was found on both somata and dendritic architecture. Twenty-nine percent of KOR positive axon terminals directly targeted TH positive neurons and of those 49% were typical excitatory asymmetric synapses. 47% of KOR positive axon terminals were also positive for preprodynorphin. KOR activation in the DR does not effect GABA_A IPSPs [162] nor does it modulate NMDA evoked IPSCs [166] and KOR does not seem to influence cell excitability directly in the LC [161].

In an interesting study by Kreibich et al. [208], the investigators examined the effects of KOR activation on various afferent inputs into the LC using an *in vivo* electrophysiology in an anesthetized animal. In this study the researchers took advantage of the well-characterized relationship between behavioral stimuli and activation of specific afferent pathways. For example, both sciatic nerve stimulation and auditory stimuli activates EEA containing fibers that results in phasic discharge within the LC. Whereas, hypotensive stress is known to stimulate corticotropin releasing factor (CRF) containing fibers that results in an increase in tonic LC activation. Infusion of U50,488 into the LC attenuated phasic discharge rates associated with activation of EEA fibers, without disrupting tonic discharge. In addition, increases in tonic LC discharge associated with engagement of CRF positive fibers was attenuated by similar U50,488 infusion. This suggests that KOR can inhibit excitation of this nucleus and presumably inhibit release of norepinephrine into the forebrain following a variety of stimuli and through a number of pathways.

There have been some studies done examining the effects of KOR activation on 5-hydroxytryptamine (5-HT) or noradrenaline (NA) release in the forebrain using radioligand binding assays and *in vivo* microdialysis. In a comparative study, the effects of U50,488 on NA release from neocortical slices of rat and human were assessed using tritiated NA. In rats, the mu agonist DAMGO inhibited NA release

in the neocortex, yet neither KOR nor DOR agonists had any effect. However, in human neocortical slices, DAMGO had no effect on NA release, but KOR and DOR agonists were able to produce a small depression in NA release [167]. *In vitro* assays that employ electrical or K^+ evoked release of 5-HT showed that in rats, U50,488 application inhibits 5-HT release in the medial septum, lateral septum and diagonal band [168], but has only minimal effects in the hippocampus [169]. It should be noted that there is a discrepancy in results from the hippocampi from different species, namely in between ground squirrels (KOR increases 5-HT release) and rats (KOR slightly decreases 5-HT release) [169].

In vivo KOR had no effect on 5-HT release in rat hippocampal slices [170]. Comparisons of opioid modulation of 5-HT in the neocortex (using tritiated 5-HT) of rat vs. human subjects showed that U50,488 depresses 5-HT in the neocortex of both species. However, in rats, U50,488 only causes inhibition of 5-HT release in the presence of antagonists against the 5-HT autoreceptor, 5-HT_{1B} [167]. These data suggest that in rodents, 5-HT autoreceptors may play a more critical role than opioids in directly regulating 5-HT release.

Finally, *in vivo* microdialysis studies have shown that U50,488 infusion into the DR, median raphe (MR) and NAcc inhibits 5-HT release in those respective sites independent of activity in glutamatergic or GABAergic efferents, suggesting that KOR directly modulates 5-HT release at pre-synaptic terminals in target regions [46, 163]. Recently, it has been shown that inactivation of KORs in either the DR or NAcc is sufficient to block U50,488 induced CPA. Interestingly, this study also showed that rescue of KORs in the DR of KOR^{-/-} mice was sufficient to produce a U50 aversion. The authors propose that KOR aversion is dependent on reduction of 5-HT at the terminals located in the NAcc [171]. This is particularly interesting with regard to the accumbens in that it begs the question, does KOR modulation of glutamate, GABA, DA and 5-HT in NAcc occur simultaneously to produce a behavioral output or is the modulation at different synapses selective for different behaviors (see Fig. 11.2)? Moreover, how does one develop therapeutic targets for disorders that affect multiple monoamine systems?

11.9 KOR and Pain Circuits

The effects of KOR on neuropathic pain responses as well as on cell excitability in regions important for nociception have already been discussed above. However, there have also been other studies done on KOR effects on synaptic transmission in the NRM and RVM, two regions important for nociception. In both regions, different populations of cells have been identified. KOR activation in the NRM antagonizes the analgesic effects of MOR agonists in that region. In the NRM, there are primary cells, which inhibit spinal pain transmission, and secondary cells, which facilitate spinal pain transmission [172].

Activation of MOR results in direct inhibition of secondary cells and inhibition of GABAergic synaptic transmission onto primary cells (a disinhibition); both

actions would result in an antinociceptive response. In contrast, KOR agonism had no effect on secondary cells, but directly inhibited primary cells, which is the potential mechanism of KOR antagonism of MOR analgesia [173]. In a more recent study, U69,593 inhibited glutamatergic synaptic transmission pre-synaptically at both primary and secondary neurons [172] and this is required for functional antagonism of MOR driven behavioral responses.

Similar to the NRM, neurons of the RVM can be segregated into different populations based on electrophysiological properties and neurochemical content. In the case of the RVM, there are serotonergic and GABAergic neurons, which exert a tonic inhibition onto serotonin containing cells. KOR is primarily localized to GABAergic neurons, but to a lesser extent is also present on 5-HT containing neurons [174, 175]. Consistent with histological findings, U69,593 application has been shown to hyperpolarize serotonergic neurons while inhibiting excitatory currents in GABAergic neurons, producing a disinhibition [176]. This is yet another example of how KOR activation results in a disinhibition at certain synapses. Other examples of KOR inhibition of GABAergic inhibitory transmission have been discussed above and occur in the VTA, NAcc and possibly the CA1 region of the hippocampus [29, 99, 143]. This is also another region in which KOR is influencing 5-HT release.

11.10 Dynorphin-KOR and Other Neuropeptides

The last section of this chapter deals with recent evidence that dynorphin interacts and oftentimes is co-expressed with other peptides in hypothalamic and limbic nuclei. Specifically dynorphin has been shown to co-localize with vasopressin in the supraoptic nucleus (SON) [177], orexin in the lateral hypothalamus (LH) [178] and kisspeptin in the arcuate nucleus (ACN) [179, 180]. The functional and behavioral ramifications of this have been most studied in the vasopressin system, but work in orexin-containing cells will also be discussed. In this section, KOR and corticostriatal pathway, KOR modulation of both the NAcc and VTA will be discussed in detail.

Interestingly, dynorphin containing neurons of the NAcc also contain substance P. As stated above, dynorphin is released following stressor exposure. Similarly, substance P is also released following stressor exposure. Yet substance P appears to produce opposite effects from dynorphin, increasing VTA excitability and dopamine release. This will also be discussed in further detail below. There are several behavioral and anatomical studies which posit an interaction between dynorphin and other neuropeptides. Localization of dynorphin and kisspeptin in the ACN is a very recent finding. There is also quite a bit of behavioral evidence suggesting that dynorphin and CRF interact and specifically that CRF causes the release of dynorphin [63, 181–183]. However, this chapter will limit the review of this material to cellular physiological studies providing evidence for dynorphin expression and its interaction with other neuropeptides.

11.10.1 *Dynorphin and Vasopressin*

Vassopressin or arginine vasopressin (AVP) release into the PNS acts primarily at the nephrons of the kidney to stimulate reabsorption of water across the collecting duct epithelium and therefore inhibits diuresis. Vassopressin release can occur during stress, pain or exercise [184–186]. This mechanism evolved as part of the PNS's fight/flight response. Centrally, it has been shown to play a critical role in social interactions, for example in monogamous voles it is critical for pair bonding behavior [187, 188]. Several studies have demonstrated that KOR agonism facilitates diuresis. Systemic administration of U50,488 facilitates diuresis, which can be blocked by KOR antagonism with norBNI or naloxone [189–193].

Dynorphin and vasopressin are co-packaged into dense core vesicles present in magnocellular secretory neurons of the SON [177]. The majority of work done on the effects of dynorphin in the SON has mainly used *in vivo* electrophysiological techniques. Thus, the mechanisms underlying dynorphin modulation of firing are not completely defined. Endogenous dynorphin regulates phasic burst firing patterns in vasopressin containing neurons of SON, but not oxytocin neurons also present in this region [194]. In the SON, vasopressin neurons exhibit depolarizing after-potentials (DAPs). Moreover, during bursting, phasic spikes are superimposed on plateau potentials that are produced by DAPs. KOR activation reduces post-spike excitability by inhibiting DAPs [177, 194, 195]. As described above, KOR is coupled to several different voltage-gated K⁺ channels including the Shaker channel that underlies I_A [121]. This in turn terminates spontaneous phasic bursting by reducing the plateau potentials [196]. KOR does not seem to be important for regulating silent periods in between burst firing [177].

The time course of release of vasopressin and of dynorphin differ. Release of dynorphin from dendrites and subsequent KOR inhibition emerges in an activity-dependent fashion [177], much like what has been shown in the hippocampus [17, 27, 97]. While dynorphin appears to mediate stress-induced negative affect by acting within the limbic system, it also seems to play a role in returning the animal to homeostasis in terms of peripheral effects of stress by opposing vasopressin and acting more slowly and only after prolonged stimulation.

11.10.2 *Dynorphin and Orexin*

Orexin release is generally considered an arousal signal. Through observation of different types of orexin knockout animals, the discovery of co-expression of dynorphin and orexin emerged. Mice lacking orexin peptides displayed narcolepsy, yet mice that had orexin neurons destroyed displayed hypophagia and obesity as well as narcolepsy [178]. This finding suggested that these orexin neurons contained other neurotransmitters and subsequently it was shown that in the lateral hypothalamus there is remarkable co-expression of orexin with dynorphin (over

90%) [16, 178]. It is well established that KOR activation by various agonists produces hyperphagia [197]. These observations suggest that stress-induced dynorphin release may contribute to the overeating response and that KOR antagonists may have antiobesity effects in specific circumstances.

In a significant study by Li and van den Pol [16], the researchers demonstrate using *in vitro* slice preparations of various hypothalamic nuclei, differential functional actions of dynorphin and orexin. Orexin expressing neurons have collateral contacts onto themselves, yet orexin seemingly does little to directly affect cellular excitability of orexin expressing neurons. However, exogenous dynorphin A application directly inhibits orexin expressing neurons by decreasing firing rate via a GIRK dependent mechanism and depresses excitatory synaptic currents by ~50%. This effect is norBNI sensitive and in fact, norBNI alone was shown to increase firing rate, suggesting that dynorphin may exert tonic control over orexin neuronal firing. Microslices only containing LH were pooled and subjected to field stimulation; following stimulation the perfusate was analyzed by ELISA.

Stimulation significantly increased dynorphin levels, suggesting that dynorphin is endogenously released in this area. Li et al. (2006) were also interested in how co-application of orexin and dynorphin affected projection neurons. Interestingly, there appears to be a difference in time course and differential responses elicited by the two neuropeptides. In recordings made from melanin-concentrating hormone (MCH) containing neurons that also express LH, dynorphin produced a hyperpolarization that desensitized more rapidly than the orexin mediated inward current. Suggesting that co-release of dynorphin and orexin onto these neurons produces an early inhibitory effect that shifts to an excitatory effect with prolonged stimulation.

In contrast, dynorphin and orexin appear to have an additive excitatory effect in recordings made from NPY containing neurons, with dynorphin inhibiting GABAergic synaptic activity and orexin increasing NPY neuronal firing directly. Both MCH and NPY are orexigenic peptides (promote feeding behavior). The effects of orexin and dynorphin on MCH neuron are consistent with the idea that stress would first suppress feeding and then stimulate feeding behavior as a homeostatic mechanism. This also consistent with the hypophagia phenotype of orexin lesioned animals. While synergistic stimulation of NPY is consistent with the obesity phenotype observed in orexin lesioned animals, the physiological relevance in normal animals is puzzling. However, the role of NPY in feeding is not well understood, and therefore, the result of modulation of NPY neurons behaviorally remain poorly understood as well.

11.11 Dynorphin and Substance P

Dynorphin and substance P (SP) are co-expressed in GABAergic medium spiny neurons of the direct pathway of dorsal striatum, which send projections back to the substantia nigra (SN) as well as in D1 expressing neurons of the NAcc which send

projections to the VTA [137, 139]. Importantly, dynorphin and SP are co-localized in other brain regions such as the hippocampus [198]. The differential effects of dynorphin and SP on the mesolimbic and mesocortical systems have been most well-studied, and will be the focus of this section. GABAergic release from MSNs onto either the SN or VTA act as a negative feedback mechanism.

As summarized above, dynorphin acts at KOR to inhibit the VTA excitability and subsequently dopamine release, as well as inhibiting dopamine release in the VTA terminal region. In contrast, SP has been shown to excite VTA neurons and increase extracellular dopamine levels in both mesolimbic and mesocortical brain regions in a dose-dependent fashion [199–203]. Interestingly, intra-nigral injections of SP at low doses (0.07–7 pmol) produced an increase in striatal DA, where at the high dose of 7.0 nmol, SP decreased striatal DA [199]. This data suggests a differential sensitivity or expression level of either NK1 or NK3 receptors on dopamine SN neurons compared to inhibitory interneurons.

As described above in detail, dynorphin is released following stressor exposure [63, 65]. Substance P is also released following stressor exposure in several limbic areas [204–206]. How does the release of these two neuropeptides into the same dopaminergic nuclei encode different behavioral responses to stress? Recall the observation that pharmacologic increase of dopamine levels following administration of a psychostimulant such as cocaine or apomorphine increases dynorphin levels (recall the “brake” mechanism described in KOR and the corticostriatal pathway), but not SP or enkephalin levels [141, 207].

It is possible that a similar brake mechanism seen during administration of drugs of abuse, may occur endogenously following stress exposure as a shut-off mechanism, returning the animal to a homeostatic state. substance P is thought to act as an arousal mechanism, increasing both norepinephrine and dopamine in the forebrain via excitation of the LC and VTA/SN respectively. Perhaps an increase in dopamine in the striatum up-regulates dynorphin expression and release, negatively regulating the further release of dopamine. The difficulty in this explanation is that we do not fully understand the time course of either dynorphin or SP action during or following the stress experience. Moreover, there is no clear information on the time course of dynorphin mRNA up-regulation and dynorphin release, or if increases in dynorphin mRNA confer to differences in release at all. Unlike work done in the hippocampus with glutamate and dynorphin, there is also no information on differences in stimulation requirements or probability of release for SP and dynorphin. These are just a few of the challenges that face future investigators in truly trying to understand this circuit and how it relates to the behavioral experience.

11.12 Conclusions and Future Directions

In the last decade there has been a new focus for the role of the dynorphin-KOR system in mediating behavior. Historically, interest in KOR was confined to its potential therapeutic value in treating epileptic and chronic pain states. Now, it is

clear that KOR activation occurs during stress and dysphoria-like states, and thus there is new interest in understanding the role of KOR in psychiatric diseases such as addiction and depression. This new behavioral evidence has to lead to the examination of KOR function in several different brain regions including the striatum, cortex and monoaminergic midbrain nuclei. Moreover, given evidence of the potential long term effects of KOR on emotion-driven behaviors, more attention has been given to KOR dependent downstream signaling events in addition to the classic KOR mediated inhibition of synaptic release and cell excitability.

Clearly there is a large body of evidence suggesting that KOR activation modulates the excitability of neural regions both through pre- and post-synaptic mechanisms. KOR inhibits release probability of neurotransmitter through coupling to Kv and CaV as well as inhibiting adenylyl cyclase. KOR can also modulate the excitability of cells post-synaptically, mainly through activation of Kv and Kir channels. While KOR is classically considered an inhibitory receptor, activation can also cause disinhibition of a circuit when KOR expression is localized to inhibitory (GABAergic) interneurons (e.g., in the NRM). Much of what we know regarding acute KOR modulation of neuronal excitability has come from work done in the hippocampus and nuclei of the descending pain pathway, and these insights have recently been extended to other regions.

While these mechanisms of acute KOR modulation are important to understand, a new and exciting avenue of research is understanding how KOR dependent activation of divergent MAP kinase pathways, namely ERK 1/2 and p38 MAPK, lead to changes in network excitability and ultimately behavior. This activation of various MAP kinases may be critical for de novo gene transcription as well as long lasting changes (hours to days) in cell excitability due to phosphorylation events at membrane proteins, and MAPK activation may underlie long term changes in cognition and emotion.

A recurring theme across this analysis of multiple brain nuclei in which KOR modulation has been studied is the idea that KOR modulation is confined to certain subregions of a circuit. In hippocampus, KOR modulation of excitatory synaptic transmission seems to be predominantly occurring within the dentate gyrus and CA3 regions, but not in CA1. In the NRM, KOR activation is confined to primary cells, and not secondary cells. In the VTA, KOR modulation is segregated to certain dopaminergic neurons based on target projection of the neuron.

This type of detailed circuit analysis is useful to know and understand, yet a major challenge is to further investigate how this selectivity contributes to certain behavioral outputs. Studies using a global gene knock out animal or using systemic administration of antagonists cannot answer these questions. While region specific injection of agonist and antagonists are useful, as the electrophysiology has demonstrated, KOR modulation is more temporally and spatially refined. For example, even if KOR modulates both VTA neurons targeted to the amygdala and the nucleus accumbens, the behavioral consequences of KOR activation of these discrete subpopulations are likely different.

Therefore, understanding this circuit more fully requires manipulations that are more refined than injection of norBNI into the entirety of the VTA. Key to this

investigation is the use of viruses, channel-rhodopsins and inducible knockout technology that allows for targeting of specific circuits or cell types. This technology will allow investigators to drive either expression or excitation of specific circuitry within a region.

Finally, as alluded to above, an important area of future research is investigation of the physiological consequences of co-expression and co-release of neuropeptides from one cell. It has been established for some time that neuropeptides (contained in dense core vesicles), and fast acting excitatory transmitters such as glutamate (contained in small synaptic vesicles) are expressed in the same neurons, yet require different stimulation intensities and likely different machinery to be released into the synapse.

Despite several documented instances of neuropeptide co-expression using *in situ* hybridization and immunohistochemistry, little is known regarding the physiological or behavioral results of co-release and mechanisms of disparate release. The example of dynorphin and orexin co-expression demonstrates co-release of these two peptides can either have opposing or synergistic effects depending on which target neuron is studied. However, the mechanisms driving opposition or synergy are not well understood. Nor is it understood how co-release ultimately changes behavior in normal animals.

The seemingly ubiquitous yet discrete nature in which KOR activation occurs in the brain poses complex circuit questions that we as scientists are just beginning to understand. From a neurobiological standpoint, a combination of old tools such as microdialysis and electrophysiology with new tools such as viral expression of genes and light-driven excitation of specific neurons will bring the field to a new, more detailed level of understanding the function of KOR in a wide set of behaviors. From a therapeutic standpoint, we need to develop better tools that can be used to probe the role of KOR in the human brain. The therapeutic potential of either KOR agonists (neuropathic pain) or antagonists (stress-resilience) remain to be realized. Only when we elucidate the change in function of KOR in pathological states, will we be able to translate this knowledge into the correct use of different agonists and antagonists at dosages that are therapeutically efficacious.

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Chapter 12

Delta Opioid Receptor Function

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Abstract Delta (δ) opioid receptors (DORs) are part of circuits involved in many physiological functions including the modulation of pain. Activation of DORs appears to mediate antinociception and antihyperalgesia in many conditions including stress, chronic pain, opioid-induced hyperalgesia (OIH), and opiate tolerance. δ Opioid signaling also extensively interacts with the mu (μ) opioid system, resulting in modulation of pain transmission. Multiple mechanisms may underlie DOR modulation of pain including the synergistic interaction between μ and δ opioid receptors and between spinal and supraspinal sites of action, enhanced endogenous enkephalinergic tone, potential μ - δ oligomers and modulation of plasma membrane receptor trafficking.

Keywords δ Opioid receptor • Expression • Regulation • Neurotransmission • Antinociception • Hyperalgesia • Allodynia • Chronic neuropathic pain

12.1 Introduction

In early studies, localization of the δ opioid receptor (DOR) in the brain and in other parts of the nervous system was investigated by autoradiographic methods using somewhat selective δ opioid ligands. With the cloning of the DORs [1–4], in situ hybridization techniques allowed a higher-resolution identification of cell types expressing the DOR. In addition, the cloning of the DOR also facilitated the preparation of selective antibodies for the DOR protein. The use of these antibodies together with advanced immunohistochemical techniques enabled visualization of

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the receptor protein at the cellular and subcellular level. In conjunction with the studies of DOR localization the distribution of putative endogenous δ opioid agonists (such as enkephalins) in the nervous system was also investigated. A more detailed knowledge of anatomical distribution of the DOR and its potential endogenous ligands provided a basis for the better understanding of the cellular and physiological functions of the δ opioid system.

12.1.1 Localization of the δ Opioid Receptor in the Central and Peripheral Nervous Systems

In the rat brain, high concentrations of binding sites for the radiolabeled δ -selective opioid agonists [D -Thr²,Leu⁵,Thr⁶]enkephalin (DTLET) [5] and [D -Pen², D -Pen⁵]-enkephalin (DPDPE) [6] have been detected in the olfactory bulb, nucleus accumbens, caudate putamen, olfactory tubercle and in deep layers of the cortex. Lower levels of δ opioid binding sites have been found in the hippocampus, substantia nigra pars reticulata; and very low or no detectable levels were found in the thalamus, hypothalamus, periaqueductal gray (PAG) and the cerebellum. Later *in situ* hybridization studies, in mouse [7] and rat [8] brain, showed generally good correlation between DOR mRNA distribution patterns and the findings of autoradiographic studies.

In some brain areas, however, considerable discrepancies were observed. For example in the substantia nigra pars reticulata and the globus pallidus no DOR mRNA was detected while DOR binding sites were clearly present. These observed discrepancies suggest that DOR protein is transported from the site of synthesis in the neuronal cell bodies to the receptor's functional localization site in the nerve terminal. Thus, the DOR binding sites in the substantia nigra and globus pallidus may correspond to the termini of neuronal cells with cell bodies localized in the striatum.

Immunohistochemical experiments using DOR-specific antibodies have also confirmed the presence of DOR immunoreactivity in the substantia nigra, periaqueductal gray matter and in other brainstem nuclei including spinal trigeminal nuclei, raphe nuclei and parabrachial nuclei [9]. In addition, these experiments demonstrated that DOR immunoreactivity displays a punctuate pattern within the cytoplasm, dendrites and axonal termini of the neurons. Double labeling experiments have revealed that DOR-containing termini are frequently found in close proximity to serotonergic (e.g., in the raphe complex) or noradrenergic (e.g., in the A5, A7 regions and the locus coeruleus) neurons, indicating that the DOR may modulate presynaptic neurotransmitter release from serotonergic and noradrenergic neurons in the descending pain modulatory pathway [9]. Interestingly, some cells in the midbrain (intermediolateral nucleus) were double labeled with anti-DOR and anti-pro-enkephalin antibodies suggesting that in certain brain areas the DOR may also regulate presynaptic enkephalin release by an autoregulatory feedback mechanism [9].

DORs are also present in the spinal cord. DOR binding sites [5] and immunoreactivity [9] were found throughout the spinal gray matter with highest

concentrations in the superficial dorsal horn, around the central canal and in the ventral horn motor nuclei. Autoradiographic methods suggested that the majority of DOR positive neurons in the spinal cord are located on the termini of afferent neurons, with lower DOR concentrations on spinal interneurons and second order neurons [10, 11]. Laminae I and II of the dorsal horn exhibit increased δ -selective radioligand binding sites and DOR immunoreactivities [12] relative to the deeper layers of the spinal cord. In contrast, DOR mRNA levels are comparable in all layers of the dorsal horn [8, 13].

Moreover, dorsal rhizotomy leads to a decreased amount of DOR staining in the superficial lamina [14]. DORs accumulate in the proximal, and to a lesser extent, distal part of the sciatic nerve after nerve ligation [15]. Taken together, these findings indicate that functional transport from the dorsal root ganglion (DRG) neuron cell bodies is the main source of the DOR protein in the superficial dorsal horn. Enkephalin containing fibers have been found in close proximity to DOR-positive terminals in the dorsal horn supporting the notion that enkephalins are the physiological ligand for the DOR in the spinal cord [14].

DOR immunoreactive cells represent about 14% of total DRG neuronal population, while MOR is expressed in about 21% and KOR in about 9% of DRG neurons [15]. The cells expressing the DOR in the DRG are mostly small diameter neurons [14], corresponding to the cell bodies of the nociceptive A δ and C-fibers. Many DOR containing DRG neurons co-express excitatory peptide neurotransmitters, such as calcitonin gene-related peptide (CGRP) [14] or substance P [16, 17].

Based on the cellular distribution of the DOR and its co-localization with excitatory pain neurotransmitters on the axonal termini of the sensory neurons, it was proposed that DORs regulate excitatory pain neurotransmitter release from small diameter primary sensory neurons. Substantial evidence has demonstrated that spinal administration of DOR agonists produced antinociception, usually following administration of thermal stimuli to the tail of mice [18]. Recently, a DOReGFP receptor knock-in mouse has been generated [19]. Evaluation of expression of the DOR in this mouse suggests that the receptor may instead be present in myelinated and non-peptidergic unmyelinated afferents [20], a finding that was correlated with an apparent activity of DOR agonists selectively against noxious mechanical, but not thermal, stimuli.

Traditional antibody studies suggest that the DOR is also present in other peripheral sites, such as the intestine [21], immune cells [22] and the heart [23], indicating that, in addition to the regulation of pain neurotransmission, the DOR may also have important physiological roles in immunomodulation, cardioprotection and in opioid-mediated regulation of the digestive system.

12.1.2 Cellular Effects of δ Opioid Receptor Activation

Stimulation of DORs by endogenous and exogenous agonists leads to regulation of a number of intracellular signaling pathways such as ion channels (GIRK type K $^{+}$

channels, Ca^{2+} channels), second messenger-regulated protein kinases (PKA, PKC, MAPK) and transcription factors (NF κ B, CREB, AP1). The resulting effect of δ agonists is neuronal hyperpolarization and the inhibition of presynaptic neurotransmitter release [24].

DORs predominantly couple to the pertussis toxin-sensitive $G_{i/o}$ proteins. Intensive studies using antisera directed against various G protein subunits [25–27] have established the involvement of G_{ia2} and G_{ia3} in the antinociceptive effect of selective δ agonists [DPDPE, Deltorphin II, [d -Ala², d -Leu⁵]-enkephalin (DADLE)] in mice. These results were also confirmed using antisense oligodeoxynucleotides complementary to G_{ia2} and G_{ia3} [28, 29].

Interestingly, intrathecal (i.th.) administration of antisense oligodeoxynucleotides to the stimulatory G_{sa} was also found to block DPDPE-mediated antinociception indicating an unanticipated complexity of the DOR-G protein coupling. Promiscuous coupling of opioid receptors to multiple G protein types has also been observed in heterologous expression systems [30]. Thus, the final cellular effect of δ agonists in the given cellular context may depend on the availability of specific G protein types and their downstream effectors within specific cells.

DORs have been shown to inhibit adenylyl cyclase, regulate voltage gated Ca^{2+} channels, and activate inwardly rectifying K^+ channels in a pertussis toxin-sensitive manner. Short-term application of δ agonists results in inhibition of basal cAMP levels in the rat brain [31]. δ Opioid agonists also inhibited forskolin and G_s protein-coupled agonist (such as prostaglandin PGE₂)-stimulated cAMP levels in cultured neuronal cell lines (e.g., NG108-15 neuroblastoma \times glioma hybrid cells) [32] and in recombinant cell lines expressing the cloned DOR [33]. In addition to regulation of the cAMP/protein kinase A (PKA) pathway, DORs have also been demonstrated to stimulate protein kinase C (PKC) [34] and mitogen-activated protein kinase (MAPK) [35].

Different types of voltage-gated calcium channels can be modulated by the δ opioid agonists. For example, activation of the endogenous DOR in the NG108-15 cells was shown to inhibit Ca^{2+} entry via N-type Ca^{2+} channels. In addition, DPDPE inhibited L-type Ca^{2+} channels in transfected GH₃ cells resulting in the inhibition of prolactin release [36]. Interestingly, in human neuroblastoma SK-N-BE cells, activation of the DOR led to an increase in intracellular Ca^{2+} concentration by promoting Ca^{2+} release from the intracellular stores [37]. Finally, [d -Ser², d -Leu⁵, d -Thr⁶]enkephalin (DSLET) was demonstrated to promote extracellular calcium influx through L-type Ca^{2+} channels in a neuroblastoma \times DRG hybrid cell line (ND8-47) [38]. Activation of the DOR also regulates G protein-coupled inwardly rectifying potassium channels in neurons, leading to presynaptic hyperpolarization. This response is also pertussis toxin-sensitive and is thought to be mediated by the $\beta\gamma$ -subunits of $G_{i/o}$ proteins.

Acute activation of DOR signaling leads to neuronal hyperpolarization and attenuation of Ca^{2+} -dependent neurotransmitter release in multiple sites of the CNS pain regulatory pathway and other brain areas. Acute DOR signaling is extensively regulated at the cellular level by receptor phosphorylation, desensitization, internalization, degradation and expression [39]. In addition, sustained opioid

exposure (hours) results in long term compensatory adaptations regulating the concentration and activity of both the DOR (down-regulation) [40–42] and its intracellular signaling partners. Thus, sustained δ opioid agonist treatment was shown to increase forskolin-stimulated cAMP production after drug removal (adenylyl cyclase superactivation) in NG108,15 cells as well as in recombinant cell lines, such as human DOR-transfected Chinese hamster ovary (CHO) cells [43] and in primary cultures of DRG neurons [44].

It was demonstrated that while acute DOR agonist-induced inhibition of cAMP formation is mediated by G_{ia} subunits, adenylyl cyclase superactivation upon chronic DOR agonist treatment involves G protein $\beta\gamma$ subunits [45]. Adenylyl cyclase superactivity may play a role in opioid tolerance and the manifestations of opioid withdrawal.

12.1.3 Physiological Effects of δ Opioid Receptor Activation

The presence of the DORs on most major components of the pain transduction pathway indicates that modulation of pain may be an important function of the DOR in the central nervous system. Accordingly, as noted above, numerous behavioral studies have demonstrated that intrathecally administered DOR-selective opioid agonists produce antinociceptive effects [46, 47]. Khan et al. [48] have directly measured the effect of DPDPE on the activity of ascending dorsal horn neurons using electrophysiological recordings in anesthetized rats. In these studies the response of dorsal horn neurons to mechanical stimulation by von Frey filaments was inhibited by local spinal application of DPDPE. The antinociceptive effects of spinal δ agonists could be blocked by δ -selective opioid antagonists and were attenuated by antisense oligodeoxynucleotide knock-down of DOR protein levels in the spinal cord [49, 50]. Collectively, these experiments provide evidence for the role of the spinal DORs in the inhibition of nociceptive transmission.

The supraspinal expression pattern of the DOR indicates that the receptor may also play a role in the processing and descending regulation of the primary nociceptive stimulus. Behavioral measurements of antinociceptive effects of selective δ ligands microinjected into the PAG or regions of the rostral ventromedial medulla (RVM) provide ample support for the functional role of the DOR in modulating the descending inhibitory pathway [51, 52]. The inhibitory function of δ agonists in the RVM on tail flick latencies have been directly linked to their effects on the activity of ON and OFF cells [51]. These cells located in the RVM have been shown to either facilitate (ON cells) or inhibit (OFF cells) nociceptive transmission in the spinal cord dorsal horn [53].

In addition, high expression of the DOR in the motor circuitry of the basal ganglia indicates that this opioid receptor type may also be involved in the regulation of locomotion and nigrostriatal dopamine release. Indeed, altered opioid transmission was observed in animal models of Parkinson's disease (PD) as well as in human PD patients [54, 55].

The observed enhanced endogenous enkephalinergic signaling was suggested to function as a compensatory mechanism to delay the motor impairments associated with dopamine depletion in Parkinson's disease [56]. Importantly, δ opioid agonists attenuated dyskinesias in both rodent and primate models of PD [57]. Tonazocine, a partial DOR agonist, also augmented the anti-Parkinson's effects of L-DOPA [58]. These studies suggest that δ agonists, may have an important therapeutic use in treatment of Parkinson's disease. The presence of the DOR in the limbic system indicates a role of the DOR in the regulation of emotions and addictive behavior. Accordingly, DOR knock-out mice demonstrate enhanced levels of anxiety and depression-like behaviors [59]. In addition, these mice show increased alcohol intake, indicating that δ opioid system may function in reinforcing behavior by altering mood states.

Distribution of the DOR in other brain areas and in the periphery suggests additional roles of the DORs in other physiological functions. Based on DOR localization potential physiological roles have been suggested for the DOR in the regulation of visual, auditory and somatosensory neurotransmission [8]. DOR is also expressed in cells of the immune system and it was shown that δ opioid agonists regulate activation, proliferation and differentiation of immune cells, such as leukocytes [60]. Finally, DOR is the predominant opioid receptor in the heart. Activation of the cardiac DOR was shown to mediate the cardioprotective effects of a brief ischemic preconditioning [61, 62], raising a possibility for a therapeutic use of peripheral δ agonists as cardioprotective agents in myocardial infarction.

In summary, the anatomical distribution of the DOR indicates that the DOR may have important functions in numerous physiological effects such as nociceptive transmission and pain modulation, locomotion, reinforcing properties, antidepressant effects, sensory neurotransmission, immune response and cardioprotection.

12.1.4 Endogenous δ Opioid Agonists

[Met⁵]enkephalin and [Leu⁵]enkephalin are the endogenous opioid peptides with the highest affinity for the DOR. These pentapeptides are synthesized from a common precursor gene pre-proenkephalin. Neurons containing proenkephalin-derived peptides are distributed throughout most of the brain areas associated with pain modulation, neuroendocrine regulation, memory, drug reward, and tolerance [63, 64]. Thus, high levels of the peptides were seen in many regions of the cortex, caudate-putamen, nucleus accumbens, amygdala, hypothalamus, PAG, raphe nuclei, as well as in the cell bodies of dorsal horn neurons.

Endogenous enkephalins may function as a defensive mechanism against acute and chronic pain, stress or exercise. For example, a conditioning noxious thermal stimulation of the rat hindpaw resulted in an increase in the animal's tail-flick reaction time. The effect was attenuated by pre-treatment with the specific δ antagonist, H-Tyr-Tic psi[CH₂NH]-Phe-Phe-OH (TIPP[ψ]), indicating that the antinociceptive effect of a brief noxious thermal stimulus on the tail withdrawal reflex is mediated

in part by the endogenous release of ligands acting at the DOR [65]. Chronic pain conditions such as inflammation and nerve injury are also associated with enhanced enkephalinergic signaling.

Pretreatment of rats with intraperitoneal naltrindole or i.th. antisera to [Leu⁵]enkephalin resulted in a significant enhancement in formalin-induced flinching behavior [66]. In another study, naltrindole inhibited an enhancement of morphine antinociceptive potency resulting from carrageenan-induced inflammation [67]. Indeed, it was found that levels of [Met⁵]enkephalin and [Leu⁵]enkephalin were increased in the RVM of rats with inflammatory injury [68]. These findings suggest the presence of an enkephalinergic inhibitory tone in response to inflammation that may represent a compensatory adaptation to the increased pain transmission. Exposure of rats to psychogenic stress was also shown to cause an increase in the number of enkephalinergic neurons in regions of the hypothalamus and medulla involved in response to stressful conditions [69].

The presence of a tonic basal level of endogenous opioids was suggested based on the ability of naltrindole alone to enhance basal outflow of substance P from the whole spinal cord of anesthetized rats [70, 71]. Endogenous enkephalins serve complex functions in establishing basal homeostasis and modulating responses to stress or acute and chronic pain.

12.2 δ Opioid Receptors in Pain Transduction Pathways

The development of selective δ agonists DPDPE and [D-Pen²,L-Pen⁵]enkephalin (DPLPE) [72, 73] made it possible to investigate the involvement of the δ receptors in spinal and supraspinal opioid antinociception [74, 75] and to establish the likely physiological role of this receptor in ascending and descending circuitries of pain transmission and modulating pathways. Detailed pharmacological investigation of δ -opioid antinociception led to a hypothesis that two DOR subtypes, termed $\delta 1$ and $\delta 2$, may exist in the CNS that differ in their selectivity to specific δ ligands. The $\delta 1$ receptor subtype was identified as the one displaying higher preference for DPDPE and an irreversible antagonist [D-Ala²,Leu⁵,Cys⁶]-enkephalin (DALCE). On the other hand, the $\delta 2$ receptor preferentially interacted with deltorphin and naltrindole 5'-isothiocyanate (5'-NTII) [76]. To this point, however, molecular evidence for the existence of two subtypes of the DOR has not been found.

Later studies with the DOR knock-out mice that demonstrated no DPDPE or deltorphin binding surprisingly showed an analgesic effect after intracerebroventricular (i.c.v.) administration of these agonists. The effect was only partially blocked by naltrindole, but not by selective μ (β -funeltrexamine) or κ (nor-BNI) receptor antagonists [77]. The authors explained the data by the existence of a secondary δ system in the DOR-KO mice. Thus, variety of opioid receptor subtypes may be present in different regions of the ascending and descending pain pathways resulting in large plasticity and complexity of δ opioid mediated antinociception.

12.2.1 Ascending Pain Pathways

Diverse modalities of pain originating in the periphery are detected by the peripheral nerve endings of specialized neurons (nociceptors) and carried along the nociceptive unmyelinated C fibers and thinly myelinated A δ fibers to the neuronal cell bodies in the dorsal root ganglia. The central projections of the DRG neurons terminate on second order projection neurons in the dorsal horn of the spinal cord. Single-unit extracellular recordings from the rat lumbar dorsal horn neurons provide evidence that spinal cord transmission neurons can be activated by electrical stimulation of C-fibers and by innocuous and noxious stimuli [78].

Opioid receptors are expressed on both peripheral and central projections of many primary nociceptors [17, 79]. Accordingly, it was demonstrated that the selective δ opioid agonist, DPDPE inhibits the C-fiber-evoked neuronal activity in a dose dependent manner [78]. The activity of ascending dorsal horn neurons in response to mechanical stimulation within the receptive field of the neuron by light touch, pressure, pinch and von Frey filaments was also found to be modulated by δ selective opioid agonists [48].

Two main classes of small nociceptive fibers have been described [80]. One type of neurons express peptide neurotransmitters substance P and/or CGRP and form synaptic connections with secondary neurons in the outermost laminae I, II of the dorsal horn. After crossing the midline of the spinal cord, projections from these neurons ascend via the spinoparabrachial tract to the parabrachial area. The nociceptive neurons from this region project to nuclei in the amygdala and the hypothalamus. The second class of nociceptive small fibers label positive for IB-4, FRAP and P2X3 and synapse on secondary neurons in lamina II that ascend to the thalamus via the spinothalamic tract. These parallel pathways are interconnected at every level and also project back to the lower regions. Final projections of the ascending pathways extend to the forebrain where conscious awareness and processing of the pain takes place. DORs are highly expressed in the parabrachial nuclei, amygdala and also in the thalamus and the forebrain [6, 9].

The most extensively studied region of the ascending pain pathway is the spinal dorsal horn, the site where the primary nociceptors make synaptic connections to secondary neurons. Immunohistochemistry experiments demonstrate the presence of numerous neurotransmitters in the spinal superficial dorsal horn, where they can either mediate or modulate nociceptive transmission [81]. Activation of the primary nociceptive afferent fibers by capsaicin stimulates the release of excitatory neurotransmitters, such as glutamate [82], substance P [83], and CGRP [84] from the central nerve termini located in the dorsal horn.

Substance P and CGRP, by activating their respective G protein-coupled receptors, produce a slow depolarization of spinal dorsal horn neurons. Glutamate, by activating the AMPA type ligand gated ion channels, mediates fast synaptic transmission. Prolonged glutamate stimulation results in activation of NMDA receptors thought to play a role in neuronal plasticity. It has been demonstrated that activation of presynaptic NMDA receptors enhances the release of substance P,

suggesting that the NMDA receptors facilitate prolonged nociceptive transmission [85]. Importantly, it was found that δ selective opioid agonists inhibit neurotransmitter release from peptidergic and glutamatergic primary afferents in the dorsal horn [86].

In summary, anatomical studies demonstrate the presence of DOR-expressing neurons and enkephalinergic interneurons at all levels of the spinal ascending pain transduction pathway. Primarily presynaptic localization of the DOR in the spinal dorsal horn [14] indicates that the δ opioid agonists modulate nociceptive signals in ascending pain transmission pathways. Indeed, numerous physiological studies, including behavioral measurements, provide strong evidence that endogenous and exogenous δ opioid agonists inhibit nociception, excitatory neurotransmitter release and pain-induced electrical activity in the spinal cord.

12.2.2 Descending Pain Modulatory Pathways

In the supraspinal nociceptive circuit, regions of the frontal cortex, amygdala, and hypothalamus are implicated in the descending modulation of the nociceptive signal. Neurons from these areas make connections into the PAG area of the midbrain, which in turn projects to several regions including the nucleus raphe magnus (NRM) and nucleus paragigantocellularis (PGi) in the RVM [87]. The RVM plays a critical role in integrating signals from the higher brain centers and giving rise to descending pain facilitation and pain inhibition pathways [88–90]. Neurons from the NRM and the adjacent reticular formation in the RVM form direct projections to the trigeminal nucleus caudalis and spinal cord dorsal horn, where they positively (i.e., enhance) or negatively (i.e., inhibit) modulate afferent nociceptive transmission [53].

Electrical stimulation of the PAG region was shown to excite many NRM cells and to suppress nociceptive responses at the dorsal horn neurons, providing electrophysiological evidence for functional connections between these regions of the descending pain pathway [87]. Electrical stimulation of the NRM was also shown to inhibit responses of the dorsal horn neurons to noxious heat [91]. It is thought that in the descending pain modulatory pathway, brainstem serotonergic and norenergic neurons descend through the dorsolateral funiculus (DLF) to the spinal dorsal horn, where they control the activity of nociceptive neurons [81]. Indeed, electrical stimulation of the NRM leads to the release of monoamine neurotransmitters in the spinal cord [91].

The DORs are present in both the PAG and RVM, although DOR expression in the PAG is much lower than that of the MOR. Thus, microinjection of δ selective opioid agonists into the PAG produced only minimal analgesic effects in naïve rats. Interestingly, it was found that in morphine tolerant animals the PAG neurons become more responsive to δ opioid agonists [92]. Furthermore, it was suggested that the analgesic action of μ opioid agonists in the PAG requires δ opioid function in the RVM, since analgesia produced by injection of morphine or the μ agonist

DAMGO in the PAG was reduced by local RVM microinjection of naltrindole [93] or of the highly selective δ antagonists TIPP[ψ] and naltriben [94]. These data suggest that μ opioid agonists in the PAG stimulate release of endogenous opioids in the RVM that act at the DORs in the descending pathway and inhibit nociceptive transmission in the dorsal horn.

In the RVM, three classes of neurons have been described: the ON cells increase, the OFF cells decrease and NEUTRAL cells exhibit no change in their electrical activities at the onset of the tail-withdrawal reflex [53]. Deltorphin, microinjected into the RVM, delays and reduces the tail-flick related electrical activity in the ON cells and delays and shortens the OFF cell pause [51]. Because DOR immunoreactivity is found on the termini but not on the cell bodies of the RVM neurons [9], deltorphin is thought to exert its antinociceptive effects predominantly by pre-synaptic inhibition of neurotransmitter release [53]. Accordingly, it was suggested that in the RVM deltorphin and endogenous enkephalins inhibit GABAergic input to the OFF cells, leading to increased OFF cell firing [94].

Taken together these findings indicate that DOR stimulation in the RVM may activate descending pain inhibitory pathways by shifting the ON and OFF cell firing rate and increasing inhibitory serotonergic and adrenergic input onto the dorsal horn. Moreover, experimental data suggest that μ opioid agonist-mediated antinociception may also require the presence of an active endogenous DOR system in the RVM. It is likely that the same δ -mediated antinociceptive mechanism is also activated during conditions such as stress, injury and acute or chronic pain (see Sect. 12.4 and 12.5 below).

12.3 Spinal δ Opioid Antinociception

12.3.1 Antinociception Induced by Intrathecal Administration of δ Opioid Agonists

Effects of spinally administered δ selective agonists have repeatedly been demonstrated in behavioral antinociceptive tests, electrophysiological properties of nociceptive neurons as well as in biochemical measurements of neurotransmitter release. Thus, intrathecal injection of DPDPE and DPLPE was shown to produce an increase in hot plate paw withdrawal latencies in mice and this effect was reversed by the selective δ opioid antagonist ICI-174,864 [74]. In the rat, spinal deltorphin exhibited full efficacy comparable to the μ -selective opioid agonist DAMGO in the thermally-induced tail-withdrawal (tail-flick) test.

However, in the same animals deltorphin only modestly increased latencies in the hot-plate test [46, 95]. Recently it was reported that in mice an i.th. administered DOR agonist SNC80 dose dependently increased mechanical pain thresholds, but was ineffective against heat pain [20]. On the contrary, in the same study DAMGO, a MOR agonist, reduced heat pain responsiveness but did not significantly alleviate

mechanical pain. The authors attribute this dissociation of different pain modalities to different localization of opioid receptors on unmyelinated peptidergic (MOR) and unmyelinated non-peptidergic or myelinated (DOR) fibers. δ -Selective opioid agonists, such as DPDPE and deltorphin, also produced a dose-dependent inhibition of formalin-induced flinching behavior in rats, which was antagonized by co-administration of 7-benzylidenenaltrexone (BNTX) or naltriben [96]. Spinal administration of δ agonists has also proved effective for alleviation of chronic inflammatory [97], neuropathic [98], or opioid-induced [99] pain. At the cellular level, spinal δ opioid agonists were shown to inhibit the activity of nociceptive neurons in the dorsal horn [100]. The inhibitory action of δ opioid agonists in the spinal cord is thought to result in decreased pain neurotransmitter release and antinociception.

12.3.2 Role of δ Opioid Agonists in Neurotransmitter Release

A number of experiments investigated the effects of δ agonists on neurotransmitter content and/or release in the spinal cord of anesthetized animals and in spinal cord slices. Both capsaicin (which specifically activates the vanilloid TRPV1 receptor on nociceptive fibers) and electrical stimulation of the sciatic nerve were shown to elevate substance P-like immunoreactivity in spinal superfusate collected from anesthetized cats [101]. In that study, the δ -selective opioid agonists DADLE and DPDPE produced a dose-dependent, naloxone-reversible reduction of the evoked release of substance P. Collin et al. [70] demonstrated that spontaneous outflow of substance P from the whole spinal cord of halothane-anesthetized rats was enhanced by the δ -selective opioid antagonist naltrindole, suggesting a tonic inhibitory role for the endogenous δ opioid system in the spinal cord. Moreover, in slices of rat dorsal lumbar spinal cord, capsaicin evoked a Ca^{2+} -dependent overflow of substance P which was attenuated by DTLET [83]. The inhibitory effect of DTLET was antagonized by a selective δ antagonist, ICI-154,129.

Capsaicin and K^+ also evoke the release of CGRP from perfused rat spinal cord slices [84, 102]. Because such evoked CGRP release could be prevented by dorsal rhizotomy, it was suggested that primary afferent fibers are the main source of CGRP in the dorsal horn. Importantly, DTLET and DPDPE reduced capsaicin and K^+ evoked spinal CGRP release and this effect was prevented by ICI-174,864. Deltorphin also inhibited K^+ evoked CGRP release from isolated spinal cord synaptosomes [17] suggesting a presynaptic site of action of the DOR.

In addition to peptide neurotransmitters, capsaicin also stimulates glutamate release in rat spinal dorsal horn slices. Capsaicin-evoked glutamate release was reduced by administration of DPDPE but not in slices pretreated with naltrindole [82]. Collectively, these results demonstrate that δ -opioid agonists are likely to modulate pain transmission in the spinal dorsal horn by inhibiting the release of excitatory neurotransmitters from primary afferents.

12.4 Supraspinal δ Opioid Antinociception

12.4.1 Antinociception Induced by Intracerebroventricular Administration of δ Agonists

Substantial evidence indicates that δ opioid agonists also modulate nociceptive responses by activating the DORs at supraspinal sites. Thus, i.c.v. administration of DPDPE or DPLPE produced dose-dependent antinociception in mice, as measured by hot-plate [74] and hot water tail-flick tests [18]. This antinociception was inhibited by the selective δ antagonist ICI-174,864 [18]. I.c.v. injection of DPDPE also resulted in an increase in the nociceptive thresholds to mechanical stimulation, as measured using the Randall–Selitto paw withdrawal test [103, 104].

Microinjections into specific brain regions identified nuclei in the medulla as the primary supraspinal sites for the antinociceptive effects of δ agonists in rodents. For example, deltorphin administered in the NRM or NGC α (nucleus gigantocellularis pars alpha) significantly increased tail-flick latency in rats [105]. Similarly, microinjection of deltorphin in the medullary reticular formation (MRF), but not in the PAG, increased the hot-plate (55°C) paw withdrawal latency in rats [52].

In another study, it was found that microinjection of DADLE or [D-Ser²-D-Thr⁶] leucine enkephalin (DSTLE) into the rat PAG produced a dose dependent increase in tail-flick and hot-plate tests [106]. Considering the multiple sites of action of δ opioid agonists within the brain and the spinal cord, it can be expected that several of these areas will contribute to antinociceptive effects.

12.4.2 Supraspinal/Spinal Antinociceptive Synergy and the PAG-RVM Pain Modulatory Circuit

Serotonergic and noradrenergic neurons in the bulbospinal pathway are implicated in the regulatory action of RVM administered opioids on nociceptive neurons in the dorsal horn [81]. Thus, it was suggested that δ opioid agonists may produce their antinociceptive effects by a dual mechanism: by direct inhibition of nociceptive neurons in the spinal cord and by actions in the RVM which are conveyed to the spinal cord via descending pain modulatory pathways. These two sites may interact either additively or supra-additively (synergistically) to produce the antinociceptive effect.

Indeed, it was demonstrated that in rats concurrent administration of [D-Ala²,Glu⁴]deltorphin into the RVM and the spinal cord produces synergistic antinociception in the tail-flick test [105, 107]. Interestingly, careful isobolographic analysis using a fixed ratio of spinal and supraspinal (ventromedial

medulla) deltorphin indicated that the antinociceptive synergism observed at lower doses of deltorphin converts to additivity at higher doses [107]. Microinjection of deltorphin into the medulla causes release of norepinephrine from the termini of bulbospinal neurons, probably activated indirectly by afferent projections from the NRM or NGC α to A5 and A7 catecholamine neurons that descend to the dorsal horn.

The synergistic antinociception (in tail-flick but not in hot-plate tests) produced by medullary and spinal co-administration of deltorphin was inhibited by the α_2 -adrenergic receptor antagonist yohimbine [107]. In addition, deltorphin microinjected into the RVM interacted synergistically with a spinally administered α_2 adrenergic receptor agonist, dexmedetomidine, in the rat tail-flick test. Therefore, the synergistic effect is hypothesized to result from the combined effects of endogenous norepinephrine, released into the spinal cord due to the disinhibitory action of deltorphin in the RVM as well as the direct action of the exogenously administered deltorphin at spinal cord DORs. The antinociceptive action of DPDPE microinjected in the RVM is also antagonized by i.th. yohimbine. Furthermore, it was found that the increase in tail-flick latency produced by microinjection of DPDPE into the NRM or NGC α is antagonized by i.th. pretreatment with a 5HT_{1/2} antagonist, methysergide [108].

These results indicate that δ selective opioid agonists stimulate the release of endogenous norepinephrine or serotonin from bulbospinal neurons that, in turn, synergistically interact with i.th. administered δ opioid agonists. However, DPDPE administered concurrently i.th. and i.c.v [109]. or i.th. and in the RVM [110] produced antinociception in an additive manner in the tail-flick test in the mouse. The effect of i.c.v. and i.th. DPDPE was synergistic in a test of mechanical nociception in the rat [104]. Thus, antinociceptive synergism observed between spinally and supraspinally administered δ agonists may be dependent on the dose and nature of the agonist and on the modality of the antinociceptive test.

Interestingly, spinal co-administration of deltorphin and the α_2 adrenergic receptor agonist clonidine also produced antinociceptive synergy in a thermal nociceptive test in mice [102]. Moreover, the DORs colocalize extensively with the α_2 adrenergic receptors in the superficial dorsal horn and synergistic interaction between these two receptors has been shown in isolated spinal cord synaptosomes [17]. Therefore antinociceptive synergy between δ opioid and α_2 adrenergic receptors may occur within a single subcellular compartment in the dorsal horn.

12.4.3 Modulation of μ Opioid Analgesia by δ Opioid Ligands

It was suggested that δ opioid agonists may also modulate the antinociceptive potency or efficacy of the μ opioid agonists. For example, in mice, i.c.v. co-administration of morphine with a sub-antinociceptive dose of DPDPE resulted in a leftward shift of the morphine antinociceptive dose-response curve accompanied by

an increase in its maximal effect [111, 112]. In contrast, i.c.v. co-administration of [Met^5]enkephalin decreased morphine's potency and intrinsic activity [113]. Importantly, these positive or negative effects of δ opioid agonists on morphine mediated antinociception were antagonized by the δ -selective antagonists ICI-174,864 or 5'-NTII [114]. These findings suggest the existence of functional interactions between the μ and δ opioid systems.

Using co-immunoprecipitation experiments it was demonstrated that μ and δ opioid receptors form hetero-oligomeric complexes in cells co-expressing both opioid receptor types [115, 116]. Bioluminescence resonance energy transfer (BRET) assays confirmed the existence of such functional heterodimers in living cells [117]. Importantly, μ - δ heterodimers exhibit unique ligand binding and G protein-coupling properties [115, 116]. Therefore, it is possible that some aspects of the μ - δ synergy observed when μ and δ agonists are co-administered in the same brain area may be related to the formation of μ - δ hetero-oligomers with altered pharmacological and functional characteristics. However, physiological interaction between μ and δ opioid systems is also suggested to originate from activation of μ and δ receptors at different sites of the brain.

For instance, it was shown that antinociception elicited by microinjection of morphine into the PAG is significantly reduced by pretreatment of the RVM with either naltrindole (a δ -selective antagonist) or β -funaltrexamine (β -FNA; a μ -selective antagonist) [93]. Selective DOR antagonists (such as TIPP[ψ] or naltriben) microinjected into the RVM also significantly attenuated the analgesic effect of PAG administration of a μ opioid agonist DAMGO. These results demonstrate that μ opioid agonists in the PAG may produce their analgesic effects in part by activating DOR receptors in the RVM through the release of endogenous opioids. Endogenous opioids in the RVM also play a profound role in chronic pain states (see below).

Additive or synergistic antinociceptive effects were also reported when μ and δ agonists were given simultaneously into supraspinal and spinal sites. Thus, i.c.v. morphine injections along with i.th. DADLE administration resulted in a synergistic antinociceptive effect as measured using the tail-flick test in mice [110]. Similarly, synergistic interaction was also found in rats using the Randall–Selitto paw-withdrawal test when i.c.v. DAMGO or DPDPE was co-administered with i.th. DPDPE. Thus, it was concluded that descending antinociceptive pathways activated by supraspinal administration of either μ or δ opioid agonists interact with spinal δ opioid sites [103].

To summarize, numerous studies demonstrated that μ and δ opioid systems are closely interconnected at many levels. Apart from the poor selectivity of most endogenous and naturally occurring opiates, even synthetic ligands that are highly selective for one opioid receptor can modulate the activity of the other receptor. The mechanism by which this occurs is unknown but the existence of μ - δ heterodimers has been suggested [115]. In addition μ and δ opioid pain pathways are interconnected resulting in the complex functional relationships observed in vivo.

12.4.4 The Role of the δ Opioid System in Stress-Induced Antinociception

Stress is a complex physiological response that involves changes in endocrine, autonomic and behavioral functions. The major hormonal response to stress includes stimulation of the hypothalamic-pituitary-adrenal (HPA) axis, responsible for the secretion of corticotrophin-releasing factor, adrenocorticotrophic hormone and glucocorticoids [118]. Stress is also accompanied by a decrease in pain sensitivity (stress-induced analgesia). Opioid mechanisms have long been implicated in regulating certain stress responses [119]. In particular, DORs are thought to play a prominent role in stress-induced analgesia.

In mice, an increase in tail-flick latencies mediated by cold-water swim-stress was antagonized by pre-treatment with a δ -selective antagonist, naltriben [120]. Stress-induced analgesia was also observed in μ opioid receptor knockout mice demonstrating that μ opioid receptors are not essential in this process [121]. Further analyses using knockout mice deficient in individual (μ , δ , or κ) receptor types and mice lacking all three opioid receptors indicated that both endogenous μ and δ opioid systems contribute to stress-induced analgesia [122]. Interestingly, the mechanism of stress-induced analgesia was apparently dependent on the gender and the genetic background of the mice.

Additionally, it was shown that the analgesic dose-response curve of the exogenous opioid agonists, such as morphine, shifts significantly to the left in mice exposed to stress (cold-water swim test). Based on the ability of a δ -selective antagonist, ICI-174,864 to block this leftward shift, DORs were also implicated in the stress-induced enhancement in morphine's antinociceptive potency [123]. Of note, diabetic mice exhibit significantly greater swim-stress-induced analgesia compared to control mice, which is thought to be produced mainly by DOR-dependent mechanisms [124, 125].

12.5 The Role of the DOR in Chronic Pain States

Under normal conditions, pain has important physiological functions as a protective mechanism from injury and tissue damage. Long-lasting chronic pain states, on the other hand, are associated with persistent hyperalgesia (pain from a noxious stimulus), and allodynia (pain from a normally non-noxious stimulus). Interestingly, a similar state of hypersensitivity was also described in patients after prolonged exposure to opioids such as morphine, a phenomenon termed opioid-induced hyperalgesia (OIH, see below).

Evidence suggests that the efficacy of δ opioid agonists is augmented in chronic inflammatory [126–128], neuropathic [98], or OIH [99] pain states. The mechanisms of enhanced DOR efficacy in chronic pain conditions may include an increased endogenous opioid tone, translocation of the DOR protein into the

plasma membrane or formation of μ - δ heterodimers, as addressed below. Different pain conditions probably share some common molecular and neural system mechanisms. Due to the apparent enhanced activity of agonists at DORs, such compounds might provide a better alternative to currently used analgesics for the treatment of inflammatory and neuropathic pain or in chronic pain patients tolerant to morphine.

12.5.1 δ Opioid Analgesia in Chronic Inflammatory Pain

Chronic inflammation due to prolonged infection or a chronic autoimmune disease such as rheumatoid arthritis is a complex pathological condition that leads to tissue destruction and nociceptive hypersensitivity. Inflammatory pain can be alleviated by systemic, spinal or RVM administration of μ or δ opioid agonists [67, 96]. Interestingly, the antinociceptive effects of opioids are enhanced in animals with experimental inflammation [67, 68, 126, 129, 130].

For example it has been shown that morphine is more potent in hot-plate and tail-flick tests in rats with carrageenan-induced inflammation of the hindpaw than in control rats [67]. Chronic inflammation induced by intraplantar injection of complete Freud's adjuvant (CFA) increased the antihyperalgesic and antinociceptive effects of both DAMGO and deltorphin microinjected into the RVM [126]. These effects were reversed by administration of a δ -selective antagonist naltriben, indicating that the enhanced efficacy of both μ and δ agonists are mediated by DORs [68].

The mechanism of such enhanced δ opioid tone in chronic inflammatory pain might include increased synthesis and release of endogenous enkephalins, upregulation of the number of membrane-bound DORs and/or increased coupling of the DOR to second messenger signaling pathways. In support of the former mechanism, increased levels of endogenous opioid peptides have been detected following peripheral tissue inflammation in the rat spinal dorsal horn, ipsilateral to the site of injury [66, 131–133].

Peripheral inflammation has also been shown to enhance the levels of [Met^5] enkephalin in the RVM [68] and the PAG [134]. Inhibition of this tonic opioid input into the RVM neurons by local injection of naltriben enhanced behavioral manifestations of nociception in CFA-treated (but not naïve) rats [68]. In primary nociceptors, the mechanism of enhanced effects of opioids may be mediated by transport of opioid receptors to the site of injury (inflammation) [135]. Chronic inflammatory pain was shown to enhance plasma membrane expression of DORs in DRG neurons [136] and the dorsal spinal cord neurons [128] of injured animals. Intraplantar injections of CFA, capsaicin or the pro-inflammatory substances prostaglandin E2 and bradykinin were shown to promote the trafficking of the DOR to the plasma membrane of the primary afferent neurons [137, 138].

Therefore, it was concluded that increased plasma membrane expression of DORs in a model of chronic inflammation enhances DOR-mediated spinal

antinociception [128, 136]. In contrast, in other studies a decrease in the number of DOR immunopositive DRG neurons was found following peripheral inflammation [15]. In the RVM, no significant changes in the number of DORs or receptor coupling to G proteins was detected [129].

Electrophysiological measurements in the RVM of polyarthritic rats have demonstrated that both the inhibitory and facilitatory modulation of spinal nociceptive transmission by supraspinal nuclei is enhanced during peripheral inflammation [139, 140]. Persistent inflammation has been linked to activation of the facilitatory pain pathway leading to hyperalgesia and allodynia. An increase in endogenous “anti-opioid” cholecystokinin (CCK) may mediate some aspects of the descending facilitation [141]. On the other hand, the increase in enkephalin synthesis and plasma membrane trafficking of the DOR activates the inhibitory pathway. Such endogenous inhibition may represent a compensatory mechanism to alleviate inflammation-associated hypersensitivity. Elevated endogenous opioid tone also functions to enhance the effects of exogenous opioids through synergistic or additive mechanisms.

12.5.2 *δ Opioid Analgesics and Neuropathic Pain*

Neuropathic pain is a chronic disorder initiated by a peripheral nerve injury and characterized by spontaneous burning pain and often hypersensitivity to innocuous stimuli as well as to experimental noxious stimuli [142]. Currently used opioid analgesics are fully effective for neuropathic pain [143] but limited in practice due to dose-limiting side effects. Animal studies suggest that DOR agonists may provide some advantages over μ agonists in alleviating neuropathic pain [98, 144, 145].

Thus, spinal administration of [D-Ala²,Glu⁴]deltorphin produced dose-dependent, naltrindole-reversible blockade of tactile hypersensitivity in rats with spinal nerve ligation (SNL) [145]. Similarly, i.th. administration of deltorphin II reversed cold hyperalgesia and attenuated mechanical allodynia in rats 14 days after chronic constriction injury (CCI) of the sciatic nerve [146]. Peripherally acting loperamide, an opioid agonist which does not readily penetrate the blood–brain barrier, reversed thermal hyperalgesia but not cold or mechanical allodynia. This effect was blocked by a δ , but not μ , opioid receptor antagonist indicating that peripheral δ agonists may be useful for treatment of some aspects of neuropathic pain [147]. Nevertheless, in other studies DOR agonists were not effective in attenuating symptoms of neuropathic pain [148].

The initiation of neuropathic pain is believed to be associated with persistent activity of primary afferent fibers from the injured or adjacent nerves [149–151]. This prolonged activity results in central sensitization and engagement of the pain facilitatory pathway arising from the RVM [90]. Maintenance of nerve injury-induced central sensitization in the spinal dorsal horn depends, in part, on descending pain facilitation mechanism [152]. Microinjection of lidocaine into the RVM

blocked the already established nerve injury-induced pain [88, 141]. Lesions of the DLF which eliminate the descending pain pathway also prevented neuropathic pain [153]. Selective ablation of μ receptor expressing neurons in the RVM (which constitute the majority of pain facilitatory ON cells) also inhibited neuropathic pain [154].

Descending facilitation may be mediated in part via enhanced endogenous levels of CCK [155, 156]. CCK, acting at the CCK_B receptor, inhibits the actions of endogenous enkephalins. Thus, i.th. co-administration of morphine with the CCK_B antagonist L365,260 significantly reduced tactile hypersensitivity in rats with SNL, while each compound alone was without effect [145, 157]. This enhancement in morphine efficacy was blocked by naltrindole, suggesting a role for endogenous δ opioid peptides in modulating nerve injury-induced pain.

Following sciatic nerve ligation, DOR knock-out mice displayed enhanced mechanical and thermal allodynia and thermal hyperalgesia [158]. Interestingly, in another study, rats with spinal cord injury exhibited allodynia after administration of naltrindole, while naltrindole had no effect in uninjured rats [159]. In addition, Kabli et al. recently reported an up-regulation of DOR protein in neuropathic DRG neurons [98] although other reports found a decrease in DOR immunoreactivity following nerve injury [160]. Together these results indicate that in neuropathic pain a delicate balance may exist between descending pain facilitation (mediated in part by CCK) and pain inhibition (mediated by endogenous enkephalins and DORs).

The outcome of this complex relationship may depend on the severity and duration of the injury, perhaps explaining some inconsistencies between different reports. Whether or not the endogenous δ opioid system is up-regulated, there is new potential for therapeutic use of δ agonists along with CCK receptor antagonists in treatment of neuropathic pain.

12.5.3 Opioid-Induced Hyperalgesia and Morphine Tolerance

Chronic opioid (morphine) treatment of patients has been shown to result in development of abnormal pain distinct from the initial pain complaint. Thus, in addition to their analgesic effects, opioids paradoxically induce a hypersensitivity characterized by allodynia and hyperalgesia [161], a phenomenon termed *opioid-induced hyperalgesia* (OIH). As a result, patients require higher doses of morphine to maintain a constant level of analgesia [162]. Therefore, it has been suggested that OIH may underlie many features of opioid antinociceptive tolerance.

OIH has repeatedly been demonstrated in animals [161]. Studies show that even a single dose of morphine, heroin or fentanyl produces a long lasting hyperalgesia unmasked by naloxone treatment [163–165]. Importantly, subcutaneous infusions of DAMGO or morphine through osmotic minipumps for 6 days produced tactile allodynia and thermal hyperalgesia even in the continuous presence of the opioid [166, 167]. These findings indicate that OIH pain does not result from opioid

withdrawal and additionally, the diverse structures of opioids (peptidic and non-peptidic) that can elicit hyperalgesia suggest that this does not occur as a consequence of metabolism to an excitatory mediator.

The precise mechanism of OIH is not known and conflicting reports exist regarding the role of opioid receptors in the initiation of the neuroplastic changes occurring in OIH. In one study, sustained spinal administration of the active (–) but not the inactive (+) enantiomer of oxymorphone produced thermal and tactile hypersensitivity and antinociceptive tolerance [168], indicating that pronociceptive actions of sustained opiate administration require specific interaction with opiate receptors. In contrast, other studies using triple knock-out mice lacking all three opioid receptors have shown that while these mice have no detectable analgesia to morphine, oxymorphone or fentanyl, they still develop hyperalgesia following sustained infusion of these opioids [169, 170]. Interestingly, injecting the NMDA receptor antagonist MK-801 during opioid infusion reversed hyperalgesia in control, but not knock-out mice, potentially suggesting unexpected adaptations in the triple knock-out mice or differential mechanisms of OIH in the presence and absence of opioid receptors.

Chronic opioid exposure results in the development of neuroadaptive changes in the primary afferents, spinal cord and in several supraspinal sites, including the RVM. The importance of descending facilitatory inputs in OIH was demonstrated by the ability of lidocaine microinjection into the RVM to block both tactile allodynia and thermal hyperalgesia in morphine-tolerant rats. Similarly, disruption of spinopetal projections from the RVM by lesions of the DLF also prevented the development of OIH [166].

CCK is thought to play a critical role in the development of descending pain facilitation from the RVM [171, 172]. Accordingly, microinjection of a CCK_B antagonist L365,260 into the RVM abolished tactile allodynia and thermal hyperalgesia in sustained morphine-treated animals [173]. Importantly, it was found that morphine-induced cortical CCK outflow was blocked by naltrindole and naltriben, indicating the role of the δ opioid system in this effect [174].

It is hypothesized that endogenous opioid nociceptive signaling is up-regulated as a result of persistent pain in chronic morphine-treated animals. Thus, an increased neuronal plasma membrane DOR concentration was found in the dorsal horn [99, 175], nucleus accumbens and the dorsal neostriatum [176] of animals after chronic treatment with morphine or fentanyl. Importantly, it has been demonstrated that enhanced DOR expression has functional consequences, because δ opioid agonists inhibited GABA neurotransmitter release in the brainstem neurons from morphine-tolerant rats, but not from opioid naïve rats [177]. PAG neurons in slices taken from morphine-treated (but not untreated) mice exhibited DOR-mediated presynaptic inhibition of GABAergic synaptic currents [92]. Therefore, chronic μ opioid agonist treatment induces up-regulation of functional DORs. Such increased plasma membrane DOR concentrations may negatively affect MOR function by formation of MOR-DOR heterodimers, leading to reduced antinociceptive effects of μ-agonists. On the other hand, up-regulation of the DOR may facilitate δ-opioid antinociception.

Indeed, chronic morphine treatment potentiated i.th. [D-Ala²]deltorphin II-induced antinociception [99, 137, 178]. Similarly, activation of DORs in the brainstem which had no effect in opioid naïve rats produced antinociception and reduced tolerance in chronic morphine-treated rats [177]. These experiments indicate that δ opioid agonists might be useful analgesics in morphine-tolerant patients. In addition, the use of mixed μ/δ opioid agonists together with compounds that block central sensitization may further attenuate the development of analgesic tolerance.

12.5.4 Mechanisms of Hyperalgesia and Allodynia Associated with Chronic Pain

Sustained activation of pain facilitatory mechanisms may be a common characteristic contributing to hyperalgesia and allodynia observed in chronic pain conditions such as inflammatory and neuropathic pain and in OIH. Pain facilitation occurs at supraspinal, spinal and peripheral sites and includes activation of the descending pain pathway from the brainstem, spinal neuroplasticity and changes in the primary afferent fibers [90, 162]. Accumulating evidence suggests a pivotal role of the RVM in the descending modulation of nociception. Manipulations that disrupt transmission from the RVM prevent the development of chronic inflammatory [179], neuropathic [180] and opioid-induced [166] hyperalgesia. An enhanced release of endogenous CCK within the CNS during chronic pain states was observed [141, 171, 174].

It is proposed that in the RVM CCK reduces the activity of OFF cells [53] leading to decreased pain inhibition. The net effect of CCK activation therefore is enhanced pain facilitation within the spinal cord. At the spinal level descending pain facilitation results in up-regulation of spinal dynorphin [181]. Dynorphin acts as a pronociceptive agent [182] by stimulating release of pronociceptive neurotransmitters including CGRP [181] and substance P [183] from the primary neurons. Interestingly, the expression of the NK1 receptor, the preferred receptor for substance P, was enhanced in the dorsal horn of the spinal cord following persistent opioid treatment [183]. Ablation of spinal NK1 receptors with i.th. injection of substance P-saporin prevented morphine-induced up-regulation of spinal dynorphin and behavioral signs of hypersensitivity and tolerance in morphine-treated rats [184].

Thus, central sensitization activated by sustained afferent stimulation during many types of pain drives additional neuroplastic changes in the spinal cord and peripheral afferent fibers resulting in hypersensitivity. The consequent tactile allodynia and hyperalgesia becomes independent of the initial painful stimulus and relies on the descending facilitation from the RVM. Emerging evidence supports a hypothesis that an increase in the enkephalinergic inhibitory tone may occur as a compensatory mechanism to the hypersensitive state. This compensation may be mediated in part by enhanced concentrations of enkephalins or by trafficking of the DOR to the plasma membrane.

12.5.5 Regulation of DOR Concentration in the Plasma Membrane and Its Functional Consequences

Responsiveness of the cell to extracellular stimuli is modulated by regulation of plasma membrane expression of the receptor for a particular stimulus [185, 186]. In the case of the DOR, the synthesis of the receptor protein, its insertion into the membrane and removal from the membrane are all tightly regulated.

Until recently, DORs were assumed to be delivered to the plasma membrane by the constitutive biosynthetic pathway. In this pathway, native protein is synthesized and assembled in the endoplasmic reticulum and delivered to the Golgi apparatus, where it undergoes post-translational modifications such as glycosylation. Mature protein is then packaged into exocytic vesicles and constitutively inserted into the membrane. While most GPCRs, including the MOR, are processed primarily by this mechanism, data suggest that newly synthesized DORs may be inserted into the membrane by a stimulus induced pathway [137].

According to this concept, a fraction of DORs is packaged in the Golgi network into neuropeptide-containing secretory vesicles and stored in the cytoplasm. For example in the DRG neurons it has been demonstrated that the DOR is sorted into protachykinin-containing large dense-core vesicles (LDCV) [187]. Direct interaction of the DOR with the substance P domain of protachykinin was required for sorting the receptor into LDCVs, since it was abolished in protachykinin gene knock-out mice. In the basal state, these LDCVs are distributed in the cytoplasm of the neurons and are transported to the plasma membrane upon various stimuli [137].

Exocytosis of the secretory vesicles and membrane insertion of the DORs can be stimulated by activation of some G protein-coupled receptors, ion channels or increased intracellular Ca^{2+} . For example, δ opioid ligands were shown to promote maturation of DORs and their trafficking to the plasma membrane, thereby acting as “chaperones” for DOR delivery [188]. Prolonged *in vivo* stimulation of the MOR results in targeting of the DOR to the plasma membrane [99, 178]. Inflammatory and pronociceptive agents, such as capsaicin, bradykinin, and ATP, also promote trafficking of the DOR from intracellular stores to the plasma membrane [138, 189].

This up-regulation of plasma membrane DORs was not observed in MOR $-/-$ mice indicating that MORs play a crucial role in this process [178]. Inserted DORs are functional, able to couple to signaling pathways and induce analgesic effects *in vivo*. In PAG slices from naïve mice, the neurons do not respond to DOR stimulation. However, chronic morphine treatment results in functional potentiation of the DOR as measured by DOR-mediated inhibition of GABAergic synaptic currents [92]. Moreover, δ -selective opioid agonists did not inhibit Ca^{2+} currents in DRG neurons under basal conditions, but up-regulation of cell surface DORs following incubation with a μ opioid antagonist CTAP (D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH₂) and a brief exposure to DPDPE induced their coupling to Ca^{2+} channels [190]. Functional competence of the DOR was not observed in MOR or in β -arrestin-2

knock-out mice after chronic morphine, suggesting that induction of DOR-mediated actions in PAG requires expression of MOR and β -arrestin-2.

Thus inflammation [128, 191], nerve injury [98], and chronic morphine treatment [99] all may promote membrane insertion and functional potentiation of the DOR resulting in enhanced antinociceptive effects of δ agonists. This view has been challenged recently by studies with DOReGFP knockin mice showing that in the DRG, the green fluorescent protein (GFP) labeled DOR is trafficked to the plasma membrane under resting conditions independently of substance P [20].

At the plasma membrane, the DORs are subjected to regulation by δ agonists. Activation of the DOR by an agonist facilitates the binding of G protein-coupled receptor kinases (GRK) [192] recruited to the plasma membrane by $\beta\gamma$ subunits of the activated $G_{i/o}$ protein [193]. GRKs then phosphorylate the activated receptor at several residues within the C-terminal region of the DOR causing receptor desensitization to further agonist stimulation. Agonist-bound and phosphorylated receptors display high affinity for the cytosolic adaptor proteins, β -arrestins. Binding of β -arrestins facilitates the targeting of the receptor to clathrin-coated pits and receptor internalization via the endocytosis of the clathrin-coated vesicles.

Sorting of internalized receptors from degradation into the recycling pathway is regulated by a complex multi signal mechanism [194, 195]. Additional sorting may take place during trafficking of the receptors into separate digestive systems: lysosomes or proteasomes [196]. The internalization process may serve either to degrade the receptor in cellular digestive organelles, thereby terminating receptor function (down-regulation), or to reintroduce the receptor to the plasma membrane (resensitization).

Based on these findings, it is evident that plasma membrane expression of the DOR is a dynamic process affected by the presence of inflammatory and pronociceptive mediators as well as endogenous and exogenous opioids. Therefore, the total expression of the DORs at the cell surface is determined by the balance between receptor synthesis, delivery, internalization, recycling and degradation.

12.6 Possible Therapeutic Uses of δ Ligands

In most cases of acute pain, δ opioid agonists provide only a moderate antinociceptive effect compared with μ opioid agonists. However, earlier it was suggested that δ -selective opioids may exhibit therapeutic benefits superior to μ opioid agonists, since they may exhibit a more limited side effect profile, namely less tolerance, respiratory depression and constipation.

In addition, as discussed above, DORs become more functional in chronic pain conditions. This property may provide a possible therapeutic use of δ agonists for the treatment of neuropathic or chronic inflammatory pain and in patients tolerant to conventional μ opioid therapy. Unfortunately, highly selective non-peptidic δ opioid agonists were found to present a potential risk for the development of convulsions [197, 198] though this possibility has never been conclusively established

(it should be noted that stimulation of DOR with peptidic opioids has not been reported to elicit convulsions). As a result, no predominantly δ acting drugs are currently used for the clinical treatment of acute pain.

Due to multiple interactions between the μ and δ opioid systems, ligands with actions at both receptors (agonist/agonist or agonist/antagonist) are being developed in the search for an analgesic with reduced side effects [199]. Another approach to design a better analgesic is to combine analgesic properties of opioids with blockade of pro-nociceptive pathways which are activated during chronic pain conditions (CCK [200, 201], substance P [202, 203]). In addition to pain relief, other potential uses for δ ligands have also been suggested in the treatment of Parkinson's disease [57], seizures [204], depression [205], ischemic heart disease [61] or immune deficiency [60]. In summary, although much progress has been made in a quest to elucidate the mechanisms of δ opioid effects in acute and chronic pain states and its interaction with other antinociceptive and pronociceptive pathways, the conclusion as to the potential therapeutic efficacy of δ opioid agonists still awaits a robust human trial.

12.7 Summary

DORs perform important functions in regulating pain transmission. Exogenous δ opioid agonists produce modest antinociceptive effects in several animal models of pain when administered systemically or supraspinally. However, the antinociceptive effects are complex and affected by cross-reactivity between μ and δ opioid systems or by adaptations occurring in some conditions such as stress, chronic pain or morphine tolerance. The mechanisms of such complex behavioral responses involve enhanced endogenous enkephalinergic tone, plasma membrane trafficking of the DORs, formation of μ - δ oligomers, and synergistic interaction between μ and δ opioid receptors and between different sites of action (spinal-supraspinal). Increased and emerging understanding of mechanisms of opioid function in various pain situations may lead to the development of appropriate DOR-based therapeutics as pain relievers.

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Chapter 13

Genetic Studies of Opioid System Function in Mice

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Abstract Several mechanisms for altering gene expression of opioid system genes have been used to enhance our understanding of this complex system. In particular, production and analysis of classic opioid system knockout (KO) mice deficient in one or more of the four opioid family receptors and precursors encoding respective ligands have been particularly valuable. These strains have been used to examine the contributions of this system to baseline function of multiple systems as well as providing novel insight into actions of exogenous compounds. Results from these studies should help focus analysis of genomic and proteomic alterations that occur in these mice as well as inform analysis of conditional mutations and siRNA treatments that are emerging to provide more regional and cell-specific analysis.

Keywords Gene targeting • Strain background • Nociception • δ KO • μ KO • κ KO

13.1 Introduction

Since the identification of the initial peptides and receptors for the opioid system, scientists have been searching for the optimal methodology to artificially regulate their expression. Cloning of the genes of endogenous opioid peptides and receptors in the 1970s and 1980s provided the tools to allow knockdown or knockout of different genes of the opioid system. Initially, antisense RNA technology was utilized to artificially knockdown receptor and ligand expression *in vivo*. Although not optimal at silencing gene expression, this method was able to significantly reduce

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gene expression, and the results confirmed and extended many of the initial hypotheses generated by classical pharmacology.

In the late 1980s, antisense technology became supplanted by gene knockout techniques. Unlike antisense, gene knockout completely silences gene expression. Rapid production and analysis of opioid system knockout mice confirmed opioid involvement in many behaviors such as reward and respiration and began to clarify receptor–ligand specificity in vivo, as illustrated by morphine being μ opioid receptor specific. However, as is common with any great expansion in knowledge, more questions arose from this new approach, for example, the specificity of ligands such as DPDPE – previously thought to be δ selective – and the role of receptor complexes in receptor function. These new questions require more subtle methods of gene regulation. Novel techniques such as siRNA knockdown of gene expression and conditional gene knockout allow silencing of gene expression in a temporal and spatial manner, whereas knock-in and transgenic mice allow up-regulation of gene expression or expression of investigator-altered gene transcripts. These new methodologies will provide the tools required to answer the questions identified by antisense and gene knockout.

13.1.1 Non-Genomic Mechanisms for Altering Gene Expression

13.1.1.1 Antisense Technology

The advent of antisense technology began a new phase in pharmacological research [1]. Briefly, specific DNA/RNA sequences are designed complementary to mRNA transcripts of interest. These complementary “antisense” DNA/RNA sequences are then injected into animals, usually into the ventricular system, subsequently enter cells, and hybridize with the mRNA transcripts they were designed to complement. This hybridization inhibits translation and targets mRNA to the degradative pathway, effectively silencing production of the targeted protein. This technology has some potential advantages over the gene knockout technology that will be subsequently reviewed. For example, antisense can reduce/eliminate gene expression in adult animals, which have developed normally, can be site specific in targeting, and is relatively inexpensive. However, antisense technology also has some significant drawbacks: For example, antisense can reduce but usually not silence gene expression [1]. In addition, identification of antisense sequences that significantly reduce gene expression can be difficult, and most importantly, selectivity of the antisense sequence is limited by gene sequence similarity. The antisense probes can cross-react between sequences with even moderate homology so that multiple silencing sequences and numerous controls are needed. Finally, antisense techniques, like other approaches including temporal conditional KOs, encounter an additional complication when attempting to silence G protein-coupled receptor expression in that opioid receptor turnover is relatively slow compared to other proteins [2]. Thus chronic administration of antisense probes is necessary to have a substantial effect

on opioid receptor protein expression. Nonetheless, following cloning of the opioid receptor/ligand genes in the early 1990s, this approach could be informatively applied to study opioid ligand selectivity as mentioned in comparison to gene knock-out studies below.

13.1.1.2 siRNA

siRNA represents a more recently developed and more specific way to regulate gene expression [3]. These probes function in a similar manner to traditional anti-sense RNA, but may be driven by viral promoters, can hybridize to nascent transcripts before they leave the nucleus, and can be of greater length. Thus, this approach provides a dramatic increase in specificity and a greater ability to silence a gene compared to traditional antisense RNA, which makes siRNA the current mechanism of choice for localized, substantial knockdown of protein. Currently this method has successfully been used to regulate μ and δ opioid receptor expression [4–7] with a loss of agonist potency of receptor-specific ligands observed following spinal, peripheral, and supraspinal siRNA treatment.

13.1.2 *Genomic Mechanisms for Altering Gene Expression*

In the late 1980s, the development of gene knockout technology provided a unique new method by which the function of genes could be analyzed [8–10]. Briefly, creation of knockout mice uses homologous recombination of a vector containing a mutated version of a gene with the genome of embryonic stem cells to create modified ES cells containing the mutated gene at the endogenous locus. The altered ES cells are injected into blastocysts, which are inserted into pseudopregnant female mice with resulting offspring chimeric for cells containing the mutated gene. Mating of a germ line-transmitting chimera will produce mice heterozygous for the mutated allele. Further mating will generate mice homozygous mutant for the allele and lacking functional protein for the specific gene mutated. Additional matings can then be performed to generate mutant mice on specific genetic backgrounds. Direct mating of chimeras to the line from which ES cells were derived allows isogenic lines to be produced immediately while backcrossing of mixed strain mice to other inbred strains produces congenic lines. Importantly, C57Bl6 ES lines have been produced [11], which now allows direct production of isogenic lines from this extensively studied strain. While ten generations of backcrossing from strain to strain is typically used to derive inbred congenic lines, investigators must be aware of the potential for closely linked genes to influence phenotypes [12].

Gene knockout has transformed the discipline of pharmacology in a profound way [13]. Study of knockout mice has allowed specific psychological and physiological behaviors directly impacted by a receptor system to be identified. Likewise,

analysis of drug specificity has also been dramatically altered in that analysis of drug specificity in vitro can be directly compared with in vivo measurements, in some cases with unexpected results.

Knockout mice are the ultimate control for drug selectivity and when drug fails to act in a knockout, the target gene must be required for its action. Nonetheless, knockout technology is also accompanied by complications in data interpretation. In some cases, for example, the relevance of novel sites of drug action observed in knockout mice lacking the presumptive primary target need to be assessed. In addition, the occurrence of alterations in gene expression in response to the lack of the targeted gene can limit interpretation [14, 15]. Finally, the lack of effects on a behavior in a knockout mouse may result from compensatory alterations in other receptor systems or redundancies within the endogenous control of the behavior. Nonetheless, KO mice have provided substantial new insight into opioid pharmacology.

13.1.3 Opioid System Knockout Mice

13.1.3.1 Opioid Receptor Gene-Targeted Knockouts/Knock-Ins

So far a total of fourteen opioid receptor KO mouse strains (Fig. 13.1) and eight opioid receptor knock-in mouse strains (Fig. 13.2) have been produced. Six strains of MOR-1 KO mice have been developed: three contain deletions of different positions of exon 1 [16–18]; one contains a replacement of exon 2 with the neomycin cassette [19]; one contains deletion of both exon 2 and 3 [20]; and in one there has been replacement of exon 11 with a lacZ promotor [21]. In mutant mice from all five strains containing deletions of exon 1, 2, and/or 3, *in situ* hybridization signals for the MOR-1 mRNA are greatly reduced or eliminated, and all binding of classic, pharmacologically defined μ opioid receptor agonists is abolished. Finally, [35 S] GTP γ S binding using prototypical μ opioid agonists such as DAMGO or morphine on tissue extracts from these mutants was also abolished in all lines tested.

Six knock-in MOR-1 strains with modified MOR-1 mRNA transcripts have also been reported [22–25] (Fig. 13.2). Two of these knock-ins have modified binding pockets with the consequence that classic μ antagonists now act as agonists [22]. In addition, two knock-ins have been designed to examine the effects of including premature stop codons in the *Mor-1* gene. Due to the placement of the stop codons, receptor level is greatly reduced in one strain while in the other strain, receptor level is relatively unchanged [24]. One knockin swapped the δ opioid receptor cytoplasmic tail for the μ opioid receptor cytoplasmic tail [23] resulting in mice with a μ opioid receptor that internalizes and recycles in response to morphine.

Most recently, the capability to produce both a traditional KO lacking exons 2 and 3 as well as a similar conditional KO has been created by inserting loxP sites before and after exon 2 and 3 [25]. Additionally, null MOR-1 KO mice can be used

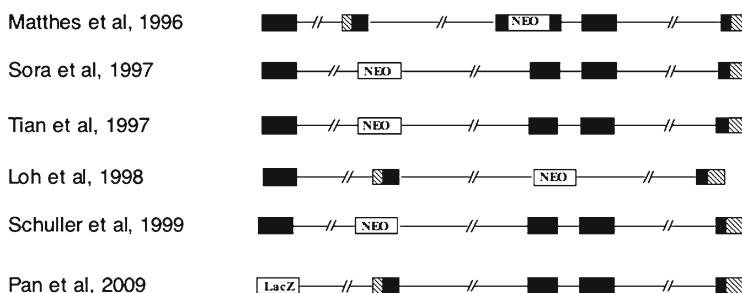
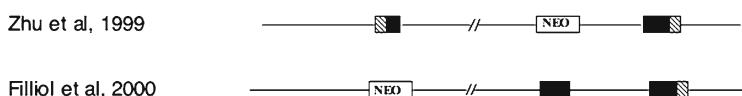
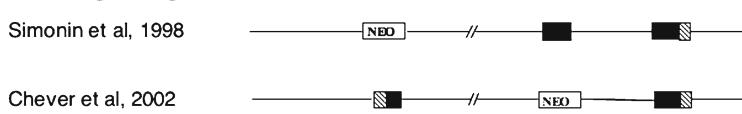
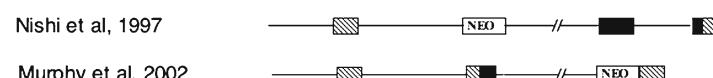
MOR-1**Wild type locus****Targeted genomes****DOR-1****Wild type locus****Targeted genomes****KOR-1****Wild type locus****Targeted genomes****NOR-1****Wild type locus****Targeted genomes**

Fig. 13.1 Production of opioid receptor knockout mice. The wild-type locus and targeted genomes are indicated for μ (top), δ (top middle), κ (bottom middle), and nociceptin (bottom) receptors. Introns are indicated by solid lines. Exons are represented by boxes and show coding (black boxes) and non-coding (striped boxes) regions of the genes. Transcription start sites are indicated by a line with the word "start" underneath. The neomycin-resistance and LacZ cassettes are indicated by a white box containing the word "NEO" or "LacZ," respectively

MOR-1**Wild type locus****Targeted genomes**

Yang et al, 2003

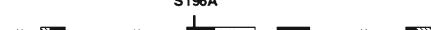
Yang et al, 2003

Contet et al, 2007

Contet et al, 2007

Kim et al, 2008

Van Rijin and Whistler, 2009

**DOR-1****Wild type locus****Targeted genome**

Scherrer et al, 2006

Van Rijin and Whistler, 2009

**KOR-1****Wild type locus****Targeted genomes**

Van Rijin and Whistler, 2009

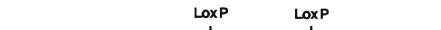
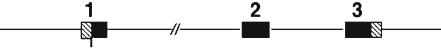


Fig. 13.2 Production of opioid receptor knock-in mice. The wild-type locus and targeted genomes are indicated for μ (top), δ (middle), and κ (bottom) receptors. Introns are indicated by solid lines. Exons are represented by boxes and show coding (black boxes) and noncoding (striped boxes) regions of the genes. Transcription start sites are indicated by a line with the word “start” underneath. Site-specific mutations are indicated by a line above the location of the change, indicating either a serine to alanine mutation (S196A), insertion of a premature stop codon (PTC), or lox P site. The neomycin-resistance enhanced green fluorescence protein and δ opioid receptor domain cassettes are indicated by a white box containing the word “NEO,” “eGFP,” or “DOR,” respectively

as a starter strain to produce transgenic mice expressing modified receptors in absence of the cognate endogenous receptor. This approach is illustrated by expression of a FLAG-tagged *MOR-1* gene in *MOR-1* KO mice, which was used to study receptor trafficking *in vivo* following ligand administration [26].

For the *DOR-1* gene, two different classic KO strains have been produced (Fig. 13.1) containing replacements of exon 1 [27] or exon 2 [28] with the neomycin cassette. Similar to the results in the MOR-1 KO mice, *in situ* hybridization confirmed loss of the *DOR-1* gene transcripts in the mutant mice. In both strains of DOR-1 KO mice, binding of radiolabelled prototypical δ agonists such as DPDPE or deltorphin 1 and 2 is absent in mutant mice. In addition, the binding of the δ selective antagonist naltrindole shows significant alteration in both density and affinity suggestive of interaction at an alternative site [28]. Also two DOR-1 knockins have been reported (Fig. 13.2). In the first, the gene for enhanced green fluorescence protein has been inserted into the third exon of the *DOR-1* gene allowing functional imaging and trafficking of the δ opioid receptor to be studied *in vivo* [29]. In the second, loxP sites have been inserted before and after exon 2 to allow both traditional as well as conditional DOR-1 KOs to be produced [25].

Two mutant strains of the *KOR-1* gene exist (Fig. 13.1) with either exon 1 [30] or exon 3 [31] replaced with the neomycin cassette. *In situ* hybridization showed that *KOR-1* gene transcript accumulation was abolished in both strains while binding density for either the highly-selective κ agonist [^3H] CI-977 or U-69,363 was undetectable by either homogenate or autoradiographic binding. In addition, one knockin strain has been crafted for *KOR-1* with insertion of loxP sites before and after exon 2 allowing production of both traditional and conditional KOR-1 KOs [25].

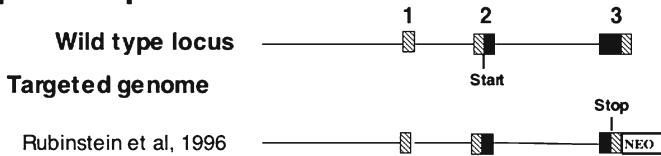
Finally, two mutant strains of the *NOR-1* gene have been produced (Fig. 13.1) with either exon 2 [32] or exon 3 [33] replaced with the neomycin cassette. *In situ* hybridization for the NOR-1 transcript and OFQ binding density was abolished in both strains. Importantly, these data demonstrate that KO mice for each individual opioid receptor have been produced with no obvious effects on viability or fertility, and with no major morphological changes reported.

13.1.3.2 Opioid Ligand Knockouts

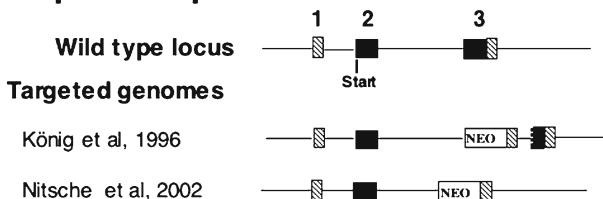
In addition to the strains targeting the opioid receptors, KO mice have also been produced for all known endogenous opioid ligands. Since most precursor peptides encode multiple peptide products, greater precision has been applied when designing some of these KOs. This was most elegantly demonstrated in the production of a mouse specifically lacking β -endorphin by adding a stop codon into the POMC precursor. This stop codon was introduced at the β -melanocyte-stimulating hormone (MSH) B-EP junction and did not alter production of β -MSH and corticotropin (ACTH) (two other peptide products produced from the POMC precursor) levels, while β -endorphin was eliminated (Fig. 13.3).

A strain lacking the entire POMC coding region has also been produced (Yaswen et al., 1999) [254]; however, since POMC encodes multiple peptides, the dramatic phenotypic changes have not been able to be accurately attributed to any specific POMC peptide. Two KOs of the *preproenkephalin* gene have been reported (Fig. 13.3) [34, 35]: either the 5' region of exon 3, which contains the enkephalin-coding

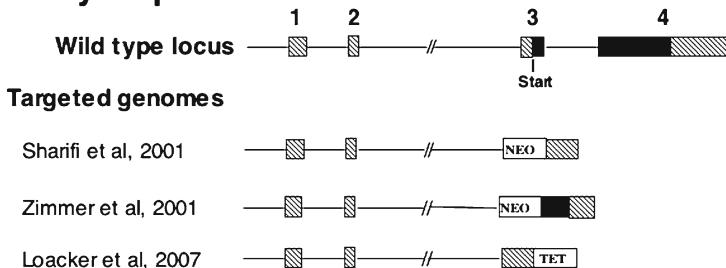
β -endorphin/POMC



Preproenkephalin



Prodynorphin



Prepronociceptin

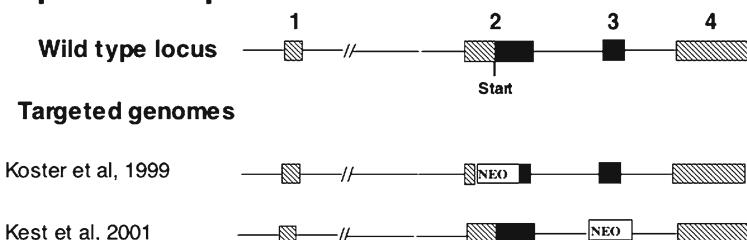


Fig. 13.3 Production of opioid ligand knockout mice. The wild-type locus and targeted genomes are indicated for END (top), ppENK (top middle), pDYN (bottom middle), and ppOFQ (bottom). Introns are indicated by solid lines. Exons are represented by boxes and show coding (black boxes) and noncoding (striped boxes) regions of the genes. Transcription start sites are indicated by a line with the word "start" underneath. The neomycin-resistance and the transactivator of the Tet-on system cassettes are indicated by a white box containing the word "NEO" or "TET," respectively

region, was truncated and duplicated, resulting in no detectable met-enkephalin; or the entire 5' part of exon 3 was replaced by the neomycin cassette, also resulting in complete loss of functional peptide.

Three mutants for the *prodynorphin* gene have been reported (Fig. 13.3): Either deletion of the whole coding region for dynorphin, spanning exons 3 and 4 [36, 37], or deletion of selective parts of exon 3 and exon 4 [38]. Both approaches resulted in mice lacking dynorphin peptides. Finally, two mutants for the *prepronociceptin* gene have been produced (Fig. 13.3), which either replaced almost all of exon 3 and part of exon 4 with the neomycin cassette [39] or inserted a neomycin cassette into exon 2 of the *prepronociceptin* gene [40]. Thus, mouse strains with mutations in each gene encoding opioid system peptides have been produced. Similar to receptor KO mice, opioid ligand KO mice show no major phenotypes other than lack of the endogenous peptides.

13.1.3.3 Combinatorial Knockouts

Combinational KO mouse strains containing two or more individual opioid receptor or ligand mutations have also been produced with no major alterations in development or survival. Two strains of mice lacking all three distinct individual receptor mutations have been reported [41, 42] and include either the alleles reported by Schuller et al., Zhu et al., and Ansonoff et al. or Matthes et al., Simonin et al., and Filliol et al. One strain of triple mutant mice lack [³H] DAMGO, [³H] CI-977, and [³H] DPDPE binding sites in homogenate assays as expected from the parental phenotypes [42], while the second strain shows a complete absence of binding to naloxone, the prototype opioid antagonist, as well as a significant decrease in baseline nociceptive latency [41]. In addition, this second triple KO mouse strain has been used to produce a quadruple mutant lacking the *DOR-1*, *MOR-1*, *KOR-1*, and *NOR-1* genes (unpublished observations). Similar to the individual mutants, all double, triple and quadruple opioid receptor mutant mice are viable and fertile. At present, only a single triple opioid ligand KO mouse on a mixed genetic background has been produced, which includes alleles reported by Rubenstein et al., Nitsche et al., and Zimmer et al. Similar to the triple receptor KO strains, this strain is also viable and fertile with no dramatic phenotype (unpublished observations).

13.1.3.4 Strain Versus Genotype Effects

During analysis of opioid system KO mice both genotype and strain background need to be considered. The common laboratory mouse belongs to the genus and species, *Mus musculus*, which was divided into 11 subspecies based on morphological characteristics more than 50 years ago [43]. Most knockout mice have been produced as hybrid strains (usually from 129 ES cells crossed onto C57Bl6 backgrounds). These mutant alleles have now usually been backcrossed to different strains (C57Bl6, 129S6, etc.). Nonetheless most early data reviewed here have used KO mice on a mixed hybrid background with only more recent studies usually using congenic mice. Thus, some caution is still needed when considering some of the earliest data that has not been repeated in congenic KO mice, since genetic

background can greatly alter the resulting phenotypes of mice containing targeted gene disruptions [12, 44, 45].

A now-classic example is that lethality of epidermal growth factor receptor KO is remarkably strain dependent. Dependent on the background strain of the knockout mouse, mice may die during early embryonic stages (CF-1 background), mid-gestation (129S6 background), or several weeks after birth (CD-1 background) [46]. A good example of ways to use the phenotypic differences between the same knockout on different strains is illustrated by the Otx2 KO mice. Heterozygote mice for the Otx2 KO allele on the C57Bl/6 strain show significant reduction or loss of mandible formation while backcrosses to the CBA strain shows little or no phenotypic difference from wild-type mice. Subsequent experiments using the drastic phenotypic difference isolated genetic loci critical in the craniofacial formation [47].

Similar experiments to identify genetic loci crucial for pain regulation may be possible for opioid system KO mice. Indeed, novel responses seen in MOR-1 and DOR-1 KO mice are remarkably strain dependent. For example, the induction of analgesia by morphine-6-glucoronide is retained in the MOR-1 KO mouse on a mixed 129/B6 background [17] and present in isogenic MOR-1 129S6 KO mice but absent in congenic MOR-1 C57Bl6 KO mice (unpublished observations).

13.2 Baseline Behavioral Changes in Opioid Knockout Mice

13.2.1 Nociception

13.2.1.1 Basal Nociception

The most well-studied behavioral consequence of opioid agonist treatment is the anti-nociception elicited by this class of compounds. Activation of opioid receptors by these exogenous agents produces anti-nociception generally by inhibiting the neural signaling responsible for nociception [48], and these responses are generally effectively antagonized by general or class-specific antagonists. In contrast, using opioid receptor antagonists to study basal responses has not produced as clear a picture in that some studies show pro-nociceptive activities for opioid antagonists, which indeed suggests basal inhibition of nociceptive processing [49], while others show no effect on nociception. Thus, one major focus in analysis of opioid system KO mice has been to identify the effect of endogenous opioids on basal nociception in several behavioral assays.

Mutations of the *MOR-1* gene are primarily pro-nociceptive (see Table 13.1 for specific references). MOR-1 KO mice show enhanced paw withdrawal to thermal pain in the plantar test as well as increased responses to thermal pain in the tail flick and hot plate tests. In contrast, MOR-1 KO mice do not show increased nociception for thermal pain in the tail immersion test. MOR-1 KO mice exhibit a pro-nociceptive increase in the tail pinch test for mechanical pain, but no change in von Frey tests.

Table 13.1 Alterations in basal nociception in opioid-system knockout mice

	MOR-1	DOF-1	KOR-1	Triple	NOR-1	END	ppENK	PDYN	ppOFQ
Thermal Pain	No change in TI [19]	No change in TH/HP [27, 28]	Increase in TF/HP [30, 31]	Increase in TF/HP [30, 31]	No change in TH/HP/TF [239]	PT/HP/TF [32, 64, 241]	Increase in TF [34, 35, 73]	No change in TF [34, 35, 73]	No change in PT/HP/PPT/TF [241]
	Increase/no change in TF/HP [18, 19, 131, 238]	Increase in females in TI [239]	Increase in females in TF/HP [18, 19, 131, 238]	Increase in females in TF/HP [30, 239, 245]	Increase in HP [239]	No change in TF in PT [239]	No change in TF in PT [239]	TI [36, 243]	Decrease in TF [40]
Mechanical Pain	No change in VF [244]	No change in TP [27]	Increase in TP [239]	Increase in TP in females [239]	Increase in TP [239]	VFT/TP [32, 64, 241]	No change in VF [63]	Decrease in VF [63]	No change in VF [243]
Chemical Pain	Reduction in W [51]	No change in W/EF [27, 28, 239]	Increase in EF males [239]	No change in W/EF [27, 28, 239]	No change in W [30, 239]	No change in EF [32, 241]	ND	Reduction in EF [35]	No change in EF [243]
	No change in W/EF [50, 246]	No change in W/EF [30, 239]	No change in EF males [239]	No change in EF [30, 239]	Increase in W [30, 239]	Increase in EF [247]	No change in EF [243]	No change in EF [243]	No change in EF [241]
Inflammatory Pain	Increase/no change in LF [239, 246]	No change in LF [28]	Increase in LF in females [239]	Increase in LF in females [239]	No change in LF in females [239]	Increase in LF [239]	ND	ND	ND
						241, [247]		LF [243]	LF [241]

EF early phase of formalin; HP hot plate; LF late phase of formalin; PT plantar test; TF tail flick; TI tail immersion; TP tail pinch; VF mechanical stimulation using von Frey filaments; W acetic acid writhing; CRD colorectal distension; ND not determined
References: Sora, et al. [18]; Matthes et al. [19]; Filliol et al. [27]; Zhu et al. [28]; Simonin et al. [30]; Ansonoff et al. [31]; Nishi et al. [32]; Nitsche et al. [34]; Konig et al. [35]; Zimmer et al. [36]; Koster et al. [40]; Sora et al. [51]; Petraschka et al. [63]; Bertorelli et al. [64]; Bilkei-Gorzo et al. [73]; Matthes et al. [131]; Hall et al. [238]; Martin et al. [239]; Clarke et al. [240]; Depner et al. [241]; Mogil et al. [242]; Wang et al. [243]; Fuchs et al. [244]; Larsson, et al. [245]; Ide et al. [50]; Zhao et al. [246]; Rizzi et al. [247]

Chemical and inflammatory pain tests provide more variable responses with formalin tests showing either no effect or enhanced nociception (Table 13.1). Nonetheless, although not completely consistent, these results do provide a reasonably clear picture of the role of the *MOR-1* gene in basal nociception.

Because mutation of the *MOR-1* gene primarily enhances nociception in the basal state, μ opioid receptors in wild-type mice inhibit basal nociceptive processing. However, the contribution of μ opioid inhibition to basal nociception is most likely relatively small. The variability of the results from transgenic studies indicate that pain intensity, test conditions, the contribution of other systems of this behavior, and perhaps strain background play a significant role in whether *MOR-1* mutation results in pro-nociceptive consequences in some tests. One potential reason for variability is that much early data were produced from mixed strain mice. Some responses, such as the decreased acetic acid writhing response seen in mixed strain *MOR-1* KO mice is absent in subsequent studies using congenic mice containing the same mutant *MOR-1* allele [50, 51]. Thus, the initial effect in this case could be a false positive due to the variability inherent to mixed strain animals or a strain specific basal excitation of chemical nociception by loss of the *MOR-1* gene.

Studies of *KOR-1* and *DOR-1* KO mice suggest that κ and δ opioid receptors also inhibit basal nociceptive processing. Although not demonstrated as consistently or in as many studies as effects in the *MOR-1* KO mouse, both *KOR-1* and *DOR-1* KO mice show enhanced basal nociception compared to wild-type mice (Table 13.1). Interestingly, these effects appear to be magnified in females. Furthermore, studies of *MOR-1/KOR-1/DOR-1* KO mice confirm the pro-nociceptive effects of opioid receptor mutation and suggest that mutation of one gene may be partially compensated for by remaining opioid receptors (Table 13.1). Specifically, although the pro-nociceptive effects of *MOR-1* KO mice on thermal and mechanical nociception are not present in each study, all studies of *MOR-1/KOR-1/DOR-1* KO mice show enhanced thermal and mechanical nociception that appears to be greater than the effect following *MOR-1* mutation alone. Finally, a recent study tentatively separates opioid receptor modulation of nociception even further, identifying δ opioid receptors as modulators of mechanical pain while μ opioid receptors influence thermal nociception [52].

Because of limited data (often reported from mixed-strain mice), opioid ligand KO mice have not yet provided as clear a picture regarding basal nociception as have opioid receptor KO mice. Similar to opioid receptor KO mice, *ppENK* KO mice show enhanced thermal and mechanical nociception. However, the *END* KO mouse shows a decrease in thermal nociception compared to wild-type mice, opposite of what would have been predicted from the opioid receptor KO studies. Mutation of the *pDYN* gene has so far shown no significant alteration of basal nociception. Finally, similar to *MOR-1/KOR-1/DOR-1*, *END/ppENK/pDYN* KO mice are pro-nociceptive to a greater degree than any individual ligand KO mouse (Table 13.1). Thus, in general, opioid ligand KO mice confirm that the opioid system inhibits basal nociceptive processing. However, mutation of *pEND* is anti-nociceptive, suggesting that opioid action on basal nociception may be more complex than first hypothesized. In addition, very little data is yet available examining

chemical and inflammatory nociception in ligand KO mice making comparison with receptor deficient mice largely incomplete.

Finally, discussion of the nociceptin system on basal nociception is warranted. Unlike opioid agonists, nociceptin agonists have primarily pro-nociceptive effects. Injection of OFQ ICV can reduce baseline values for all types of pain. This effect of OFQ has been attributed to nociceptin reversal of anti-nociceptive actions. It is thought that the nociceptin system has no effect on basal nociception, but only inhibits anti-nociceptive actions of other receptor systems. Analysis of nociception in OFQ and NOR-1 KO mice confirms this hypothesis in that neither mutation appears to effect basal nociception (Table 13.1). However, the number of studies is limited especially in regards to chemical and inflammatory pain, which show, in one out of three studies performed, enhanced nociception in NOR-1 KOs.

In conclusion, most mutations of opioid receptors and ligands result in primarily pro-nociceptive effects in most types of basal analgesic testing. These effects appear to be enhanced in mice lacking all three opioid receptors or ligands suggesting that different opioid systems may compensate for mutation of another system. Nonetheless, the overall effects of opioid system inhibition on basal nociception are significant, but not dramatic, and thus this system appears to be one of several components that regulate basal nociception.

13.2.1.2 Chronic Pain Syndromes

Several studies have now begun to identify the role of the opioid system following induction of chronic pain syndromes or neuropathies in which normally non-painful stimuli become painful (allodynia) or enhanced nociception arises from painful stimuli (hyperalgesia). Prior work in animal models indicated that the altered behavioral nociceptive states following induction of chronic pain syndromes could be explained by a variety of nonexclusive mechanisms. Thus, release of nociceptive processing from descending inhibition, permanent increases in receptor activation triggered by acute increases in the extracellular excitatory amino acids, deafferentation of spinal neurons and/or thalamic neurons resulting in hyperexcitability, and disinhibition and/or increased efficacy of previously ineffective synapses have all been shown to contribute to the development of chronic pain [53–55]. Of most relevance, many neuropathies demonstrate a loss in opioid analgesic efficacy [55, 56] reinforcing the concept that the opioid system is involved in the development of chronic pain.

The most well-studied neuropathy in opioid system KO/transgenic mice has been the peripheral sciatic nerve ligation model (PNL). In terms of opioid effects on this neuropathy, the κ opioid receptor system is most well-studied and has revealed interesting differences between receptor and cognate ligands in this system. Thus, following PNL, KOR-1 KO mice demonstrate enhanced mechanical and thermal nociception compared to wild-type controls, whereas pDYN KO mice show reduced sensitivity to mechanical and thermal stimulation compared to wild-type controls. These results, in conjunction with additional studies, suggest that the

κ opioid system has both pro- and anti-nociceptive actions in the formation of neuropathy following PNL.

Specifically, following injury, dynorphin is elevated in the affected area [57, 58], which activates κ opioid receptors to minimize pain (anti-nociceptive), though chronic stimulation of this receptor leads to receptor inactivation. This κ inactivation disinhibits spinal neurons normally inhibited by κ receptors and enhances mechanical and thermal nociception [59]. In addition, this example illustrates that dynorphin can also act in a non-opioid receptor fashion via NMDA and GABA receptors to enhance neuronal excitability and thus exert pro-nociceptive as well as anti-nociceptive effects. Thus, mutation of dynorphin eliminates the pro-nociceptive actions of dynorphin via NMDA and GABA receptors as well as the anti-nociceptive actions of dynorphin via the κ opioid receptor. The loss of pro-nociceptive actions predominate, resulting in a less severe neuropathy compared to KOR-1 KO mice, where dynorphin expression can contribute to pro-nociceptive actions while the loss of κ opioid receptors eliminates any anti-nociceptive actions of dynorphin and mimics the pro-nociceptive actions of dynorphin via receptor desensitization to produce a more severe phenotype (Table 13.2).

The μ and opioid receptor systems also appear to be involved in chronic pain developed following PNL. In DOR-1 KO mice, both thermal and mechanical nociception is elevated compared to wild-type controls suggesting a similar role for δ opioid receptors as outlined above for κ opioid receptors (Table 13.2) [60]. Interestingly, MOR-1 KO mice show only enhanced mechanical nociception following PNL compared to wild-type controls suggesting that thermal hyperalgesia and mechanical allodynia may form via different mechanisms (Table 13.2) [61]. One possible explanation for the differences is that thermal hyperalgesia is produced via a peripheral mechanism and mechanical allodynia is via central mechanism; thus μ opioid receptors may only be involved in the central mechanism [62]. Alternatively, a recent study suggesting that μ opioid receptors alter mechanical nociception only seems relevant [52]. END KOs do not show any significant enhancement or inhibition of the elevated nociception induced by PNL suggesting that this peptide, unlike dynorphin, has only anti-nociceptive effects through stimulation of its respective receptor subtypes (Table 13.2) [63].

Finally, the nociception receptor system does not appear to be involved in the formation of chronic pain in that NOR-1 KO mice following PNL show no effect on the changes in nociceptive thresholds compared to wild-type mice (Table 13.2) [64]. Thus, in the PNL model of chronic pain, study of opioid system KO mice has identified significant roles for dynorphin and the three classical opioid receptors. Whether this complement is maintained in all models of neuropathy is still to be determined.

13.2.2 Emotion/Anxiety

Mood is a complex behavior encompassing many differing behavioral aspects [65]. The level of anxiety and motivation are crucial determinants in the display of mood.

Table 13.2 Alterations in chronic pain following partial sciatic nerve ligation in opioid-system knockout mice

	MOR-1	DOR-1	KOR-1	NOR-1	END	ppENK	pDYN	ppOFQ
Thermal hyperalgesia/ allodynia	No effect in PT either hot or cold stimulus	Enhanced in PT for both hot and cold stimuli [60]	Enhanced in PW [59]	No effect in PT [64]	No effect in PT [63]	ND	Inhibited in PW [59]	ND
Mechanical hyperalgesia/ allodynia	Enhanced in VF [61]	Enhanced in VF [60]	Enhanced in VF [59]	No effect in VF [64]	No effect in VF [63]	ND	Inhibited in VF [59]	ND

ND not determined; PT plantar test; VF mechanical stimulation using von Frey filaments; PW, Paw Withdrawal

References: Xu et al. [59]; Nadal et al. [60]; Manisikka et al. [61]; Petraschka et al. [63]; Bertolli et al. [64]

Although not well-documented, early pharmacological studies with opioid ligands demonstrated that activation/blockade of opioid receptors can alter emotional state [66–69] such as decreasing aversion to noxious stimuli. Behavioral tests of mood have been divided into two distinct groupings: anxiety measurements and depressive behaviors with opioids shown to influence both types of tests [70, 71].

Several studies have demonstrated that mutation of μ and δ opioid systems results in substantial alteration of anxiety levels in mice. Specifically, mutation of DOR-1 produces increased anxiety as measured by the light-dark box and elevated plus maze [27]. Conversely, MOR-1 KO mice show decreases in anxiety using the similar assays [27, 72], while KOR-1 KO mice show no change in baseline anxiety [27]. Similar to DOR-1 KO mice, ppENK KO mice show increases in anxiety [73–75], though the prevalence of these differences is both sex and strain dependent [73]. Further linkage of the δ opioid receptor and its ligand enkephalin in regards to mood was demonstrated in a recent study showing that wild-type and MOR-1 KO mice show similar responses in the presence of an elevation of endogenous enkephalin [76]. Likewise, END KO mice show a decrease in anxiety compared to wild-type mice similar to effects seen in MOR-1 KO mice [74]. Surprisingly, pDYN KO mice do not react in the same manner as KOR-1 KO mice. In the only published study examining anxiety related behaviors in pDYN KO mice, pDYN KO mice show significant increases in anxiety compared to wild-type mice in two out of three assays used to measure anxiety [74]. Thus, although relatively few studies have examined anxiety in opioid KO mice, all studies so far demonstrate that mutation of the μ opioid receptor or its endogenous ligand, endorphin, reduces anxiety while mutation of the δ opioid receptor or its endogenous ligand, enkephalin, increases anxiety.

In contrast, the contribution of the κ opioid system remains less clear with differing effects seen in the receptor and ligand KOs. Interestingly, there appears to be a correlation between altered levels of stress hormones and anxiety responses in opioid ligand KO mice with pDYN and ppENK KOs showing prolonged stress responses and increased anxiety and END KOs demonstrating lower anxiety and trends for lower/shortened stress responses [74] though such a correlation has only been demonstrated for the decreased anxiety in MOR-1 KO mice [77, 78]. In any case, the mechanism underlying those instances where opioid modulation of anxiety has been demonstrated remains unclear.

Study of depression-like behaviors in opioid system KO mice remains less well-studied than anxiety-like behaviors, though the main effects on these behaviors again appears to be primarily through the μ and δ opioid systems and, again, in different directions. For example, mutation of the *MOR-1* gene decreases immobility in the forced swim assay a behavioral test of depression [27, 72], while mutation of the *DOR-1* gene increases immobility time [27]. Acutely, mutation of the κ opioid system has no role in depression-like behaviors, although several studies suggest that mutation of both the *pDYN* or *KOR-1* genes eliminates characteristic increases in depressive-like behavior following repeated exposure [27, 79–81]. Thus, the relatively limited studies with receptor KO mice indicate that μ and δ opioid systems regulate depressive-like behaviors analogous to their effects on anxiety.

In conclusion, analysis from KO mice has confirmed a role for the opioid system in regulation of anxiety/depressive-like behaviors. However, one limitation of several of the prior testing paradigms is that while testing anxiety/mood behaviors, one is often also including contributions of locomotion, nociception and basal effects of opioid system KO on these behaviors, which can make interpretation of alterations in mood/anxiety difficult. In addition, examination of effects on opioid system mutation on more traditional receptor systems linked to anxiety/depression, such as the serotonergic system [82], will shed light on the pathways and mechanisms by which the opioid system regulates anxiety/depression.

13.2.3 Immunology

Over the past three decades, it has become clear that the opioid system participates in a wide variety of functions of multiple immune cell types. For example, the commonly used analgesic, morphine, has been extensively reported to be an immunosuppressive agent [83–85]. To date, all four members of the opioid family of receptors have been functionally detected on the surface [86–88] of a variety of immune cells, suggesting that the endogenous opioid peptides targeted to immunocytes may be involved in communication between the central nervous system and the immune system, which can thus be considered to be an important neuroendocrine target tissue. Also, lines of evidence suggest that the opioid receptors expressed by immunocytes are often similar, if not identical, to their neuronal form in most cases [87, 88]. Since the opioid receptor system has a certain degree of promiscuity, genetic approaches using KO mice have become a valuable and indeed indispensable tool to explore the complicated opioid regulations of multiple immune functions.

An initial study using opioid system KO mice was performed using MOR-1 KO mice and showed that the absolute numbers of femoral bone marrow granulocyte macrophage (CFU-GM), erythroid (BFU-E), and multipotential (CFU-GEMM) progenitor cells in bone marrow were significantly higher in mutant mice compared to homozygotes [16]. This initial study linked the μ opioid system to hematopoiesis, which is associated with general peripheral immune functions. Additionally, morphine regulation of several innate immunity parameters, including macrophage phagocytosis and macrophage TNF- α production, was abolished in MOR-1 KO mice [89, 90].

In an independent study using a distinct MOR-1 KO strain, no difference between wild-type and mutant mice was found regarding multiple immune parameters in the naïve state when no opioid was given [91]. After morphine administration, previously identified effects of chronic morphine treatment such as lymph organ atrophy, decreased percentage of CD4(+)CD8(+) cells in the thymus and impaired natural killer cell activity in WT mice were not seen in this MOR-1 KO mouse [91]. It is worthwhile to note that immunosuppression property of opioids can be beneficial. The anti-inflammation characteristics of μ opioid receptor drugs can be utilized to alleviate the attacks on self-organ by our own immune system.

Recent work showed that preventive administration of the selective μ opioid receptor agonist DAMGO enhanced hepatoprotective-signalling pathways in an *in vivo* hepatitis mouse model, which were blocked by using naloxone methiodide. Consistently, genetic disruption of MOR-1 has enhanced the severity associated with experimental hepatotoxin-induced hepatitis [92]. Therefore, the genetic study of acute inflammation suggests that μ opioid receptor antagonism may open a new avenue to maintain remission in both chronic inflammatory bowel and liver diseases, which might be extended to other autoimmune diseases.

δ and κ opioid system KO mice have not been as extensively studied for immune effects. However, recent studies do suggest that both the *KOR-1* and *ppENK* genes play a role in immune response. *KOR-1* KO mice show a reduced thymus cellularity [93] and an increase in immature thymocytes (CD4-CD8 double(+)). Furthermore, *KOR-1* KO mice have enhanced humoral response compared to wild-type controls [93]. In *ppENK* KO mice, the relationship between enkephalin and airway inflammation has been studied. *ppENK* mutation correlates with reduced IL-5 production and eosinophil infiltration suggesting that enkephalin has a role in Th2 response development [94]. In addition, *ppENK* KO attenuates the proliferation response of T lymphocyte [95]. In contrast, spleen cellularity and splenocyte proliferation stimulated by ConA is not affected in *DOR-1* KO mice [96]. While the antibody response against KLH [97]. These results indicate that the often preferential relationship between *DOR-1* and enkephalin may not be maintained in the immune system.

The immunological function of the novel orphan opioid receptor NOR-1 remains less clear. NOR-1 KO mice produce a lower level of inflammatory cytokine TNF- α and chemokines following endotoxin exposure compared with WT. Moreover, mutant mice show attenuated *in vivo* leukocyte trafficking (Wen et al., submitted) [255]. These data indicate that the NOR-1 is associated with innate immunity and plays a role in inflammation regulation.

13.2.4 Reproduction

Pharmacological studies demonstrated a role for the opioid system in regulation of reproduction [98, 99]. In female rodents, the hypothalamus controls two major reproductive functions, GnRH release and reproductive receptivity or lordosis [98, 100]. Pharmacological studies implicate the opioid receptor system in regulation of both these functions [100–102]. Specifically, stimulation of hypothalamic opioid receptors directly and indirectly modulate the release of GnRH by inhibiting hypothalamic GnRH neurons [103–105]. Additionally, stimulation/inhibition of hypothalamic μ opioid receptors has been shown to be inhibitory to female receptivity, while δ and κ opioid receptors appear to be primarily excitatory [106–109]. Pharmacological studies have also implicated the opioid system in male reproductive activity. Specifically, opioid receptors modulate both male sexual receptivity [99] as well as sperm quality [110, 111].

Analysis of opioid system KO mice has allowed a more direct measurement of the basal requirement for specific opioid components in specific aspects of reproductive behavior. μ Opioid receptor is expressed on human sperm cells [112]. In males, *MOR-1* gene mutation results in decreased mating behavior, sperm motility, and reduced litter size [16, 110]. Since enkephalin peptide and opioid receptors have been detected in sperm [110, 113], it is surprising that no major change in male reproductive behavior has been noted in either the DOR-1, KOR-1 KO mice or the opioid ligand KO mice. In females, ppENK KO mice show equivalent lordosis quotients, but increased approach behavior [75]. In contrast, *MOR-1* KO females show reduced lordosis quotients, but no change in both active and passive approach behaviors [114]. Similar to males, no reported enhancement/deficit in female reproductive behavior (litter size or mating behavior) has been seen in DOR-1, KOR-1, END, and pDYN KO mice [27, 28, 30, 31, 36, 115].

In conclusion, opioid KO mice confirm a role for μ opioid receptors in normal male and female reproductive physiology and behavior with one study suggesting that enkephalin may be the endogenous ligand responsible for opioid action on female sexual behavior. However, in contrast to pharmacological studies of opioid involvement in reproductive behavior, mutation of most of the components of the opioid system appears to have little effect on reproductive function. Yet, the importance of the opioid system for optimal reproductive behavior is still unclear because in-depth studies examining female fertility (via egg production or estrous cycle variability) and behavior (via lordosis quotients) or male fertility (via sperm quantity or quality) or behavior (via approaches) have not been carried out for most opioid system knockout mice.

13.2.5 Feeding

The endogenous opioid system is involved in regulation of both food intake and body weight homeostasis [116–118]. Earlier pharmacological studies convincingly demonstrated that opioid receptor agonists stimulate food intake and body weight gain while opioid antagonists are anorectics [119, 120]. However, because opioid ligands often are pharmacologically nonspecific, identifying the receptor(s) involved in regulation of food intake has been difficult. More recent work has confirmed the involvement of individual opioid receptors in feeding using antisense mRNA techniques [121]. However, it is still unclear which opioid receptor(s) are crucial in modulation of feeding in the rodent and whether opioids regulate basal food intake and body weight homeostasis.

Generation of the opioid system knockout mice has allowed analysis of endogenous basal modulation of food intake and body weight homeostasis. The initial data from the opioid ligand KO mice confirmed that basal opioid activity is crucial for these parameters, but not in the manner predicted by pharmacological studies. Specifically, END KO are hyperphagic and obese [122, 123], suggesting that, unlike pharmacological stimulation of opioid receptors, which results in enhanced

food intake, endogenous opioid tone mediated by END reduces food intake helping to maintain energy homeostasis. Conversely, ppENK and pDYN KO mice show no change in food consumption as measured by sucrose intake [75, 124] but enhanced food preference towards consumption of sucrose [124].

The opioid ligand studies have been complemented by studies of a series of opioid receptor KOs. Similar to END KO mice, MOR-1 KO mice show increased body weight [125, 126]. However, the elevation in body weight does not correlate with increased food consumption like the END KO mice. KOR-1 KO mice show no change in either body weight or food intake when maintained on regular chow [127]. However, if maintained on a high-fat diet, KOR-1 KO mice gain significantly less body mass as well as having increased energy expenditure than wild-type mice [127]. DOR-1KOs, similar to their corresponding preferential ligand KO, do not appear to alter food consumption or body weight. Most recently, a comprehensive study of the three classic opioid receptors has been completed using single and combinatorial opioid receptor KO mice. The major findings of this study were that LY 255582, a general opioid antagonist, interacts with both μ and δ opioid receptors to reduce sweetened milk consumption, and that mutation of parts of the endogenous opioid system can alter basal consumption of this highly palatable food [128].

In conclusion, analysis of opioid system KO mice confirms that endogenous opioid tone regulates body weight homeostasis and food intake. Specifically, opioid system KO studies identify endorphin and the μ opioid receptor as normally responsible for opioid effects on basal body homeostasis. However, unlike studies using antisense RNA or pharmacological antagonists which cause hypophagia and weight loss, loss of the *END* or *MOR-1* gene results in obese mice. Conversely, the other classic opioid ligands and receptors have no role in regulating basal food consumption or body homeostasis, although they may influence food palatability via opioid effects on the reward pathway and the *KOR-1* gene can influence energy expenditure in mice exposed to an altered diet.

13.2.6 *Respiration*

Pharmacological agents provided evidence that the opioid system modulates respiratory activity [129], but they did not indicate the importance of the endogenous opioid tone on respiratory function. Studies of MOR-1 KO mice show no change in basal respiration between wild-type and KO mice [130, 131]. So far there are no reports reporting altered basal respiratory parameters in mice lacking functional genes for either κ or δ opioid receptor or any of the opioid ligands. Thus the limited analysis indicates that endogenous opioid tone does not significantly impact on basal respiration.

Conversely, opioid system KO mice have successfully identified/confirmed the receptors and sites of action for respiratory depression induced by opioid analgesics. Both KO and antisense techniques confirmed the μ opioid receptor system as

the receptor system responsible for the respiratory depression following morphine treatment [132–134]. Interestingly, δ analgesics fail to inhibit respiration in MOR-1 KO mice suggesting that δ opioid receptor mediated respiratory depression may be due to the non-selective nature of the δ ligands (see also Sect. 13.3.3.2) rather than action at δ opioid receptors [131]. Conversely, κ analgesics retain respiratory suppressive activity in MOR-1 KO mice suggesting that κ agonists suppress respiration via an alternative site of action, most likely the κ opioid receptor though this has not been tested genetically.

13.2.7 Gut Transit

Since the initial clinical use of opioids to produce analgesia, constipation has been a major side effect experienced by patients while diarrhea has been a common sign observed during opioid withdrawal [135]. Pharmacological studies identified μ and κ opioid receptors as the primary actors on GI transit [136, 137] with both μ and κ agonists inhibiting GI transit in a naloxone-reversible manner [136–138]. Although some δ agonists also inhibit GI transit, the role of DOR-1 receptors was thought to be minor because δ opioid receptors, unlike μ receptors, appear to be only localized in the circular muscle of the GI tract. Recent pharmacological studies also in GI transit show that OFQ enhances GI transit, thus also implicating NOR-1 receptors in this process [139–142].

While such pharmacological studies provided strong evidence that activation of opioid receptors inhibits GI transit [136, 137], opioid antagonists failed to enhance GI transit [136, 137], suggesting that basal GI transit is not regulated by the opioid system. Thus, based on pharmacological data, the μ , δ , and κ opioid systems as well as the nociception receptor system were thought to modulate GI transit, but endogenous opioid tone was not critical for this process.

Use of opioid system KO mice provided new insight into these processes and showed that basal GI transit is dependent on the endogenous opioid system. Specifically, basal GI transit is enhanced in MOR-1 KO and MOR-1/DOR-1/KOR-1 KO mice ([143], unpublished observations). In addition, NOR-1 KO mice show enhanced GI transit suggesting that nociceptin receptor tone also influences basal GI transit (unpublished observations). Furthermore, in MOR-1 KO mice, κ and δ agonists fail to inhibit GI transit, suggesting that all opioid action on GI transit may be mediated through the μ opioid receptor [143]. Currently, no studies have examined GI transit in opioid ligand KO mice.

In conclusion, unlike opioid antagonist studies, opioid KO mice demonstrate that the μ opioid receptor system modifies basal GI transit and that opioid inhibition of GI transit is exclusively the result of action of opioid agonists on the μ opioid receptor. Finally, it appears that opioids inhibit GI transit and NOR-1 receptor ligands excite GI transit in an opposing manner similar to their respective actions on nociception suggesting that the neural network underlying opioid effects within the enteric nervous system are similar to other parts of the nervous system.

13.3 Opioid Drug Pharmacology

13.3.1 Classical View of Opioid Ligand Specificity

Opioid pharmacology begins with the behavioral classification of opioid analgesics. Initial studies identified analgesics, such as morphine, as opioids based on their inhibition of nociception and reversibility by opioid antagonists, such as naloxone. In the early 1970s, a specific saturable binding site for morphine (as well as other opioid ligands) was first identified in neural tissue [144] and soon thereafter the first of several endogenous opioid ligands were identified [145]. Following identification of a specific binding site and endogenous ligands, the number of presumptive opioid receptor types and subtypes, generally ascribed to different mechanisms, increased significantly [146, 147]. Thus, pharmacological studies identified three main opioid receptors, μ , δ , and κ , each of which was composed of at least two subtypes, (μ_1 , μ_2 , δ_1 , δ_2 and κ_1 – κ_3) as well as additional opioid receptors, such as the sigma opioid receptor (no longer identified as an opioid receptor) [148–150] and often postulated the existence of opioid complexes such as receptor dimers [151, 152]. Thus, the cloning of only three opioid receptor genes was surprising [153, 154]. Although the major postulated receptor types could be clearly distinguished, the mechanisms underlying multiple subtypes simply could not be accounted for by the number of opioid genes.

13.3.2 Knockout Confirmation of Opioid Ligand Specificity

13.3.2.1 MOR-1 KO

Initial studies with MOR-1 KO mice confirmed that DAMGO and other typical μ ligands target the *MOR-1* gene product. Specifically DAMGO binding, anti-nociceptive potency, and measured behavior is absent in MOR-1 KO [17, 19, 20]. Morphine specificity was also elucidated using the MOR-1 KO with initial studies finding that all anti-nociceptive, locomotor and respiratory action of morphine is absent in mice containing mutant *MOR-1* genes (Table 13.3) [17, 19, 20, 130], despite morphine binding to other receptors in transfection assays *in vitro* [155]. However, more recent studies have identified some actions of morphine that are retained in MOR-1 KO mice [156, 157]. Specifically, spinally injected morphine can induce anti-nociception in MOR-1 KO mice via the κ opioid receptor. However, although these studies show that morphine can act *in vivo* via opioid receptors other than μ , these effects are substantially smaller in magnitude and require significantly higher doses compared to morphine action via the μ opioid receptor.

In addition, new and significant information regarding δ and κ opioid ligand specificity has also been obtained using the MOR-1 KO mice. While typical δ opioid ligands DPDPE and deltorphin II showed no or minimal anatomic change

Table 13.3 Alterations in morphine responsiveness in opioid system knockout mice

	MOR-1	DOR-1	KOR-1	NOR-1	END	pPENK	pDYN	ppOFQ
<i>Acute effects of morphine</i>								
Analgesia	Lost [17–20, 51, 244]	No change [28, 34]	No change [30]	No change [64, 193]	No change [242] Reduced [242]	No change [34]	No change [36]	No change [39]
<i>Chronic effects of morphine</i>								
Tolerance	N/A	Lost [28, 34]	No change	Partial [193] No change [192]	ND	Lost [34, 250]	ND	No change [39]
Withdrawal	Lost [18, 252]	No change [34]	Partial [30]	Partial [192, 193]	ND	Increased [3]	ND	Partial [251]
Reward	Lost [19, 209, 252]	ND	No change [30]	ND	No change [63, 253]	No change [250, 253]	ND	Increased [39] Decreased [251] ND

N/A not applicable; ND not determined

References: Schuller et al. [17]; Sora et al. [18]; Matthes et al. [19]; Loh et al. [20]; Zhu et al. [28]; Simonin et al. [30]; Nitsche et al. [34]; Zimmer et al. [36]; Kest et al. [39]; Sora et al. [51]; Petraschka et al. [63]; Bertorelli et al. [64]; Mamiya et al. [192]; Ueda et al. [193]; Becker et al. [193]; Chung et al. [250]; Skoubis et al. [251]; Fuchs et al. [244]; Marquez et al. [250]; Chung et al. [251]; Sora et al. [252]; Skoubis et al. [253]

in autoradiographic binding distribution in MOR-1 KO mice [158], the anti-nociceptive activity of both DPDPE and deltorphin II were altered in MOR-1 KO mice [18, 131]. Specifically, supraspinal DPDPE and deltorphin II activities were lost in MOR-1 KO mice while spinal DPDPE and deltorphin II actions were retained although at reduced potency [131, 159]. At present it is still believed that DPDPE and deltorphin II bind to δ opioid receptors *in vivo* due to the retention of the δ -like opioid receptor binding pattern as well as behavioral responses (such as depression) other than anti-nociception [27, 160–162] that appear DOR-1 dependent. On the other hand, the analgesic action of these compounds, especially supraspinally, is thought to be μ opioid receptor dependent either due to minimal δ specific anti-nociception or a requirement for μ opioid circuitry in the δ response [160–162]. Both explanations appear incomplete, however, because the nonpeptidic δ ligand SCN-80 retains both binding and behavioral activity in the MOR-1 KO mouse [160] while absent in DOR-1 KO mice [163]. κ opioid ligands such as U69,593 and U58,543 present a less confusing picture. κ opioid receptor autoradiography is unchanged in MOR-1 KO mice [158] and, in addition, all κ opioid ligand behavior is maintained in mice containing mutant *MOR-1* genes [17, 19, 20, 42].

13.3.2.2 DOR-1 KO

Traditionally, the prototypical δ ligands were DPDPE for $\delta 1$ opioid receptors and deltorphin II for $\delta 2$ opioid receptors [164]. Production of the DOR-1 KO mouse definitively demonstrated that all DPDPE and deltorphin II radioligand binding require the *DOR-1* gene [27, 28]. Further, both DPDPE and deltorphin II show reduced or loss of spinal anti-nociceptive activity consistent with this behavior being mediated by the δ opioid receptor [28]. However, both DPDPE and deltorphin II supraspinal anti-nociceptive activities are retained in DOR-1 KO mouse [28]. This result, as noted earlier, is consistent with DPDPE and deltorphin II inhibiting nociception *in vivo* via μ opioid receptors but as discussed below, these expressed activities in DOR-1 KO mice are MOR-1 independent. Conversely, non-peptidic δ ligands such as SNC-80 show both complete loss of [35 S] GTP γ S binding and anti-nociceptive potency in DOR-1 KO mice [163] (unpublished observations). Thus, DOR-1 KO studies suggest that traditional peptidic δ ligands do not have equivalently specific *in vitro* and *in vivo* selectivity (see Sect. 13.3.3.2), whereas nonpeptidic δ ligands appear to maintain their selectivity *in vivo*. Regarding behavioral responses beyond anti-nociception, both peptidic and nonpeptidic δ ligands show reductions or loss of potency in DOR-1 KO mice [27, 28].

Both μ and κ opioid receptors show minimal changes in radioligand autoradiography in DOR-1 KO mice [165]. Likewise, the potency of behavioral activities of both ligand groups appears to be maintained in DOR-1 KO mice, suggesting that μ and κ opioid ligands show appropriate *in vivo* selectivity for their corresponding receptors versus δ opioid receptors. The one major exception appears to be regarding morphine action. Morphine maintains a similar analgesic and withdrawal

profile in DOR-1 KO mice, but tolerance to morphine is absent in mixed strain DOR-1 KO mice [28]. Whether this loss of morphine tolerance may be due to loss of a primary action of morphine directly at δ opioid receptors or to a secondary action of morphine on other endogenous receptors and/or ligands which requires functioning δ opioid receptors is not known at present (see Sect. 13.4.1).

13.3.2.3 KOR-1 KO

Pharmacologically, κ opioid receptors comprise two major subtypes, $\kappa 1$ and $\kappa 2$ [166, 167]. Specific ligands, such as U69,593 and U50,488H, have been identified as specific for the $\kappa 1$ opioid receptor subtype [168, 169]. The $\kappa 2$ opioid receptor subtype is defined as the opioid binding remaining following occupancy of μ , δ , and $\kappa 1$ opioid receptor sites by specific ligands, although a putative $\kappa 2$ ligand, 6'-GNTI, has recently been discovered [42, 170]. Mutation of the *KOR-1* gene results in mice lacking all $\kappa 1$ opioid receptor binding as well as behavioral activity [30, 31, 42]. In addition, most $\kappa 2$ ligands fail to function equivalently in the KOR-1 KO mice [30, 31, 42]. However, because $\kappa 2$ opioid receptors are defined as the activity/binding remaining following occupancy of all other opioid receptors, ligands that can bind/activate $\kappa 2$ opioid receptors have activity at μ and δ receptors and therefore some functional activity in KOR-1 KO mice at these alternate sites. However, any residual activity of $\kappa 2$ ligands in KOR-1 KO mice appears to be via either μ or δ receptor mediated as shown by studies both pharmacologically and genetically [42]. In addition, studies with the 6'-GNTI, the putative $\kappa 2$ specific ligand, show almost complete loss of anti-nociceptive action in KOR-1 KO mice [171].

In addition, production of the KOR-1 KO mice has allowed identification of novel behavioral targets of κ ligands as well as new drug classes capable of binding the *KOR-1* gene product. Specifically, tests in KOR-1 KO mice demonstrate definitively that *Salvinorin divinorum*, a naturally occurring hallucinogen, can act at the κ opioid receptor [31]. These results suggest that activation of κ opioid receptors may have hallucinatory potency confirming older observations that could not be definitively proven [172]. Additionally, the active component of *S. divinorum* is salvinorin A, a compound with a novel chemical structure when compared to known κ ligands, demonstrating a new class of chemical compounds that have potential as κ ligands [173, 174].

Both μ and δ opioid receptors show minimal changes in radioligand autoradiography in KOR-1 KO mice. Likewise, behavioral activities of both ligand groups appears to be maintained in KOR-1 KO mice suggesting that μ and δ opioid ligands show good selectivity for their corresponding receptors versus κ opioid receptors. The one major exception again appears to be regarding morphine action. Morphine maintains a similar analgesic and tolerance profile in KOR-1 KO mice, but withdrawal to morphine is reduced in KOR-1 KO mice [30]. Whether this reduction of morphine withdrawal is due to loss of a primary action of morphine directly at κ opioid receptors or a secondary action of morphine on other endogenous receptors and/or ligands with require functioning κ opioid receptors is not known at present (see Sect. 13.4.2).

13.3.3 Identification of Novel Targets for Opioid Ligands

One major advance resulting from the use of opioid system KO mice was identification of novel opioid-like receptor activities. As described earlier, studies of the MOR-1 KO definitively demonstrated that the primary *in vivo* target for morphine was the μ opioid receptor. However, studies of other classic μ ligands, such as heroin and the morphine metabolite, M6G, have been less conclusive. Specifically, heroin and M6G retain almost complete potency in the exon 1 MOR-1 KO mouse [17] (Table 13.4) but show a significant loss of potency in exon 11 MOR-1 KO mice [21]. These findings suggest that heroin and M6G act through the exon 11 splice variant of *MOR-1* rather than the exon 1 splice variant. In addition, recent studies show that other μ opioid specific agonists, such as fentanyl and oxycodone, retain some *in vivo* function in MOR-1 or triple KO mice possibly through modification of ion channels, specifically NMDA receptors [175–177]. Similarly, the specificity of DPDPE, the prototypical δ agonist, was re-evaluated following generation of the MOR-1 and DOR-1 KO mice. Supraspinal DPDPE retains activity in DOR-1 KO mice, but shows reduced or loss of function in all strains of MOR-1 KO mice. Finally, analysis of ORL KO mice has renewed debate about the possible existence of an alternative site of action for OFQ that is anti-nociceptive rather than its confirmed pro-nociceptive action.

13.3.3.1 Morphine-6-Glucoronide

For over 20 years, antisense and cloning studies demonstrated that the *MOR-1* gene has multiple splice variants and two functional start sites encoded at either exon 1 or exon 11 [146, 147]. Initially produced MOR-1 KO mice showed no response when injected with heroin and/or M6G [20, 178] though both studies produced mice with mutation of the *MOR-1* gene at exon 2. Since all transcripts of the *MOR-1* gene require a functional exon 2, this design results in mice lacking all transcripts for the *MOR-1* gene. In 1999, a third MOR-1 KO mouse was generated using a *MOR-1* gene mutated at exon 1 [17], eliminating all exon 1 MOR-1 transcripts, but retaining any MOR-1 transcripts initiated at an alternate start site, exon 11 [146, 147]. Similar to the earlier MOR-1 KO mice, morphine action is lost, but heroin and M6G function is retained [17]. Examination of MOR-1 transcripts shows loss of all exon 1 transcripts, but exon 11 transcripts remain [17]. Apparently, M6G and heroin can function at almost equal efficacy in mice containing only exon 11 MOR-1 transcripts (Table 13.4).

Moreover, a recent study has generated a transgenic mouse containing LacZ under an exon 11 promotor and GFP under an exon 1 promotor [21, 179]. This study shows that both promotors generate transcripts throughout the brain and spinal cord, but have a distinct distribution pattern [179]. Thus, MOR-1 KO mice confirm that splice variants for the *MOR-1* gene exist and demonstrate that while morphine activity requires a functional exon 1, M6G and heroin can function in

Table 13.4 Identification of novel targets for “typical” opioid ligands

Genotype	Morphine-6-glucoronide (punitive μ agonist)			DPDPE (punitive δ agonist)		
	SC	ICV	IT	ICV	IT	IT
MOR-1	Lost [20, 133, 178] Reduced [17]	No change [17] Lost [20, 178] Reduced [21]	Reduced [17]	Lost [159, 244] No change [20] Reduced [131, 160, 162]	Lost [159, 244] No change [20]	Reduced [160, 249]
DOR-1	ND	No change [28]	No change [28]	No change [28, 162]	No change [28, 162]	Reduced [28]

References: Schuller et al. [17]; Loh et al. [20]; Pan et al. [21]; Zhu et al. [28]; Matthes et al. [131]; Romberg et al. [133]; Sora et al. [159]; Hosahata et al. [160]; Scherer et al. [162]; Kitanaka et al. [178]; Fuchs et al. [244]; Qui et al. [248]; Guo et al. [249]

mice containing only exon 11 MOR-1 transcripts. Furthermore, analgesics studies of these mice show a dramatic decrease in the ability of M6G and heroin to elicit anti-nociception, but no change in morphine potency or efficacy [21].

13.3.3.2 DPDPE

Classical pharmacological studies identify DPDPE and deltorphin II as prototypical δ agonists, but transgenic studies suggest that *in vivo* both DPDPE and deltorphin II can act through the μ opioid receptor as well as a possible novel alternative site of action. Specifically, supraspinal DPDPE treatment fails to elicit significant anti-nociception in MOR-1 KO mice (Table 13.4) [17, 20, 131, 159]. Furthermore, supraspinal DPDPE functions normally in DOR-1 KO mice (Table 13.3) [27, 28]. More recent studies using pharmacological antagonists for μ and δ receptors show that DPDPE action in DOR-1 KO is partially inhibited by naltrindole, a prototypical δ antagonist, but completely reversed by CTOP, a μ antagonist [162]. Thus, supraspinal DPDPE is now thought to act primarily via μ opioid receptors to elicit anti-nociception. Deltorphin II also shows reduced function in MOR-1 KO mice, but retains some action [17, 131]. Whereas deltorphin II shows equal or slight reductions in function in DOR-1 KO mice, suggesting that *in vivo* deltorphin II can act at both μ and δ opioid receptors. Interestingly, studies using combinatorial opioid receptor KO mice show that in MOR-1/DOR-1 and MOR-1/DOR-1/KOR-1 KO mice both deltorphin II, DPDPE and the nonpeptidic δ agonist, BW373U86 still function [180], although in the individual MOR-1 KO strain used to derive the double and triple KO mice, DPDPE and deltorphin II show reduced action [17] providing evidence that DPDPE and deltorphin II, act through the μ opioid receptor to induce anti-nociception, but also have a novel site of action, which is uncovered following mutation of the *DOR-1* gene [28].

13.3.3.3 Nociceptin

Initial identification of OFQ and NOR-1 receptors labeled these receptors as antiopioids [181–184], characterized by their ability to reverse anti-nociception action induced by opioids as well as other analgesics [181–184]. Further study determined that these receptors not only reduces anti-nociceptive activity but also promotes pro-nociception [185]. Antisense experiments confirmed the specificity of OFQ to ORL and showed that pro-nociceptive actions of OFQ are lost following application of exon 1 NOR-1 antisense [185], but not exon 2 or 3. These studies also uncovered an alternative site of action for OFQ that appears to be anti-nociceptive [185] and were blocked only by antisense to exon 2 and 3 of the *NOR-1* gene. Specifically, OFQ appears initially to act pro-nociceptively through NOR-1 receptors sensitive to exon 1 antisense followed by anti-nociceptive action, which is sensitive to exon 2 and 3 antisense [185]. Furthermore, the anti-nociceptive action appears strain specific and is potentiated or uncovered by haloperidol pretreatment

[186–188]. Together these antisense studies suggest that OFQ acts through specific NOR-1 receptor splice variants to cause pro-nociceptive and anti-nociceptive action. Subsequent, NOR-1 KO studies provide results contradictory to the anti-sense findings. NOR-1 KO mice lacking exon 2 and 3 show no pro-nociceptive response to OFQ (unpublished observations). In addition, the anti-nociceptive actions of OFQ are retained in these NOR-1 KO mice in the presence of haloperidol (unpublished observations). In conclusion, both antisense and KO studies confirm that OFQ has both pro-nociceptive and anti-nociceptive actions *in vivo*. However, whether *NOR-1* gene splice variants or unique gene products mediate these different functions is still unclear.

In conclusion, antisense and KO techniques have identified several novel sites of action for known opioid ligands. Specifically, M6G and heroin anti-nociceptive and GI transit action is retained in mice lacking exon 1 *MOR-1* splice variants. Several δ opioid ligands, DPDPE and deltorphin II, maintain inhibition of nociception in mice lacking a functional *DOR-1* gene. And finally, OFQ is able to inhibit nociception in mice lacking functioning NOR-1 receptors. Thus, although it is still unclear whether the retained functionality of these ligands is due to the retention of splice variants of the mutated receptors or induction of alternative gene products, it is certain that both antisense and KO studies have identified alternative sites of action for traditional ligands.

13.4 Drug-Induced Behaviors

13.4.1 Tolerance

Tolerance is defined as loss of drug potency following repeated exposure. Most pharmacological agents produce some degree of tolerance following repeated exposure, and one of the major drawbacks of opioid use as a pain reliever is the development of opioid tolerance following chronic exposure. Specifically, chronic exposure to morphine results in drastic reductions in morphine potency in a relatively short time span [189]. Typically, all drug tolerance occurs via a similar process [190]. Repeated drug exposure results in chronic activation of a receptor system. Chronic activation of the receptor system results in down-regulation and desensitization of that system. The down-regulation of the receptor system (via receptor/ligand down-regulation/desensitization) results in decreases in drug potency, i.e., more drug is required to obtain a comparable behavioral effect. Studies of tolerance of opioid drugs confirmed this pathway of tolerance formation for compounds such as fentanyl or DAMGO, but morphine, the opioid compound most commonly used clinically as an analgesic, does not induce tolerance via the standard mechanism [190, 191].

Generation of opioid system knockout mice provides some insight into the pathways responsible for morphine tolerance. As expected MOR-1 KO mice show no tolerance to morphine because morphine in these mice has no potency (Table 13.4).

However, DOR-1, ppENK and NOR-1 KO mice, in which morphine has potency equivalent to wild-type mice, show reduced/loss of tolerance to morphine following repeated exposure (Table 13.4) [28, 34, 39, 192, 193]. The reduction/loss of tolerance to morphine in these KO mice corresponds directly with two prevalent hypotheses on the mechanisms behind formation of morphine tolerance. One hypothesis is that morphine tolerance is not due to direct action of morphine on receptors, but rather the result of receptor desensitization and down-regulation induced by release of endogenous neurotransmitters following morphine treatment. This hypothesis proposes that morphine treatment results in release of endogenous enkephalin, which activates the δ opioid receptor [194–196].

Chronic morphine treatment results in chronic release of endogenous enkephalin and subsequent desensitization of δ opioid receptors, which eventually leads to tolerance. Thus, disruption of any section of the endogenous enkephalin- δ opioid receptor system should alter development of morphine tolerance. Indeed, ppENK KO and DOR-1 KO, develop reduced tolerance to morphine following chronic morphine treatment (Table 13.3) [28, 34]. Interestingly, in KOs lacking arrestin-2, a protein critical for GPCR internalization, desensitization, and resensitization, tolerance to morphine does not develop [197] though morphine potency is shifted.

An alternative hypothesis for the formation of morphine tolerance focuses on hyperalgesia present in animals chronically exposed to morphine [198–200]. This hypothesis proposes that morphine tolerance develops because chronic exposure to morphine results in hyperalgesia causing a shift in an enhancement of basal nociception. Thus, higher doses of morphine are required to obtain an equivalent inhibition of nociception making it appear that morphine has lost potency. Activation of the nociceptin receptor system results in hyperalgesia or an enhancement of nociception. Thus, up-regulation/sensitization of the nociceptin receptor system could result in an enhancement of basal nociception, resulting in changes in morphine potency that may appear as tolerance. As predicted, at least some studies show that mutation of the *NOR-1* gene results in a mouse with reduced tolerance to morphine (Table 13.3) [39, 192, 193].

Most likely, development of tolerance to morphine is a complex process involving multiple signaling pathways. The reduction/loss of morphine tolerance in both pENK/DOR-1 and NOR-1 KO mice appears contradictory. A student of morphine tolerance is left with the question if release of endogenous enkephalin is responsible for morphine tolerance, how can morphine tolerance be reduced in NOR-1 KO mice, and vice versa. Likewise, where do other alternative hypotheses of morphine tolerance such as NMDA receptors, β -adrenergic receptors and/or PKC activation [201, 202] fit into this model. The likely explanation is that all these pathways are responsible for morphine tolerance. Drug dose, route of administration and frequency of treatment, all play a role in which pathway of morphine tolerance is activated. It is interesting to note that studies of the NOR-1 KO mice have shown loss of tolerance as well as no change in morphine tolerance [39, 192, 193] (unpublished observations) as have studies of the DOR-1 KO mice [28, 52]. The difference between these studies was primarily the dose and frequency of treatment. Closer examination of morphine potency via dose-response curves rather than analysis at a single dose or reductions in morphine exposure normally allow discrimination

between mice showing complete tolerance and those showing a reduction/slowing in tolerance formation (unpublished observations). Thus, formation of tolerance to morphine is due to multiple mechanisms resulting in both reduction of an animal's sensitivity to morphine as well as changes to basal nociception.

13.4.2 Withdrawal

A major drawback of the use of opioids as analgesics is the formation of both physical and psychological dependence following chronic exposure. Similar to the formation of drug tolerance, drug dependence is typically via a similar mechanism. Chronic drug exposure results in down-regulation/densensitization of the receptor system altering the basal state of the system [190]. By altering the basal state of the receptor system, the endogenous receptor system becomes physically dependent on the exogenous drug. The subject's well-being is now dependent on the presence of the exogenous drug. Withdrawal of the exogenous drug results in failure of the endogenous receptor system to maintain well-being manifested through expression of psychological, behavioral, and physiological symptoms. The most common opioid analgesic, morphine, has been shown to induce dependence.

KO studies have been able to help elucidate the mechanism by which physical dependence to morphine occurs. As stated earlier, typical drug dependence is thought to occur via a similar mechanism as drug tolerance. Chronic drug exposure results in alterations of the signaling system resulting in a physical requirement of the drug for well-being. As described earlier in this chapter, KO of the *MOR-1* gene demonstrated that the primary target for the anti-nociceptive activity of morphine is the μ opioid receptor. Likewise, with loss of a functioning *MOR-1* gene, morphine tolerance is lost.

In agreement, *MOR-1* KO mice demonstrate no withdrawal symptoms following chronic morphine exposure (Table 13.3) [17, 19, 193]. However, morphine action on the *MOR-1* gene does not appear to be the only site of action involved in formation of physical dependence to morphine. KO studies of the *CB1*, *KOR-1*, and alpha-calcitonin genes show reduction/loss of morphine withdrawal symptoms, but no significant change in morphine tolerance (Table 13.3) [30, 203, 204]. Conversely, mutation of the *DOR-1*, *ppENK*, and *arrestin-2* genes show a significant reduction/loss in formation of tolerance to morphine, but no difference in development of morphine dependence (Table 13.3) [34, 197].

Finally, mutation of some genes, such as the *NOR-1* or β -adrenergic receptors, result in reduction/loss of both morphine tolerance and dependence (Table 13.3) [192, 193, 202]. These studies demonstrate that the mechanism for morphine dependence is not identical to the mechanism for morphine tolerance. Some overlap between signaling pathways does occur, specifically the requirement for functioning *MOR-1* and *NOR-1* genes. However, both tolerance and dependence can occur in the absence of the other showing that the major alterations in receptor signaling following morphine tolerance and dependence are distinct.

13.4.3 Reward

Opioid modulation of drug or behavior-induced reward is believed to occur via two different pathways. The classic view is that reward is regulated by dopaminergic neurons in the nucleus accumbens [205]. Opioid modulation of these dopaminergic pathways can influence the acquisition of reward [205–208]. An alternative pathway is hypothesized to exist via direct μ opioid modulation of the reward circuitry independent of dopaminergic neurons [205–208]. Obviously, the reward effects of drugs, which specifically stimulate receptors transcribed by mutated genes, are absent in their corresponding knockout mice. For example, morphine provides no reward in MOR-1 KO mice (Table 13.3) [19, 209]. Similarly, deltorphin II also shows no rewarding effects in MOR-1 KO [210], consistent with observations discussed above suggesting that *in vivo* deltorphin II can activate the μ opioid receptor. However, the reduction in reward in opioid KO mice is not limited to drugs that have lost their site of action.

In MOR-1 KO mice, for example, the rewarding properties of alcohol, cannabinoids and food are all reduced. Specifically, cannabinoid place preference is lost in MOR-1 KO mice [211]. Likewise, alcohol reward is reduced with MOR-1 KO mice showing reduced consumption and place preference [212]. Finally, food, a behavioral-induced reward is also suppressed in MOR-1 KO mice [213]. In agreement with the receptor studies, a recent study on nicotinic reward in END KO mice showed decreased reward [214]. Taken together these results suggest that a μ dependent opioid pathway plays a significant role in the formation of reward though it is not known which of the two above mechanisms mutation of the μ opioid system alters.

KOR-1 KO mice also can affect the hedonistic values of drugs and behaviors. For KOR-1 KO mice, the most well-studied reward paradigm is the hedonistic value of cocaine. Deletion of the *KOR-1* gene results in two major effects on the reward circuitry. Substantial evidence demonstrates a role for κ opioid receptor regulation of the dopaminergic circuitry. Mutation of the *KOR-1* gene results in loss of an inhibitory input on dopaminergic neurons resulting in their hyperactivity [215, 216]. Alternatively, many drugs of abuse have both positive and aversive effects. The κ opioid receptor plays a significant role in the aversive properties of drugs such as THC and alcohol, which is lost following mutation of the *KOR-1* gene [211, 217]. Thus, in *KOR-1* KO mice, the hedonistic value of many drugs is enhanced via dual mechanisms. Loss of κ opioid inhibition of dopamine neurons results in direct enhancement of the hedonistic value of the reward. Likewise, mutation of the *KOR-1* gene also enhances reward by eliminating the aversive properties of the rewarding stimulus.

Surprisingly, although a significant number of studies have demonstrated a role for the δ opioid receptor in reward no significant study has been published showing a change in the hedonistic value of various rewards in mice lacking the *DOR-1* gene. The *DOR-1* gene appears to play little to no role in reward formation [218]. Examinations of the opioid ligand knockout mice have also been sparse, but initial

findings suggest a similar pattern to that observed in the receptor knockouts. Specifically, similar to KOR-1 KO mice, DYN KO mice show reduced reward to alcohol [219], while ENK and END KO mice demonstrate reductions in the hedonic value of food similar to MOR-1 KO mice [124]. Thus, knockout studies have confirmed a role for κ and μ opioid pathways in reward though, their exact mechanism has not been totally clarified.

13.5 Perspectives

13.5.1 *Opioid Receptor Dimerization*

Over the past several years substantial evidence has documented that G protein-coupled receptors form homo- and heterodimers in cell lines (dimerization of GABA(A) receptors, 5HT receptors, adrenergic receptors, opioid receptors as well as others have all been demonstrated) [152, 220–222]. These studies in cell lines suggest that μ , δ , and κ opioid receptors can form both homodimers [170, 223] as well as heterodimers with other opioid receptors and G protein-coupled receptors from other families [222, 224–227].

Initial identification of opioid receptor dimers in vitro has led to study of their existence in vivo. Specifically, evidence in part derived from opioid receptor KO mice suggests that spinal κ/δ and μ/δ heterodimers may exist in vivo and are involved in nociception [170, 171, 228, 229]. Similarly, in the caudate, $\mu/CB1$ heterodimers have been suggested to exist and possibly influence cell proliferation [230]. The gold standard for in vitro confirmation of G protein-coupled receptor dimerization is co-immunoprecipitation of both receptors within the dimer. In vivo such confirmation is difficult due to technical limitations such as low receptor density, poor antibody specificity and a lack of tagged receptors. However, co-immunoprecipitation of two or more receptors provides minimal information regarding our understanding of in vivo receptor pharmacodynamics if dimerization/oligomerization does not alter receptor–ligand affinity/action. Thus, another critical factor regarding in vivo dimers is not whether they exist, but rather do the pharmacological changes associated with in vitro dimers occur in vivo.

In cell lines, differences in ligand-receptor affinity/action are observed by stimulating cells expressing either one receptor alone or coexpressing multiple receptors. For example, the prototypical κ ligand U69,593 has substantially greater affinity and density in cells expressing KOR-1 alone compared to cells expressing both the *KOR-1* and *DOR-1* genes, even though the level of *KOR-1* gene product is similar in both cell lines [222]. In vivo, limiting expression to only one gene in a tissue is not possible. Rather, in knockout mice one or more genes suggested to be dimer partners are removed. Thus, effects potentially mediated by dimers can be assessed. For example, similar to vitro studies, U69,593 binding density and nociceptive potency is enhanced in the spinal cord from mice containing a mutant *DOR-1* gene,

creating an *in vivo* condition similar to cells expressing KOR-1 alone [231]. Thus, through the use of knockout mice, researchers are able to confirm the pharmacological consequences demonstrated for dimers *in vitro* [222]. Furthermore, drug potency studies in knockout mice may provide clues towards which physiological functions are regulated by dimers.

For example, although not yet confirmed by testing in knockout mice, it was hypothesized that since pharmacological changes associated with μ/δ opioid receptor heterodimers exist in the spinal cord, co-administration of μ and δ agonists should produce synergistic rather than additive effects on nociception which was substantiated [228]. In addition, *in vivo* studies using KO mice are starting to provide clarification for the pharmacological identification of multiple subtypes. Studies show loss of specific opioid subtypes correlate directly with loss of the potential for specific dimer types [25, 171, 231]. Specifically, spinal $\kappa 2$ opioid receptors require both the *DOR-1* and *KOR-1* genes [231]. While the $\delta 1$ opioid subtype seems to require either the *DOR-1* and *KOR-1* genes and/or the *DOR-1* and *MOR-1* genes [25, 171].

Knockout mice provide an important initial control for *in vivo* dimer studies. If a pharmacological/behavioral effect is mediated by a specific dimer, it cannot exist in mice containing a mutated version of one of the two receptors forming the dimer. Second, knockout mice can provide a hint of which physiological behaviors may be regulated by dimers as well as which receptors may dimerize. For example, if δ and κ opioid receptors dimerize, what happens to the κ portion of the dimer in a *DOR-1* KO mouse? One possibility is that the remaining κ portion may remain as functional receptor either as a monomer, homodimer, or heterodimer with an alternate G protein-coupled receptor resulting in an increase in U69,593 binding density and increases *in vivo* potency. Such responses were observed [171]. Anatomic observations consistent with this type of regulation has also been observed in all opioid receptor knockout mice. Thus, autoradiographic studies of the *MOR-1*, *DOR-1*, and *KOR-1* KO mice consistently show a slight increase in binding density to opioid receptors whose genes have not been mutated [158, 165, 232]. This slight increase could be due to decreases in opioid receptor heterodimer expression resulting in enhanced binding affinity for the subtype specific radiolabeled ligands used.

Finally, knockout mice provide an excellent testing mechanism for putative heterodimer specific compounds. If heterodimers exist *in vivo* then it is plausible that specific physiological responses may be primarily under the control of a subset of heterodimers. If a clinically relevant drug can be designed to recognize only the heterodimer, then this specific physiological function may be targeted with limited side effects. Knockout mice provide the testing ground for these compounds. As stated earlier, if a compound is heterodimer specific, then that compound cannot function in mice lacking either one of the two receptors believed to be involved in the dimer pair.

Although knockouts are a useful tool for examining dimers *in vivo*, there are several caveats on interpretation of the results generated. First, similar to any knockout, a change in binding affinity/density or ligand efficacy in a knockout only means that the change is dependent on the mutated gene but not causal. For

example, the increase in U69,593 binding density and potency observed by Ansonoff et al. in DOR-1 KO mice is dependent on mutation of the *DOR-1* gene, but the cause for the change is unknown. All the results may be consistent with the hypothesis of δ/κ heterodimers, but there exists no direct evidence that the heterodimers existed in wild-type mice and that the κ portion formed alternative receptors in the DOR-1 KO mice. Alternative explanations are possible. For example, binding density could increase to compensate for loss of functioning δ opioid receptors. Additionally, the behavioral changes could be due to decreased basal inhibition of the neural circuitry upstream or enhanced responses downstream from the U69,593 sensitive site. However, one critique of heterodimer theory is less likely *in vivo*. It is unlikely that changes in receptor coupling due to increases in free G proteins following mutation of the *DOR-1* gene occurs. *In vivo* there exist a whole host of G protein-coupled receptors available to modulate G protein levels making it unlikely that loss of one G protein-coupled receptor gene could dramatically alter the availability of G proteins.

Development of new transgenic mice such as temporal and region specific conditional opioid receptor knockout mice as well as recent production of knockin eGFP labeled opioid receptor mice will alleviate some of these concerns [29]. At present, studies of opioid heterodimers in knockout mice provide evidence consistent with the existence of dimers, and coupling this information with *in vitro* studies showing that heterodimers can exist and have pharmacological consequences has at least provided substantial correlative data suggestive of *in vivo* heterodimers. In conclusion these studies have identified regions that possess pharmacological properties consistent with opioid heterodimer formation and will be used to evaluate the *in vivo* efficacy of heterodimer specific drugs as they become available.

Ultimately, an anatomic component will be essential. In that respect, studies using the recent the DOR-1 knock-in mice containing eGFP labeled δ opioid receptors [29] in conjunction with knock-in mice containing either labeled μ and/or κ opioid receptors should provide appropriate resolution to resolve the issue of *in vivo* opioid receptor heterodimers. In addition, knock-in mice containing labeled receptors will be useful in enhancing our understanding of receptor distribution, localization overlap, and trafficking. Finally, by backcrossing these knock-in mice to different background strains, a definitive understanding of strain sensitivity to various opioid ligands may be forthcoming.

13.5.2 Compensatory Effects

Study of opioid KO mice has identified several common compensatory effects following gene KO [14, 15, 233]. KO mice often show up/down regulation of other receptors/ligands to compensate for loss of the targeted gene. Specifically, this type of compensation can be divided into changes in expression of genes directly involved in signaling by the targeted gene and changes in signaling pathways indirectly related to the KO gene. For example, mutation of the *ppENK* gene

up-regulates μ and δ opioid receptors throughout the nervous system [234]. The concept is that by eliminating the ligand for these receptors, the organism up-regulates receptors in an attempt to enhance signaling. Although not absolute, the up-regulation of the receptor for the mutated endogenous ligand commonly occurs. Therefore, if a given receptor is promiscuous, the biological effect of other ligands will be artificially magnified. Surprisingly, the converse, up-regulation of the ligand following mutation of the receptor seldom occurs [17, 28].

Indirect compensatory effects are further subdivided into up-regulation of alternative receptors/ligands to replace the mutated gene or alteration in downstream systems normally regulated by the mutated receptor system. For example, in all the autoradiographic binding studies performed on the single opioid receptor KO mice, a small, but significant up-regulation of alternative opioid receptors is observed in many brain regions [158, 165, 232]. This up-regulation of alternative opioid receptors was attributed to heterodimer loss, but an alternate explanation may be that elevation of these receptors is compensation for gene loss. Similarly, in dopamine receptor KO mice, the dopaminergic mediated reward pathway is reduced [235, 236], but not eliminated, suggesting that alternative receptor signaling pathways must compensate for the loss of the dopaminergic system.

Conversely, receptor systems downstream of the mutated receptor may be regulated to compensate for the mutation. Specifically, dopaminergic signaling in the nucleus accumbens is regulated by the κ opioid receptor system [215, 237], dopamine release in the nucleus accumbens, but there is a corresponding compensatory increase in dopamine uptake so that there is no net change in external dopamine levels. Interestingly, acute treatment with κ opioid antagonists increases dopamine release, but does not increase dopamine uptake, resulting in a net increase in external dopamine in the nucleus accumbens and corresponding behavioral effects [215].

Compensatory effects of gene knockout are a constant source of concern when evaluating results obtained in KO mice and, in that regard, gene array expression profiling and proteomic profiling should soon provide more definitive information regarding the exact modifications in mRNA and protein expression patterns that occur following gene ablation as well as help identify when “steady state” has been re-established following conditional deletion. These types of information will be invaluable as interactions between the opioid system and other neuromodulatory circuits become identified and better understood.

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Chapter 14

Opioid Receptor Trafficking

Mark von Zastrow

Abstract The functioning of opioid receptors involves a wide range of processes. Their association with G-proteins is clearly one of the most important. However, receptor trafficking also plays a crucial role in their activation, inactivation and desensitization. This review will attempt to summarize the receptors from their synthesis to their eventual proteolytic elimination.

Keywords Opioid receptor • Biosynthesis • Endocytosis • Sorting • Desensitization • Down-regulation

14.1 Introduction

Membrane trafficking mechanisms play a fundamental role in controlling the number and subcellular distribution of opioid receptors in target neurons. The membrane trafficking of opioid receptors is remarkably specific and highly regulated, and there has been considerable progress in the past several years toward elucidating opioid receptor trafficking events at the mechanistic level. This chapter will attempt to summarize this progress by following opioid receptors through their entire cellular lifetime, from “birth” by receptor biosynthesis to “death” by proteolysis. Functional consequences of opioid receptor trafficking will then be discussed, focusing on current insights to this question generated from studies of opioid receptor trafficking in the endocytic pathway. We will then speculate on the potential significance of opioid receptor trafficking mechanisms to human physiology and disease, and highlight areas of potential interest for future translational investigation.

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14.2 Trafficking of Opioid Receptors in the Biosynthetic Pathway

Opioid receptors are typical, albeit complex, polytopic membrane proteins that traverse a highly conserved biosynthetic pathway (depicted schematically in left side of Fig. 14.1). Receptor trafficking in the biosynthetic pathway is tightly orchestrated, and can be regulated at specific stages by physiological stimuli as well as exogenous agents that influence cellular responsiveness to opioids.

14.2.1 Synthesis, Folding, and Conformational Quality Control in the Endoplasmic Reticulum

Opioid receptors are born in the limiting membrane of the endoplasmic reticulum (ER). Spliced, polyadenylated messenger RNA (mRNA) encoding opioid receptors

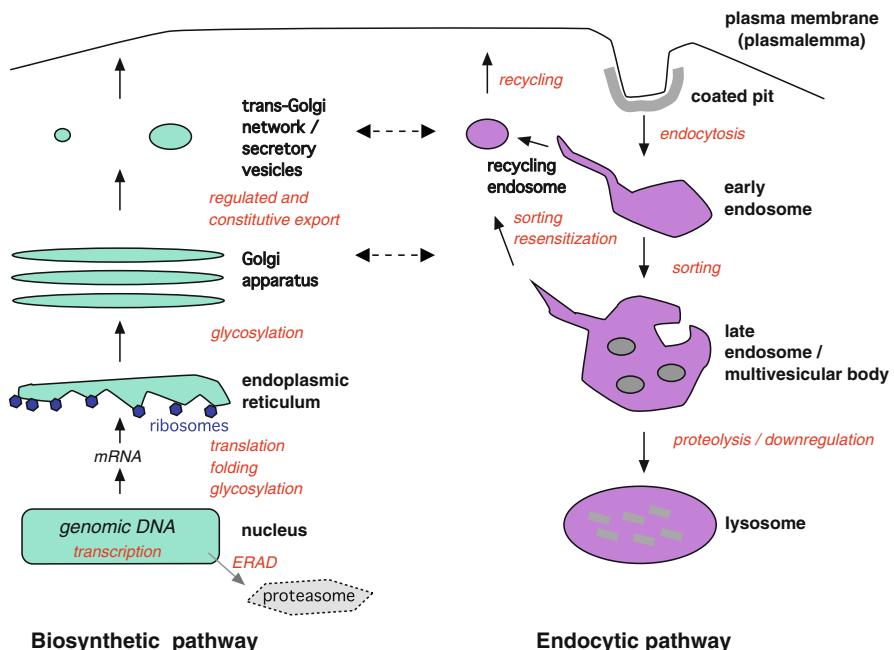


Fig. 14.1 Schematic representation of the biosynthetic and endocytic pathways relevant to GPCR trafficking. *Solid arrows* indicate predominant routes of membrane flow between individual compartments indicated in *solid black* type. *Dotted arrows* indicate vesicular connections that bridge the biosynthetic and endocytic pathways, and contribute significantly to receptor regulation in some cell types. Major processes occurring at each stage of receptor trafficking are indicated in *red italic* type

are exported from the nucleus and translated by ribosomes that associate with the translocon complex, which is embedded in the ER membrane [1]. During the process of receptor mRNA translation, the emerging polypeptide is stitched across the ER membrane to form the seven-transmembrane topology characteristic of G protein-coupled receptors. The nascent polypeptide is co-translationally glycosylated on asparagine residues located in the amino-terminal luminal domain (destined to become the extracellular domain) of the receptor protein.

A variety of ER-associated chaperone proteins assist proper folding of the nascent polypeptide into a functional heptahelical bundle structure, and do so by catalyzing specific folding reactions and/or stabilizing particular folding intermediates. In addition, a conserved disulfide bond is formed on the luminal surface of the receptor protein, driven by the relatively oxidizing conditions of the ER lumen and catalyzed by protein disulfide isomerase enzymes located in this compartment. Properly folded opioid receptors are subsequently exported from the ER by coat-assisted budding and vesicular transport to the Golgi apparatus.

The folding pathway outlined briefly above represents a complex and hazardous childhood, rife with opportunities for aberrant conformational states that represent dead ends with regard to eventually forming a functional “adult” opioid receptor. The ER possesses a highly efficient quality control mechanism that effectively prevents misfolded proteins, including opioid receptors, from proceeding further in the biosynthetic pathway [2]. This mechanism involves recognition of the aberrant receptor polypeptide by protein interactions with various resident ER proteins, the details of which remain poorly understood [3], followed by chemical tagging of receptors by addition of ubiquitin.

Ubiquitin is a 76-amino acid polypeptide that is added en bloc to lysine residues exposed on the cytoplasmic surface of the ER (by specific ligase enzymes that form an isopeptide linkage with the e-amino group of lysine). Ubiquitin attached to opioid receptors in the ER is typically extended into long poly-ubiquitin chains. Receptors are then extruded from the ER and delivered to a multiprotein complex called the proteasome, which contains a number of ubiquitin-binding proteins together with multiple proteases, forming a highly efficient machine for mediating targeted degradation of ubiquitinated receptors. In cultured cells, it is estimated that only ~50% of receptor polypeptides initially synthesized are properly folded; the remainder undergo rapid destruction by the ER-associated degradation (ERAD) mechanism described above [4].

14.2.2 Role of the Endoplasmic Reticulum in Generating the Quaternary Structure of Opioid Receptors

Many membrane proteins that exist in oligomeric complexes undergo multi-subunit assembly prior to ER export. This has been clearly documented for certain GPCRs, such as the GABA_B receptor, which functions as an obligate heterodimer between GB1 and GB2 polypeptides. GB1/GB2 complexes are formed in the ER, and an

elegant mechanism has been identified that assures proper assembly prior to ER export. A cytoplasmic sequence in the GB1 subunit retains unassembled monomers in the ER; assembly with the GB2 subunit masks this sequence, thereby facilitating selective export of the properly assembled heterodimer [5].

It is unclear whether such precise regulation of quaternary structure pertains to opioid receptors. Opioid receptors have been known for many years to self-associate in vitro [6], and oligomerization of opioid receptors can significantly affect receptor function in vivo [7]. Hetero-oligomeric complexes of opioid receptors can indeed assemble in the ER, but in contrast to results obtained previously in studies of GABA_B receptors, recent studies suggest that hetero-oligomer assembly of opioid receptors is not required for ER export [8].

14.2.3 Chemical and Pharmacological Chaperones

A number of exogenous agents have been shown to influence folding efficiency in the ER, thereby affecting the net rate at which functional receptors are produced. Some compounds (such as the small amphipathic molecule DMSO) enhance the efficiency with which various polytopic proteins are folded, with little apparent specificity, and are thought to do so by altering the overall physicochemical environment of the ER. Such compounds are typically called chemical chaperones [9]. Other compounds enhance the folding of opioid receptors specifically, and are thought to do so by binding to intermediates in the folding pathway resembling the native receptor structure, thereby increasing the proportion of nascent polypeptides that ultimately achieve a properly folded state. These compounds include membrane-permeant ligands that bind well to native opioid receptors, such as various non-peptide agonists and antagonists, and are typically called *pharmacological chaperones* [10]. Both classes of compound are of potential translational interest because they have been shown to affect the net number of functional opioid receptors present in intact cells.

14.2.4 Opioid Receptor Trafficking and Modification Through the Golgi Apparatus

In accord with the general paradigm of anterograde membrane traffic [11], opioid receptors exported successfully from the ER traverse cis, medial, and trans elements of the Golgi in which a series of further post-translational modifications occur. Of particular experimental interest, the asparagine linked glycans added to receptors in the ER are trimmed, and additional modifications (largely *N*-acetyl glucosamine and sialic acid moieties) are added by specific glycosyl transferases. These modifications can be assayed biochemically using established lectins and glycosidases that specifically recognize particular glycan structures, providing

useful tools for following the vectorial movement of newly synthesized opioid receptors from the ER (where glycans are exclusively of the *immature* or *high mannose* type) and through the Golgi apparatus (where glycans are trimmed and modified to their *mature* forms) [3].

The duration of the receptor's *adolescence* in the Golgi can vary considerably. In non-neuronal cell culture models, opioid receptors are thought to traverse the Golgi apparatus rapidly, within a few minutes, with subsequent delivery of fully processed receptors to the plasma membrane occurring in an apparently nonspecific manner via Golgi-derived transport vesicles [10]. There is increasing evidence for additional selectivity and regulation of post-ER opioid receptor traffic in neurons, however, which results in specific receptors being retained for a relatively prolonged time period in Golgi or post-Golgi compartment(s).

A significant fraction of endogenous delta opioid receptor immunoreactivity was localized to post-ER membrane compartments in medium spiny neurons of the nucleus accumbens, including smooth membranes thought to represent Golgi-associated vesicles and dense core secretory vesicles. In the same neurons, however, endogenous mu opioid receptor immunoreactivity was localized primarily in the plasma membrane [12]. Selective intracellular localization of delta opioid receptor immunoreactivity was also observed in spinal neurons [13]. In these cells, there is also evidence that the subsequent vesicular transport of delta opioid receptors to the plasma membrane is regulated by pharmacological or physiological stimuli, thereby allowing cells to rapidly modify their sensitivity to opioids *in vivo* [14].

14.2.5 Molecular Sorting and Regulation Opioid Receptor Traffic in the Late Biosynthetic Pathway

Cloned mu and delta opioid receptors were found to differ accordingly when their localization was examined in a transformed neurosecretory cell line (PC12 cells), providing a useful model for mechanistic studies [15]. Biochemical analysis of receptor glycan modification clearly established the existence of a substantial intracellular pool of mature (i.e., post-ER) delta opioid receptors, while mu opioid receptors were present predominantly in the plasma membrane.

Interestingly, the formation of this intracellular pool of delta opioid receptors was enhanced in PC12 cells after differentiation toward the neurosecretory phenotype, and could be enhanced acutely by activation of receptor tyrosine kinase signaling, suggesting that sorting of delta opioid receptors into the intracellular pool is a cell type-specific phenomenon that can be regulated by physiological stimuli. Further, chemical depolarization of cells (by increasing extracellular potassium concentration) resulted in rapid increase in delta opioid receptor number in the plasma membrane, suggesting that the intracellular pool of receptors can be rapidly mobilized to the plasma membrane in an activity-dependent manner.

A portion of the carboxyl-terminal tail of the delta opioid receptor was identified as a sequence sufficient to direct this intracellular sorting when attached to a distinct membrane protein, beginning to identify a specific sorting mechanism controlling opioid receptor export from the Golgi. The membrane compartment mediating intracellular sequestration of delta opioid receptors in these experiments colocalized with markers of the trans-Golgi network (TGN), but was largely distinct from dense core vesicles [15]. A subsequent study reported localization of delta opioid receptors specifically in dense core vesicles of PC12 cells, and proposed that receptor sorting to these vesicles is mediated by a luminal (rather than cytoplasmic) sequence in the receptor protein [16]. Thus, opioid receptor trafficking in the late biosynthetic pathway can be specifically regulated by at least one additional mechanism.

14.3 Trafficking of Opioid Receptors in the Endocytic Pathway

The importance of membrane trafficking does not end upon delivery of adult (i.e., fully processed) opioid receptors to the plasma membrane from the biosynthetic pathway. Indeed, opioid receptors present in the plasma membrane are subject to additional regulated trafficking events occurring in the endocytic pathway, which can mediate diverse effects on receptor fate and cell physiology (right side of Fig. 14.1). Molecular sorting events that determine the specificity of opioid receptor trafficking in the biosynthetic pathway are emerging as critical determinants of whether endocytosis leads to imminent *death* of receptors by proteolysis or supports a more productive and extended lifetime, in which the same receptor can mediate multiple rounds signal transduction over a prolonged time period.

14.3.1 Rapid Regulation of Opioid Receptors by Phosphorylation and Endocytosis

Pioneering studies of rhodopsin (a light-activated GPCR), and several ligand-activated GPCRs (such as the beta-adrenergic receptor or B2AR), elucidated a conserved mechanism regulating the functional activity of many GPCRs in mammals [17]. Activated receptors are selectively phosphorylated by a specific family of G protein-coupled receptor kinases (GRKs), and then associate with cytoplasmic accessory proteins called arrestins. These events effectively prevent receptor interaction with heterotrimeric G proteins, disrupting the pathway of GPCR-mediated signal transduction at the earliest stage. Opioid receptors are also regulated by this mechanism. GRK/arrestin-mediated desensitization of opioid receptor function has been examined in some detail in a variety of cell systems, including cultured mammalian cells and *Xenopus* oocytes, and in vivo experiments using gene knockout methods suggest that this mechanism affects opiate responses in the intact mouse brain [18–23].

Pharmacological studies of opioid ligand binding to intact NG108-15 cells motivated the hypothesis that opioid receptors, like the B2AR, are removed from the plasma membrane by an endocytic process after agonist-induced activation [24, 25]. Biochemical and immunochemical methods applied to cloned opioid receptors clearly verified that this is the case, and established that opioid receptors undergo agonist-induced endocytosis via clathrin-coated pits [26, 27]. There is also evidence for alternate (i.e., clathrin-independent) endocytic mechanisms contributing to regulated endocytosis of some GPCRs, including opioid receptors in some systems [28]. Nevertheless, in several neural and non-neuronal cell types examined so far in greatest detail, the predominant mechanism mediating rapid endocytosis of opioid receptors appears to be clathrin-coated pits.

Clathrin-coated pits represent a major endocytic route for many cell surface components besides opioid receptors, including membrane proteins (such as LDL and transferrin receptors) that are endocytosed and recycled continuously to extract essential nutrients from the extracellular milieu [29]. This has raised the question of how the endocytosis of opioid receptors is regulated. It turns out that GRKs and arrestins, in addition to their previously established role in mediating functional uncoupling of receptors from heterotrimeric G proteins, also promote rapid endocytosis of receptors via clathrin-coated pits. Two arrestins that are widely expressed outside the visual system, arrestins 2 and 3 (also called β -arrestins-1 and -2) can bind simultaneously to activated, phosphorylated GPCRs and to the clathrin-containing lattice structure, thereby functioning as regulated endocytic *adapters* [30–32]. The mechanism controlling opioid receptor endocytosis is thus similar to the classical mechanism of rapid desensitization mediated by GRKs and arrestins [33, 34].

14.3.2 Type-Specific Differences in Opioid Receptor Endocytosis

Studies of cloned mu, delta, and kappa opioid receptors revealed quantitative differences in the ability of these closely homologous receptors to undergo regulated endocytosis following agonist-induced activation [27]. Such *type-specific* differences in regulated endocytosis of opioid receptors can result from differences in the ability of receptors to undergo GRK-mediated phosphorylation and subsequently interact with arrestins [35]. Regulated endocytosis of opioid receptors can also be affected by alternative splicing events that modify the structure of the receptor's carboxyl-terminal cytoplasmic domain [36]. This was shown first for two splice variants of the mu opioid receptor, MOR1 and MOR1B, and has since been extended to other splice variants [37]. Oligomer formation can significantly affect the ability of opioid receptors to undergo regulated endocytosis, exerting control over endocytosis of receptors beyond that explained by direct GRK or arrestin interactions. Oligomerization with a strongly internalized GPCR can promote regulated endocytosis of opioid receptors; conversely, oligomerization with a non-internalizing GPCR can inhibit opioid receptor endocytosis [38–40].

14.3.3 Agonist-Specific Regulation of Opioid Receptor Endocytosis

Beyond different endocytic properties characteristic of distinct opioid receptor types or receptor-associated oligomeric complexes, a number of opioid agonists have been found to vary considerably in their ability to promote regulated endocytosis of the same opioid receptors. This was noted initially in comparing the regulatory effects of morphine and opioid peptides in cultured cells expressing cloned mu opioid receptors [26], and has been extended to a large number of agonists and various cell types [41, 42].

To a first approximation, quantitative differences in the endocytic effects of distinct ligands correlate with differences in intrinsic agonist efficacy, with more efficacious agonists possessing greater ability to promote rapid endocytosis of receptors [18]. This correlation makes sense in relation to the standard paradigm, as efficacious agonists are thought to be more capable of initiating the endocytic process by stimulating GRK-mediated phosphorylation of receptors. Further, over-production of either GRK or arrestin proteins can enhance the endocytic activity of an agonist such as morphine, even in cells in which morphine promotes endocytosis of receptors very poorly. Differences in the endocytic effects of distinct opioids may not be fully explained by the traditional concept of agonist efficacy, however.

An elegant early study, which correlated relative agonist efficacy with agonist-induced phosphorylation of cloned mu opioid receptors found a limited correlation for certain agonists, such as morphine [43]. Studies of this kind have been extended by several groups to direct measures of opioid receptor endocytosis, providing strong support for differences in the regulatory effects of certain opioid agonists that do not correlate strictly with differences in agonist efficacy assessed by classical estimates of receptor-G protein coupling, and can be dissociated by receptor mutation [21, 42, 44–46]. Such observations are probably not unique to opioid receptors. Pronounced differences between efficacy and endocytic activity have been noted in studies of the CB1 cannabinoid receptor, for example [43].

The biophysical basis for the apparent divergence between the endocytic potential of an agonist and its relative agonist efficacy remains unclear. One hypothesis is that there may exist multiple receptor conformations that differ in relative signaling and endocytic potential, and are differentially stabilized by distinct agonists [47]. In essence, this hypothesis proposes that agonist efficacy represents a multidimensional parameter that is only partially defined by classical estimates based on G protein-mediated signaling. Accordingly, agonists are proposed to differ in their ability to induce or stabilize a multiple conformational states (or mixture of conformers), thereby produce measurably distinct functional effects (i.e., G protein activation and receptor endocytosis) that are not directly linked to one another [48]. The concept of multidimensional agonist efficacy is gaining momentum, and potentially broad influence, based on increasing recognition of additional functional differences between agonists of a number of GPCRs [49], and based on the convincing biophysical demonstration that there exist agonist-selective conformational transitions of the B2AR [48, 50].

14.3.4 Molecular Sorting of Endocytosed Opioid Receptors to Lysosomes

In several cell culture systems, delta opioid receptors traffic efficiently to lysosomes after regulated endocytosis by clathrin-coated pits, the same endocytic mechanism that delivers mu opioid receptors to a rapid recycling pathway. Thus, it is thought that the functional consequences of opioid receptor endocytosis are determined in large part by molecular *sorting* operations, occurring after endocytosis, which direct receptors selectively among divergent downstream membrane pathways [51].

Recent studies have described a highly conserved endosome-associated mechanism that is thought to function in sorting many integral membrane proteins, including GPCRs, to lysosomes and is directed by ubiquitin-mediated tagging of receptors [52]. This function of ubiquitination in endocytic sorting to lysosomes is thought to be entirely distinct from the previously defined role of ubiquitination in directing receptors to the proteasome from the biosynthetic pathway. Opioid receptors utilize a number of the same endosome-associated sorting proteins as signaling receptors (such as the EGF receptor tyrosine kinase) that clearly require ubiquitination for lysosomal sorting. The endosome-associated scaffold protein Hrs, in particular, directly binds ubiquitinated membrane proteins and initiates their endocytic sorting to lysosomes, and is also important for sorting opioid receptors to lysosomes [53, 54].

Further, the Vps4 ATPase, another essential component of the ubiquitin-directed sorting mechanism, is required for efficient trafficking of endocytosed opioid receptors to lysosomes [54, 55]. A surprising observation, the significance of which remains poorly understood, is that ubiquitination of opioid receptors is actually not essential for their endocytic sorting to lysosomes, at least when examined in two non-neuronal cell models (HEK293 and HeLa cells) [54, 56].

Additionally, a family of cellular proteins has been identified that are abundantly expressed in the nervous system bind to opioid receptors in the absence of ubiquitination, and at least one of these proteins specifically influences endocytic sorting of receptors to lysosomes [57, 58]. Thus it is likely that the lysosomal sorting of opioid receptors involves core machinery also utilized in sorting ubiquitinated proteins, as well as additional components that are receptor-specific and may not require ubiquitination for their function.

14.3.5 Sorting of Opioid Receptors in the Recycling Pathway

A recent development in our understanding of opioid receptor endocytic sorting is the emergence of an endocytic sorting mechanism that is based on sequence-directed recycling of receptors, rather than on ubiquitin-directed sorting to lysosomes [59]. Detailed discussion of this machinery is beyond the scope of the

present chapter, and the reader is directed to a recent review for further information regarding this rapidly advancing area [60]. Nevertheless it is worth noting a few salient features.

First, the emerging sorting code promotes receptor entry to the recycling rather than degradative pathway, thereby representing a fundamentally different principle of sorting relative to previously established mechanisms (which function by directing receptors specifically to lysosomes). Second, at present there is no precedent for the occurrence of this novel sorting mechanism functioning outside of metazoa (as opposed to ubiquitin-directed sorting to lysosomes that was initially recognized in yeast and is deeply conserved). Third, the emerging opioid sorting code appears to involve multiple sorting sequences and is essential for proper endocytic regulation of a number of mammalian GPCRs that are of great clinical significance, including the mu opioid receptor but also including various catecholaminergic GPCRs [60].

14.4 Functional Consequences of Endocytic Receptor Trafficking

The functional significance of opioid receptor trafficking in the endocytic pathway is an area of intense investigation. While much of what is known about the diverse functional consequences of opioid receptor trafficking come from studies of cultured cell models, exciting advances have been made recently toward addressing these questions in near-native tissue preparations and *in vivo* using transgenic mouse models.

14.4.1 Relationship Between Rapid Desensitization and Endocytosis of Opioid Receptors

According to the classical paradigm of rapid desensitization, as discussed above, the GRK/arrestin mechanism prevents receptor association with heterotrimeric G proteins before promoting receptor association with coated pits. Thus it is generally believed that endocytosis of GPCRs is not required for functional desensitization of signaling. Early studies of this question, carried out on cloned mu opioid receptors expressed in a non-neuronal cell line (CHO cells), were surprising because they found that opioid signaling was highly dependent on surface receptor density. These results suggested the existence of low receptor reserve and, in this cell model, that endocytosis of receptors mediated functional desensitization directly [61]. Studies of another non-neuronal cell model (HEK293 cells) found that opioid receptor desensitization did not require endocytosis of receptors, and endocytosis of receptors was associated in some settings with reduced cellular desensitization [62–64].

Functional desensitization of mu opioid receptor-mediated signaling measured in native neurons, using acutely prepared tissue slices from the locus coeruleus, found that rapid desensitization occurred under experimental conditions in which endocytosis was inhibited [65]. Thus it is now reasonably clear that rapid desensitization of opioid signaling can occur in the absence of receptor endocytosis, but it is possible that endocytosis further reduces opioid responsiveness in some cells. It is also increasingly clear that additional mechanisms, which do not require either GRK/arrestin proteins or the endocytic apparatus, can contribute to functional desensitization *in vivo*. In particular, protein kinase C and MAP kinases are thought to play an important role in mediating functional desensitization of opioid signaling under various conditions [63, 66].

14.4.2 Role in Promoting Functional Resensitization of Opioid Receptors

The finding in some studies that desensitization of mu receptor-mediated signaling correlated inversely with receptor endocytosis suggested that endocytic trafficking of opioid receptors, as proposed previously based on early studies of the B2AR [67, 68], may contribute to the opposing process of receptor resensitization [2, 42, 43]. In essence, this hypothesis proposes that trafficking of receptors through a rapid recycling pathway facilitates dephosphorylation and subsequent return of opioid receptors to the cell surface in a fully dephosphorylated (and, presumably, fully functional) state.

There is considerable support for this hypothesis based on a number of studies of non-neuronal cell models [69]. There is also evidence suggesting that alkaloid drugs such as morphine, which activate opioid receptors but promote their endocytosis relatively inefficiently in HEK293 cells, can produce a phosphorylated state of the receptor in these cells that is persistently desensitized because it is unable to undergo efficient dephosphorylation in the endocytic pathway [70]. Moreover, as discussed further below, there is increasing evidence that regulated endocytosis of opioid receptors occurs in native opioid-responsive neurons, and exerts important effects on opioid responsiveness *in vivo*.

14.4.3 Role in Promoting Proteolytic Downregulation of Opioid Receptors

Perhaps the most clearly established function of opioid receptor endocytosis is in promoting the process of receptor down-regulation. Early evidence for this hypothesis came from elegant studies of ligand-induced down-regulation of endogenously expressed delta opioid receptors in NG108-15 cells, where proteolytic down-regulation of receptors was associated with the appearance of endocytosed receptor-ligand

complexes in an isolated membrane fraction enriched in lysosomes [71]. This hypothesis has been clearly verified using other biochemical and immunochemical approaches applied to various cultured cell models, although the degree to which this pathway functions physiologically remains an important unanswered question. Further, additional proteolytic mechanism(s) have not been ruled out and may function under some conditions [72].

14.4.4 Functional Consequences of Opioid Receptor Trafficking In Vivo

When compared to evidence gathered from studies of cultured cell models and tissue explants, less is known about functional effects of opioid receptor trafficking *in vivo*. Two fundamental issues have been essentially resolved, however. First, it is now clear that regulated endocytic trafficking of opioid receptors does indeed occur in the intact nervous system. Regulated endocytosis of opioid receptors has been convincingly demonstrated by several groups, and in several neuronal populations, in response to systemic administration of clinically relevant opioid drugs [73]. Most studies of this question have focused on mu opioid receptors, but an elegant recent study showed regulated endocytosis of a tagged delta opioid receptor throughout the brain of a transgenic mouse model following administration of a behaviorally relevant dose of the delta agonist SNC80 [74].

There is also convincing evidence that opioid receptor trafficking is regulated in response to physiological stimuli, at least under some conditions, such as during specific mating activities examined in freely behaving rats [75]. Second, it is increasingly clear that opioid receptor regulatory mechanisms do indeed affect physiological responsiveness of the endogenous opioid system *in vivo*. Gene knockout studies support an important role of GRK3 and arrestin 3 (or β -arrestin-2) in affecting baseline opioid sensitivity and measures of physiological tolerance [19, 76].

The *in vivo* effects of GRKs and arrestins appear to be complex, and there is evidence for considerable genetic redundancy in this system, so definitive conclusions are difficult to reach based on single gene knockouts [23]. Recently, a knock-in mutant mouse model was described that expresses an engineered mu opioid receptor with enhanced morphine-induced endocytosis [77]. Detailed behavioral studies of this mouse model suggest that endocytosis of mu opioid receptors has multiple physiological effects. Mutant mice exhibited increased baseline opioid sensitivity when initially administered morphine and, following chronic drug administration, there was attenuated development of antinociceptive tolerance as well as physical dependence. It is clear that much remains to be learned regarding specific link(s) between opioid receptor trafficking mechanisms and physiological regulatory effects *in vivo*. Nevertheless, there would now seem to be little doubt that regulated trafficking of opioid receptors does indeed occur *in vivo*, and can produce significant functional effects at the level of whole-animal physiology and pharmacology.

14.5 Conclusion and Future Perspectives

Following the molecular cloning of opioid receptor cDNAs, and with various improvements in technology available for manipulating and analyzing receptor function in living cells and intact animals, we have enjoyed an era of rapid progress in our understanding of opioid cell biology. Major features of opioid receptor trafficking in both the biosynthetic and endocytic pathways have been elucidated, and mechanistic progress has been particularly rapid in understanding the process of agonist-induced endocytosis of receptors. Studies of cell culture model systems, although advantageous for mechanistic studies, represent only a starting point and are increasingly found to only partially mimic the properties of opioid receptor trafficking in physiologically relevant neurons.

A major emphasis of current research is to extend rigorous examination of biochemical mechanisms to cell and animal models that increasingly approximate the native setting of opioid regulation that is relevant to physiology and pathology. Another exciting area of current mechanistic research is toward elucidating the basis for agonist-selective control of opioid receptor signaling and regulated endocytosis. Recent advances in biophysical and structural methods applied to GPCRs, as exemplified by the recent elucidation of a high resolution crystal structure for a ligand-occupied conformation of the B2AR [78, 79], raise great hope for accelerating progress in this important but challenging area. Further, given the potential functional significance of agonist-selective receptor regulation suggested by our present experimental models, defining the biophysical basis for agonist-selective regulation could facilitate the development of improved opioid pharmacotherapies.

Yet another promising avenue of research is toward understanding mechanisms that control opioid receptor sorting following endocytosis. Less is known about this process, when compared to the initial events leading to endocytosis of receptors via clathrin-coated pits. Based on our current functional understanding, however, these sorting mechanisms are likely to have considerable physiological and biomedical significance. This area of research has benefited enormously from advances occurring in other spheres of membrane cell biology. Conversely, rigorous exploration of opioid receptor sorting in complex mammalian cells has identified additional components of the sorting machinery, which were not anticipated from other cell biological studies and likely critical to opioid receptor regulation in mammalian neurons. In particular, there has been significant progress toward elucidating a biochemical novel *code* that determines the endocytic sorting of opioid receptors without requiring ubiquitination of the receptor. The generality of these recent observations to other areas of membrane cell biology and molecular pharmacology remains to be determined.

Nevertheless, it seems very likely that such sorting machinery is profoundly important to the cellular regulation of the endogenous opioid system, and to determining the long-term effects of exogenously administered opioid drugs. If it is

possible to define specific sorting proteins that affect endocytic trafficking of particular opioid receptors, without affecting general trafficking processes, these proteins might have great value as potential drug targets. Considerable progress has been made in the field of opioid receptor trafficking, but clearly much remains to be learned. This field continues to offer exciting scientific challenges, and holds considerable future potential for translational medicine.

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Chapter 15

Opioid Receptor Dimerization

Raphael Rozenfeld, Ivone Gomes, and Lakshmi A. Devi

Abstract Opioid receptors are the primary clinical targets for the attenuation of pain. Many opiates used for pain management have a high abuse liability due to their psychoactive and rewarding properties. In recent years, accumulating evidence obtained through an array of techniques (from biochemical to pharmacological and biophysical) demonstrate that these receptors exist as homomers/oligomers, and more importantly, that they also form heteromers with closely or distantly related GPCRs. Heteromerization of opioid receptors has profound effects on their maturation, pharmacology, signaling, and trafficking. In addition, heteromer levels are regulated by pathophysiological conditions and by chronic drug treatment, underscoring their importance in the regulation of receptor properties.

In this chapter, we describe evidence for opioid receptor homo- and heteromerization, we document the types of opioid receptor-containing heteromers and summarize their most important features, and finally, we discuss the relevance of developing selective ligands targeting heteromers. The interaction of such molecules with a specialized pool of receptors, within a given heteromer, could enhance greater therapeutic activity without undesired effects. This is particularly relevant for the treatment of pain and other related disorders.

Keywords G protein coupled receptors • Homodimers • Heterodimers • Morphine • Opioid receptors

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15.1 Introduction

It is becoming increasingly accepted that G protein-coupled receptors (GPCRs) associate to form dimers and/or oligomers. Dimerization is important, if not necessary, for proper folding and maturation of the receptors [1]. In addition, dimerization (between two distinct receptor types to form heteromers/heterooligomers) has been shown to lead to the generation of new pharmacological entities. More importantly, dimerization between opioid receptor types and between opioid and non-opioid receptors has recently been shown to be involved in the regulation of opiate action *in vivo*, and to play a role in complex biological phenomena, such as in the development of morphine tolerance [2]. Currently, efforts to identify compounds targeting opioid receptor heteromers are ongoing; such compounds could represent new analgesic drugs with enhanced specificity and selectivity. Therefore, opioid receptor heteromers represent novel therapeutic targets for the treatment of pain and other related disorders.

15.2 Historical Prospective

15.2.1 *Indirect Evidence*

Several studies carried out in the last 30 years have provided evidence that GPCRs exist and function as complexes (rather than as individual monomeric units). Early studies examining ligand binding properties of β -adrenergic or muscarinic receptors in endogenous tissues showed cooperativity between binding sites [3–8]. To explain this and other similar observations, the *receptor mosaic hypothesis* was proposed that stated that the formation of receptor complexes (called *mosaic*) at synapses represented a molecular adaptation to various patterns of transmitters released by the source neuron [9].

The functional unit within the mosaic was thought to be a receptor dimer made up of two protomers (or an oligomer consisting of a multiple dimers). Support for such an idea came from a study that showed that the gonadotrophin-releasing hormone receptor antagonist functions as an agonist in the presence of a divalent antibody against the antagonist; this could be explained by the antibody-mediated cross-linking of the antagonist bound receptor protomers [10].

From these studies emerged the idea that occupancy of the two binding sites of a receptor dimer was necessary and sufficient to put the receptor in a conformation that could elicit gonadotropin-releasing hormone activity [10]. Biochemical studies further supported the idea that GPCRs associate to form dimers/oligomers. For example, gel-filtration analysis [11], radiation inactivation analysis [12–15], and affinity labeling studies [16, 17] showed that the size of the functional unit was greater than expected, suggesting that the receptors exist as higher molecular weight complexes – consistent with the notion of dimers/oligomers as functional units.

15.2.2 Functional Complementation

Functional complementation analysis has been applied to demonstrate direct association of proteins to form functional receptors. This analysis explores the formation of a functional unit by co-expression of two nonfunctional protein partners. Maggio et al. [18] used two complementary chimeras of m₃ muscarinic and α_2 adrenergic receptors (α_2 AR), that did not contain the binding site for the respective ligands when the receptors were individually expressed, to reconstitute an adrenergic and a muscarinic binding site [18]. These results suggested a physical association between m₃ muscarinic and α_2 AR (heteromerization) leading to the formation of the ligand-binding pocket for both receptors.

In another study, Monnot et al. [19] used two different mutants of the AT_{1a} angiotensin receptor that did not bind to angiotensin. When co-expressed, the ability to bind the ligand was restored, suggesting that an association between the two AT₁ receptor protomers (homomerization) leads to the reconstitution of a functional ligand binding site [19].

15.2.3 Physical Evidence

Direct evidence for receptor dimerization has come from biophysical techniques that allow the visualization of GPCR complexes. Fotiadis et al. used atomic force microscopy (AFM) to reveal that in isolated murine disc membranes, rhodopsin is organized in linear arrays of receptor dimers [20]. The same paracrystalline organization was also observed in the opsin isolated from photoreceptors of Rpe65^{-/-} mutant mice, that do not produce the chromophore, 11-cis-retinal, suggesting that the dimeric arrangement of the receptors is independent of their activation state [21].

An independent study using cryo-electron microscopy to determine the 3D structure of squid rhodopsin from 2D crystals, reported an ordered alignment in the photoreceptor membrane with strong protein–protein contacts along the rows, and also contacts between the rows [22], reminiscent of receptor dimerization. Recently, the crystal structure of the β_2 adrenergic receptor (β_{2A} R) has been solved [23, 24].

To obtain crystals, Kobilka et al. stabilized the receptor using an Fab antibody fragment that recognizes a conformational epitope at both ends of the third intracellular loop [23] or by replacement of the third intracellular loop with T4 lysozyme [24]. This resulted in the generation of crystals that diffracted at 3.4 Å/3.7 Å [23] and 2.4 Å [24], respectively. Although the crystals of the β_{2A} R-Fab complex showed no inter-receptor contacts, in the β_{2A} R-T4 chimera crystals, lipid-mediated contacts between helices 1 and 8 suggested the possibility of receptor–receptor interactions. It is likely that association with Fab or the presence of T4 lysozyme interferes with the ability of the protomers to interact especially since in both cases most of the C-terminus of the receptor was truncated and this could affect the formation of receptor dimers.

15.2.4 Pharmacological Evidence

Recently, several studies have demonstrated that some GPCRs require heteromerization with specific partners to be efficiently transported to the cell surface. The best-documented example is that of γ -aminobutyric acid B (GABA_B) receptors that consist of GABA_{B1} and GABA_{B2} proteins that are nonfunctional when either one is expressed alone. GABA_{B1} is retained in the endoplasmic reticulum (ER) due to the presence of an ER retention signal in the C-terminal tail, whereas the GABA_{B2} protein is expressed at the cell surface, but does not bind to the ligand, GABA.

Co-expression of both proteins leads to the masking of the ER retention signal in GABA_{B1} by GABA_{B2} and this, in turn, results in the expression of the functional receptor at the cell surface [25–28]. Another interesting example of the requirement of heteromerization for cell surface expression is that of the taste receptors. In this case, in addition to enabling efficient trafficking to the cell surface, heteromerization leads to alterations in ligand specificity; for example, dimerization of T1R1 with T1R2 leads to the formation of a receptor for sweet tasting molecules, whereas dimerization of T1R1 with T1R3 results in a receptor for savory (“umami”) tasting molecules such as L-amino acids [29, 30].

Furthermore, molecular modeling and site-directed mutagenesis studies have demonstrated that heteromerization of T1R2 and T1R3 leads to the formation of several structurally distinct binding sites and this contributes to distinct binding properties for a variety of sweet-tasting compounds [31]. These studies indicate that heteromerization is a prerequisite for the activity and pharmacological function of some GPCRs.

15.3 Opioid Receptor Homomerization

The availability of cDNAs to opioid receptors allowed studies to rigorously evaluate the presence of receptor dimers in recombinant systems. By expressing differentially epitope tagged receptors in heterologous cell lines and immunoprecipitating complexes using selective antibodies, the interaction of MOR, DOR and KOR to form homomers was demonstrated [32–34]. Additional cross-linking studies, biophysical studies, computational modeling studies and physiological studies have helped characterize opioid receptor homomers; these are described below.

15.3.1 Biochemical Techniques

Crosslinking of proteins using a variety of cross-linking agents followed by Western blotting analysis to detect the cross-linked receptors was used to demonstrate the presence of DOR homomers [32] as well as KOR homomers [33]. In the case of

DOR, it was shown that treatment of intact cells with the cross-linker stabilized the interaction between two 60 kDa proteins (corresponding to DOR protomers), so that more than 50% of the total receptors appeared as a 120 kDa band [32].

An important control to confirm that the high molecular form of the receptor is a homomer and not a highly glycosylated form of the monomer is to subject the protein to deglycosylation by treatment with endoglycosidases [33]. Such deglycosylation treatments lead to the generation of lower molecular weight forms of both the 60 and 120 kDa forms, likely corresponding to deglycosylated monomeric and dimeric receptors, respectively [32], suggesting that the 120 kDa complex observed without endoglycosidase treatment corresponds to DOR homomers.

Another technique used to demonstrate the presence of receptor dimers is the coimmunoprecipitation of differentially tagged receptors. In this case, a receptor is either tagged with tag A or tag B (usually FLAG or myc epitope) and the two tagged versions are co-expressed in heterologous cells. The receptors from the cells expressing these two tagged versions are immunoprecipitated using anti-tag A antibody, subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and proteins transferred to a membrane that is immunobotted with anti-tag B antibody. If a signal is detected only in cells co-expressing both tagged versions of the receptor, and not in cells expressing only one tagged version of the receptor, or from a mixture of cells expressing each receptor individually it would then suggest an interaction between the two receptors [33]. This paradigm has been successfully used to demonstrate the dimerization of opioid receptors [35, 36].

15.3.2 Biophysical Techniques

Although crucial in establishing the concept of dimerization for opioid receptors, biochemical studies do not allow the examination of receptor–receptor interactions or their modulation by ligands in live cells. Therefore, bioluminescence resonance energy transfer (BRET), a proximity-based biophysical method has been used, to examine receptor–receptor associations under physiological conditions (in living cells). This assay is based on a naturally occurring phenomenon in which the energy generated by a luminescent donor, *Renilla* luciferase (Rluc) upon hydrolysis of its substrate, coelenterazine, is transferred to a fluorescence acceptor, a mutant form of green fluorescent protein (GFP). The energy transfer can occur only if the donor and the acceptor are less than 100 Å apart, demonstrating close proximity between the donor and the acceptor [37].

Using this technique, close proximity that would enable association between protomers has been demonstrated in the case of MOR, DOR, and KOR [38–40]. In these studies, BRET signals were found to be comparable regardless of receptor expression level (including expression level as low as 100 fmol/mg of protein) between every opioid receptor pair examined, supporting the notion that opioid receptor dimerization is a physiological phenomenon [40]. In addition, treatment

with receptor specific ligands did not lead to a change in the observed BRET signals suggesting that the receptors were constitutively dimerized (i.e., dimerized before insertion into the plasma membrane) [39–41].

15.3.3 Computational Modeling

In parallel to the above-mentioned approaches, homology modeling based on the crystal structure of rhodopsin as well as evolutionary trace correlation mutation analysis have helped generate models of dimers [41, 42], and predict their putative interacting domains [43, 44] that have been validated in the case of D2 dopamine receptors [45]. For DOR, the contacts are predicted between the TM4 and/or TM5 helices. For KOR, only TM5 has been identified as the most likely homomerization interface, whereas for MOR, TM1 and TM3 are suggested to be involved (with TM1 as the most populated interface). These predictions are very exciting and need to be validated. Such studies offer the possibility of generating opioid receptors deficient in the ability to form homomers, which would provide a valuable tool for evaluating the role of dimerization in receptor function.

15.3.4 Physiological Studies

Opioid receptor homomerization has also been indirectly demonstrated using two distinct ligands targeting MOR. Since morphine does not promote MOR internalization but DAMGO does, He and colleagues [34] examined the effect of morphine along with a non-internalizing dose of DAMGO on MOR internalization. They found a significant internalization of MOR in transfected HEK293 cells and primary hippocampal neurons. Furthermore, rats treated chronically with this combination of drugs showed reduced analgesic tolerance compared to rats treated with morphine alone [34]. Similarly, animals co-administered with morphine and a small dose of methadone retained full analgesic potency, did not exhibit morphine tolerance and had reduced dependence [45]. Taken together these studies indicate that opioid receptor dimerization could play an important role in modulating the biological responses of clinically used opiates.

15.4 Heteromerization Between Opioid Receptors Types

In addition to their existence as homomers, opioid receptors can also exist as heteromers. Ligand-binding studies using DOR selective compounds such as deltorphin II, naltriben, BNTX and naltrindole suggested the existence of multiple delta receptor sites (delta1 and delta2) [46]. Similarly, studies with KOR selective

compounds such as U69,593 indicated the presence of different subtypes of KOR [47]. Despite large-scale efforts by several laboratories, complementary DNAs corresponding to these subtypes have not yet been conclusively demonstrated.

Since studies examining the pharmacology of DOR-KOR heteromers showed that it exhibited the properties attributed to the KOR-2 receptor subtype [33] it has been proposed that some of the other reported opioid receptor subtypes could result from dimerization of opioid receptors with other opioid or non-opioid receptors. In the following sections we will discuss the evidence for heteromerization between different opioid receptor types and its functional significance (summarized in Table 15.1).

15.4.1 DOR-KOR

The DOR-KOR heteromer was the first opioid receptor heteromer to be characterized using biochemical, biophysical, and pharmacological techniques. Association between these two receptors was demonstrated by co-immunoprecipitation of differentially tagged receptors [33] and by BRET studies [38]. DOR-KOR heteromers could be immunoprecipitated only from cells co-expressing myc-KOR and FLAG-DOR but not from a mixture of cells individually expressing these receptors.

Treatment with β -mercaptoethanol resulted in the destabilization of the heteromers [33] suggesting a role for intramolecular interactions in the formation of this heteromer. BRET studies also showed that DOR and KOR were in close proximity ($<100\text{ \AA}$) for interaction in live cells [38]. Analysis of BRET signals at various receptor expression levels indicated that the DOR-KOR heteromer formed at least as efficiently as the KOR homomers and a significant signal was obtained with less than 100,000 copies of KOR-YFP as acceptor [38].

These results suggest that DOR-KOR interactions are likely to occur at physiological levels of the receptor. Functional analysis of the DOR-KOR complex has revealed a new receptor type that exhibited ligand binding, functional and trafficking properties that were distinct from those of each individual receptor [34]. The heteromers were found to have greatly reduced affinities for their selective ligands. Furthermore, selective agonists were found to cooperatively bind to heteromers and this led to synergistic functional responses including potentiation of signaling [33]. In addition, studies used to characterize the pharmacological profile of opioid receptors in neural membranes from porcine myenteric plexus that co-express DOR and KOR revealed the presence of a unique site that displayed low affinity for DOR selective (DPDPE, deltorphin II, SNC80 and TIPP) or KOR selective (U69,593) ligands and a moderately high affinity for naltrexone-derived DOR (naltriben, BNTX) and KOR (nor-BNI, 5'-GNTI) antagonists [48].

This profile is reminiscent of that described for DOR-KOR heteromers and indicates that receptors with similar properties are present in neural membranes isolated from porcine myenteric plexus [49]. Based on these findings, DOR-KOR specific bivalent ligands were synthesized where DOR and KOR antagonists

Table 15.1 Modulation of receptors properties by heterodimerization

	Pharmacology	Coupling	Signaling	Trafficking	References
DOR-KOR	↓ affinity for selective agonists; synergistic binding of highly selective ligands	NR	↓ signaling of individual ligands; synergic effect of selective ligands	Altered endocytosis of DOR	Jordan and Devi [33]
MOR-KOR	↓ affinity for selective MOR agonists	NR	NR	NR	Wang et al. [40]
MOR-DOR	↓ affinity for selective agonists; allosteric effect of DOR ligands on MOR binding	Allosteric effect of DOR ligands on MOR coupling Altered: coupling to Gz	Altered; allosteric effect of DOR ligands on MOR signaling	Delayed receptor co-internalization	Gomes et al. [39, 67]; George et al. [68]
		Altered: coupling to βarrestin2	Potentialization of MOR coupling		Fan et al. [70]; Hasbi et al. [74]
MOR-α _{2a}	NR	Potentialization of MOR coupling	Potentialization of MOR signaling; negative allosteric modulation of MOR by α _{2a} ligands	NR	Jordan et al. [80]
MOR-CB1	NR	NR	Negative allosteric modulation of MOR by CB1 ligands	NR	Rios et al. [90]
MOR-D1	↑ Binding of MOR agonists	NR	NR	NR	Juhasz et al. [93]
MOR-ORL1	↑ Affinity for MOR ligands	NR	Altered; cross-desensitization of the receptors	Co-internalization upon treatment with a single ligand	Pan et al. [101]; Wang et al. [99]; Evans et al. [100]

MOR-NK1	NR	NR	Transphosphorylation/ desensitization	Receptor co-internalization upon treatment with a single ligand; altered interaction of MOR with arrestin	Pfeiffer et al. [106]
MOR-sst2a	Decreased affinity for sst2a selective ligands	NR	↑ Potency of sst2a agonist; ↑ potency of MOR agonist; transphosphorylation/ desensitization	Receptor co-internalization upon sst2a stimulation, but not MOR stimulation	Pfeiffer et al. [107]
DOR- α_{2a}	NR	NR	Potentiation of DOR signaling	NR	Rios et al. [115]
DOR- β_{2a} DOR-SNSR4	NA NA	NA NA	NA ↓ coupling of DOR; receptor co-stimulation leads to DOR inhibition	Receptor co-internalization Impaired DOR internalization	Jordan et al. [116] Breit et al. [122]
DOR-CXCR2	NR	NR	NR	Potentiation of DOR signaling by CXCR2	Parenty et al. [118]
KOR- β_{2a}	NA	NA	NA	Impaired	Jordan et al. [116]

NA not affected under conditions used; NR not reported

(NTI and 5'-GNTI, respectively) were linked by variable length spacers [48]. One of such compound (KDN-21) exhibited a much higher affinity and selectivity for DOR-KOR heteromers relative to DOR or KOR alone when examined using competition binding studies with selective radioligands [^3H]naltrindole (for DOR) and [^3H]norbinaltorphimine (nor-BNI for KOR) [50].

In addition, the presence of naltrindole led to an increase in the binding of nor-BNI to KOR and the presence of nor-BNI led to an increase in the binding of naltrindole to DOR, suggesting a reciprocal allosteric modulation of the receptors in the heteromer [50]. As predicted from in vitro studies, the DOR-KOR heteromer in the spinal cord displayed altered pharmacological properties (i.e., DOR1 and KOR2 phenotypes) [33, 48, 50]. KDN-21 was found to have antinociceptive activity when administered directly into the spinal cord, but not into the brain supporting the notion that the relative levels of the heteromer varies in different regions and that by selectively targeting the heteromers we could increase the specificity of a drug with probable reduction in associated side-effects.

15.4.2 MOR-DOR

A number of studies over several years have suggested interactions between MOR and DOR [51, 52]. Indirect evidence obtained from ligand receptor binding studies [53–55] and autoradiographic studies in rat striatum [56] supported the existence of MOR-DOR heteromers and suggested the notion of allosteric modulation of receptor function by dimerization. In these studies, [^3H]DADLE was found to label two binding sites in vitro that could be discriminated using MOR ligands.

The authors found that the data obtained with the MOR agonist, oxymorphone, best fit a two-site allosteric model where oxymorphone functioned as a competitive inhibitor at the high affinity site and a noncompetitive inhibitor at the lower affinity [^3H]DADLE binding site. Since autoradiographic studies demonstrated that the lower affinity [^3H]DADLE binding site had an anatomical distribution identical to that obtained with [^3H] oxymorphone, and since MOR ligands decreased the apparent binding capacity of this binding site the authors concluded that the lower affinity [^3H]DADLE binding site could represent DOR physically coupled to a MOR binding site in a MOR-DOR complex [53, 54].

Further indirect support for the existence of MOR-DOR complexes in rat brain emerged from studies on the effects of selective irreversible blockade of MOR and DOR. Holaday et al. used a variety of in vivo opioid receptor assays to show that the selective irreversible MOR ligand, β -funaltrexamine, irreversibly prevented the effects of the selective DOR agonist, ICI 174864 [57–59]. Studies examining the biochemical nature of opioid receptors also supported the existence of MOR-DOR complexes.

Schoffelmeer et al. carried out cross-linking studies with human [^{125}I] β -endorphin in rat striatal membranes and identified a 80-kDa glycoprotein that exhibited the pharmacological properties of both MOR and DOR [60]. Furthermore,

immunoblotting studies using MOR specific antibodies detected multiple protein bands (up to 114 kDa) in solubilized membranes from mouse central nervous system regions that likely represented interacting complexes of MOR including dimeric forms of the receptor [61].

A number of studies exploring the pharmacological interactions of MOR and DOR ligands have reported functional interactions between MOR and DOR receptors [62]. For example, sub-antinociceptive doses of the DOR antagonist have been shown to modulate the antinociceptive responses to a MOR agonist in the tail-immersion test [63]. Also pretreatment of mice with DOR antagonists was shown to prevent the development of morphine tolerance and dependence [64]. DOR knockout animals were found not to develop analgesic tolerance to morphine [65]. Finally, treatment of animals with DOR antagonists together with morphine (spinal) augmented the acute analgesic effects of morphine, inhibited the induction of chronic tolerance and reversed established tolerance [66]. Taken together, these studies suggested the existence of MOR-DOR interacting complexes and that such complexes could have a physiological role.

The availability of cDNAs for MOR and DOR allowed the demonstration of direct interactions using co-immunoprecipitation and BRET analysis with differentially tagged receptors expressed in HEK 293 cells [39, 40, 67]. Treatment with ligands did not affect the extent of co-immunoprecipitation or BRET signal suggesting a constitutive interaction. Using receptor selective antibodies to detect endogenous MOR and DOR receptors, a complex consisting of MOR-DOR was isolated from spinal cord membranes of wild-type but not DOR knockout mice [39].

Pharmacological characterization indicated that the MOR-DOR heteromer exhibits distinct ligand binding and signaling properties [39]. Treatment with extremely low doses of DOR-selective ligands led to a significant increase in the binding of the MOR agonist. Similarly, treatment with MOR-selective ligands led to a significant increase in the binding of the DOR agonist [67]. In addition, highly selective synthetic agonists for each receptor exhibited reduced potency for the heteromer. This, taken with the report that the MOR-DOR heteromer exhibits enhanced affinity for endomorphin-1 and Leu-enkephalin, suggests that dimerization might lead to the formation of a novel binding pocket [68].

MOR-DOR heteromers also display altered signaling properties. In heterologous as well as neuroblastoma cells co-expressing these receptors, treatment with the DOR antagonist led to a significant enhancement of MOR agonist-mediated signaling [39, 67]. A similar increase was also seen in spinal cord membranes, irrespective of the nature of the DOR ligand (antagonist, inverse agonist or non-signaling dose of an agonist) suggesting that occupancy of DOR is sufficient to potentiate MOR signaling. This effect was seen only in membranes from wild-type mice and not in membranes from mice lacking DOR [39], indicating a requirement of both receptors for the observed novel pharmacology.

In spite of these studies that demonstrated the direct interactions between MOR and DOR very little is known about the domains involved in heteromerization. Homology modeling of MOR and DOR based on the crystal structure of rhodopsin

in combination with a subtractive correlated mutation method have been used to predict the interface of MOR-DOR dimers [44]. According to the prediction, the MOR-DOR heteromerization interface involves TM1 of MOR and TM4, TM5 and TM6 of DOR [43]. Further studies are necessary to test these predictions by introducing mutations at sites predicted to be necessary for heteromerization and examining the ability of receptors to dimerize; dimerization deficient receptors would prove to be valuable tools for evaluating the role of heteromerization in receptor function.

A variety of techniques have been used to evaluate the extent of alterations in signaling by MOR-DOR heteromers. Studies using opioid receptors fused to the inhibitory G protein, $G\alpha_i$, have found that the G protein fused to DOR could be activated by MOR agonists (or vice versa) [69] supporting the idea that heteromerization could lead to the formation of a novel binding pocket; this in turn could lead to the recruitment of signaling complexes distinct from those of the individual receptors [70, 71].

A study examining the inhibition of voltage-dependent Ca^{2+} channels (VDCCs) in DRGs from wild-type, DOR heterozygotes (\pm) or DOR knockout mice found that morphine or DAMGO exhibited reduced inhibition of these channels in cells lacking DOR. Introduction of DOR but not a DOR construct lacking C-terminal 15 amino acids to DRGs lacking the receptor restored the ability of MOR agonists to inhibit voltage-dependent Ca^{2+} channels [72]. Another study examined calcium channel inhibition in transfected GH3 cells [73] and found that the DAMGO-mediated inhibition of calcium signaling was significantly decreased in the presence of DOR. The change in DAMGO response induced by DOR expression seemed to involve coupling to a new signaling pathway by the MOR-DOR heteromer [73]. Consistent with this, a study examining G protein association with MOR-DOR reported that the heteromer associates with $G\alpha_z$ and this recruitment occurs in the ER [68, 74].

Finally, a recent study examining MOR-DOR mediated signaling reported a switch in signaling from a G protein-mediated to a β -arrestin-mediated pathway [71]. It was also found that this switch, in turn, results in the cytoplasmic retention of phosphorylated ERK1/2 leading to differential activation of transcription factors (as compared to MOR homomers). Thus, MOR-DOR heteromer activation appears to lead to changes in signaling by a variety of pathways. Since this also results in changes in the spatio-temporal dynamics of signaling and transcription factor activity, this is likely to result in long lasting changes as seen during chronic morphine administration and development of tolerance.

Studies examining the trafficking of MOR-DOR heteromers have yielded contradictory results. One study reported that treatment with receptor selective ligand leads to the endocytosis of its cognate receptor, but not of the dimeric partner [75], whereas another study reported that treatment with some ligands (DAMGO or deltorphin II) leads to co-internalization of the receptor complex whereas treatment with other ligands (DPDPE or DSLET) did not [74]. Differences in the maturation and cell surface trafficking properties of MOR-DOR complexes have also been reported [74, 75]. One study reported MOR-DOR to be associated with $G\alpha_i$ only after the receptors have reached the cell surface [75], whereas another study found

MOR-DOR to associate with Gα_i as early as in the ER, and to travel to the cell surface as a preassembled complex [74].

These differences could be due to the differences in the cell system or the nature of the assay used in these studies. Additional studies are needed to explore the trafficking (maturation and endocytic) properties of the MOR-DOR heteromers and compare them to receptor homomers. A recent study showed that the levels of MOR-DOR heteromers at the cell surface are regulated by a Golgi chaperone, RTP4 (receptor transport protein 4); the latter associates with MOR-DOR heteromer, protects them from ubiquitination and degradation, leading to increased heteromer expression at the cell surface [76].

The relevance of MOR-DOR heteromerization to opiate pharmacology *in vivo* has been examined using low doses of DOR ligands to potentiate morphine-mediated analgesia [39, 66]. Co-treatment with a very low dose of a DOR selective antagonist was shown to significantly enhance the analgesia induced by a submaximal dose of morphine. These results support the idea that ligands targeting the heteromer could function as potent analgesics (with fewer side-effects compared to currently used analgesics). One strategy to develop heterodimer selective ligands would involve the development of bivalent ligands using a MOR agonist and a DOR antagonist as pharmacophores. In principle, this strategy can lead to improved target selectivity since such drugs should bind preferentially to the heterodimer. Moreover, such ligands could also exhibit tissue selectivity, since heteromers are likely to be selectively expressed only in tissues that co-express both of the interacting receptors. Portoghese and colleagues synthesized a series of ligands consisting of a MOR agonist pharmacophore (oxymorphone) and a DOR antagonist pharmacophore (NTI) separated by spacers of variable lengths [75]. The authors found that the bivalent ligands functioned as agonists and were more potent than morphine. In addition, bivalent ligands whose spacer was longer than 16 atoms produced less dependence and tolerance than either morphine or a μ monovalent control. This supports the notion that the MOR-DOR heteromer represents a potentially valuable drug target.

15.4.3 MOR-KOR

Interactions between MOR and KOR have not been explored as extensively as that of MOR and DOR. Co-immunoprecipitation studies with MOR and KOR revealed that an interacting complex could not be isolated using the same immunoprecipitation conditions as that used for the isolation of the DOR-KOR heteromer [33]. This could be due to a differential detergent sensitivity of the MOR-KOR heteromer. A study examining ligand-binding pharmacology of MOR-KOR receptors found that MOR agonists (DAMGO and endomorphin-1) exhibited lower affinity for MOR when expressed with KOR. In contrast, the affinity of KOR agonists (U69593 and U50488H) was not altered [40], suggesting that MOR-KOR association leads to unique and subtle changes in their ligand binding properties.

15.5 Heteromerization Between Opioid Receptors and Non-Opioid Receptors

Opioid receptors have also been shown to interact with non-opioid receptors of family A GPCRs. Investigations have focused on receptor pairs that have been shown to co-localize in endogenous tissues or cells, or to contribute to similar physiological phenomena (such as analgesia). Each opioid receptor type (MOR, DOR, or KOR) has been shown to interact with non-opioid receptors, this association leading, in general, to changes in the properties of both interacting protomers (Fig. 15.1). These studies are described below and summarized in Table 15.1.

15.5.1 MOR- α_{2a}

A number of early studies noted functional interactions between MOR and α_{2a} adrenergic receptors (α_{2a} AR) [77, 78]. Direct evidence supporting an interaction between these two receptors has come from studies with mice lacking functional α_{2a} AR [79]. These mice exhibit a decrease in the analgesic potency of spinally administered morphine compared to wild-type mice, suggesting an interaction between opioid and adrenergic receptors [79].

Heteromerization between the two receptors was reported using co-immunoprecipitation as well as BRET assays [80]. Examination of the functional activity of MOR (GTP γ s binding and MAP kinase phosphorylation) showed that the presence of α_{2a} AR was sufficient to enhance MOR signaling. However, simultaneous activation of both receptors led to a significant decrease in signaling. Stability of the receptor complex evaluated by co-immunoprecipitation experiments indicated that co-treatment with ligands to both receptors led to a decrease in the levels of the immunoprecipitable complex suggesting changes in detergent sensitivity of the heteromer upon activation [80].

A recent study has examined early molecular events underlying the interaction between MOR and α_{2a} AR using fluorescence resonance energy transfer microscopy [81]. This study showed that morphine binding to MOR triggers a conformational change in the norepinephrine-bound α_{2a} AR; this leads to a decrease in signaling by G αi and the downstream MAP kinase cascade. Using BRET, the authors further demonstrated a conformational change that propagated from one receptor to the other, leading to a rapid inactivation of the second receptor [81]. This is in support of the idea that the decrease in immunoprecipitable complex upon co-stimulation of the two protomers demonstrated by Jordan et al. [71, 80] is due to a conformational change of the complex instead of a dissociation of the receptors.

A study examining the endocytosis properties of MOR- α_{2a} AR showed that treatment with a ligand specific to one of the receptors in the MOR- α_{2a} AR complex led

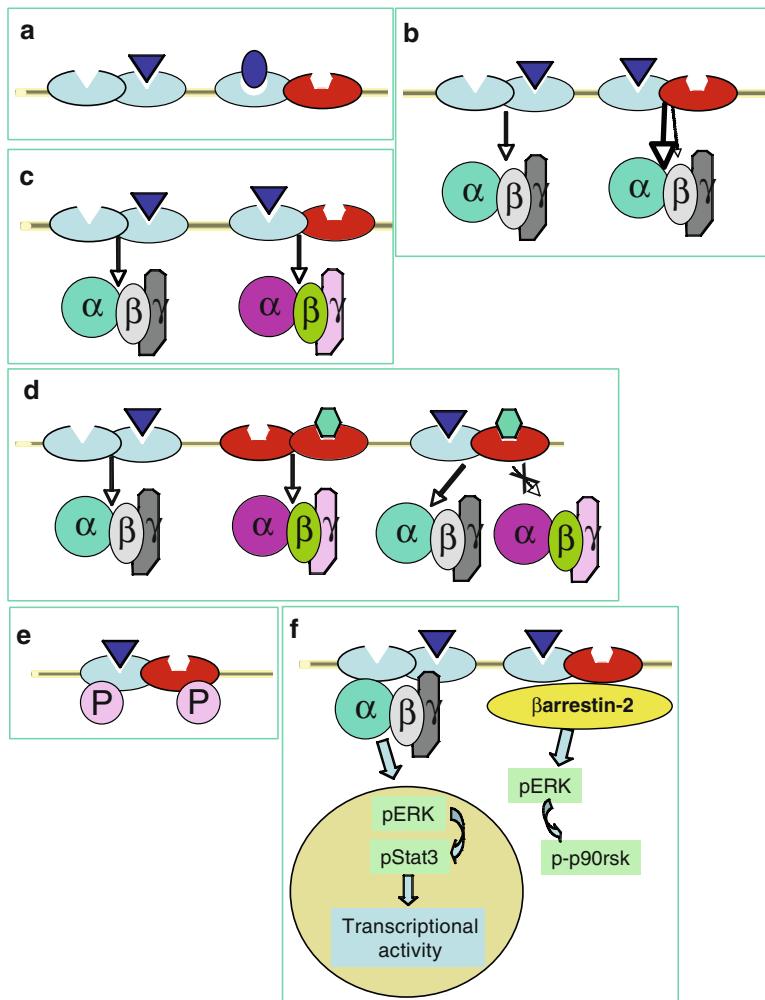


Fig. 15.1 Schematic representation of the modulations of receptor function by heterodimerization. (a) Heterodimerization leads to the formation of an altered ligand-binding site. Shown for DOR-KOR [33, 129], MOR-KOR [40], MOR-DOR [67, 68], and MOR-sst2A [107]. (b) Heterodimerization leads to an increased or a decreased coupling to the G protein. An increased coupling was shown for MOR- α_{2A} AR [80], and a decreased coupling was shown for MOR-DOR heterodimer [67]. (c) Heterodimerization leads to coupling to a new G protein. Shown for MOR-DOR heterodimer coupling to Gz [70, 74]. (d) Heterodimerization of receptors coupled to distinct G proteins leads to functional inhibition following co-activation of both protomers in the heterodimer, due to competition for G proteins and preferential coupling of the heterodimer for one of the G proteins. Shown for DOR-SNSR-4 [122]. (e) Heterodimerization leads to cross phosphorylation/desensitization. Shown for MOR-ORL1 [99], MOR-NK1 [106], MOR-sst2A [107]. (f) Heterodimerization leads to a switch in receptor coupling, from G protein to β -arrestin, and subsequent differential signaling and transcription factor activation. Shown for MOR-DOR heterodimer [71]

to the internalization of that protomer but not of the complex as a whole [82]. While it is possible that the visualized internalized receptors correspond to protomers associated as homomers and not to the protomer present in the heteromeric complex, additional studies are needed to fully evaluate these findings.

15.5.2 MOR-CB1

Behavioral studies using selective opioid or cannabinoid agonists [83, 84] or mice lacking individual receptors [85, 86] initially suggested interactions between opioid and cannabinoid receptors. Since localization studies revealed that MOR and CB1 receptors co-localize in dendritic spines in the caudate putamen and dorsal horn of the spinal cord [87–89] it is likely that a functional interaction between these two receptors by direct receptor association could occur in these brain regions. Interaction between MOR and CB1 was demonstrated by co-immunoprecipitation experiments and by BRET and FRET analysis [90, 91].

Examination of the signaling properties of the MOR-CB1 complex revealed that, while the expression of CB1 receptors did not affect MOR signaling, the occupancy of CB1 receptors by selective ligands had an antagonistic effect on MOR-mediated G protein activation and signaling [90]. Reciprocally, occupancy of MOR had an antagonistic effect on CB1 receptor signaling [90]. This was also reflected in the inhibition of neurite outgrowth following treatment with a combination of MOR and CB1 agonists in Neuro2A cells expressing both receptors [90].

Additional studies are needed to fully characterize the MOR-CB1 heteromer properties *in vivo* and to assess the physiological role of this complex in regulating MOR and CB1 functions.

15.5.3 MOR-Dopamine D1

Studies showing that chronic morphine administration did not cause an increase in locomotor activity in dopamine D1 knock-out mice and that the latter exhibited a decrease of MOR immunoreactivity in striatal patches compared to wild-type animals suggested functional interactions between these two receptors [92]. Immunohistochemistry with antibodies directed to the C-terminal region of either MOR or dopamine D1 receptors detected co-localization of these receptors in select neurons in the cortex and striatum of rats further supporting the idea that MOR and dopamine D1 receptors could form functional heteromers [93].

A unique strategy was used to demonstrate the heteromerization of MOR and dopamine D1 receptors in living cells. The introduction of a nuclear localization sequence into helix 8 of the dopamine D1 receptor led to a robust translocation of the receptor from the cell surface to the nucleus and cytoplasm as seen by confocal

microscopy of live cells. In cells expressing only MOR the latter exhibited only a cell surface expression. Translocation of MOR to the nucleus and cytoplasm was observed only in cells that also co-expressed the dopamine D1 receptor containing the nuclear localization sequence thereby suggesting the formation of MOR-dopamine D1 heteromeric complexes [93].

BRET studies using MOR-Luc and D1-GFP also confirmed that MOR and D1 dopamine receptors are in close proximity ($<100\text{ \AA}$) to interact in live cells. In these studies, the BRET signal could be blocked by over-expression of untagged dopamine D1 receptors [93]. Binding studies show that co-expression of D1 dopamine receptors causes ~76% increase in the binding of the radiolabeled MOR agonist, DAMGO. This increase is not seen with a highly homologous receptor, D5 dopamine receptor, or with chimeric constructs where the C-terminal tail of D1 receptor has been replaced with that of D5 receptor, and vice versa [93].

These results implicate the C-terminal tail and other regions of D1 receptors in increasing DAMGO binding to MOR. Although dopamine D1 receptors increase the surface expression of MOR, competition assays examining the ability of DAMGO to displace [^3H]naloxone binding in cells expressing either MOR alone or in combination with D1 receptors do not show significant changes in low and high affinity states and on the proportion of receptors in the high affinity state [93].

15.5.4 MOR-ORL1

Activation of ORL1 (opioid receptor-like 1 receptor) by orphanin FQ (also known as nociceptin) produces a variety of biological effects, including spinal analgesia, supraspinal hyperalgesia, inhibition of locomotor activity and learning, anxiolytic-like effects and antagonism of opioid-induced effects (for review, see [94, 95]). In addition, ORL1 has been shown to functionally interact with MOR [95–98].

For example, intracerebroventricular administration of a selective ORL1 antagonist potentiates MOR agonist-induced analgesia and produces a naloxone-resistant antinociceptive effect [94]. The contribution of receptor dimerization to these functional interactions was tested with recombinant receptors stably expressed in HEK293 or tsA-201 cells [99, 100].

Co-immunoprecipitation studies demonstrated that MOR and ORL1 could be isolated in interacting complexes [100, 101] in heterologous cells and DRG neurons. In cells co-expressing MOR and ORL1, treatment with the ORL1 agonist, nociceptin, caused MOR internalization. Similarly treatment with the MOR agonist, DAMGO caused ORL1 internalization [100]. In addition, the presence of MOR attenuated nociceptin (ORL1 agonist) induced inhibition of N-type calcium channels while activation of MOR induced N-type channel internalization only in the presence of ORL1 receptors [100].

Pharmacological characterization of the heteromer showed that MOR-ORL1 heteromerization affects the potency of DAMGO to inhibit adenylyl cyclase activity and MAP kinase phosphorylation. Interestingly, nociceptin pre-treatment not

only caused the desensitization of ORL1 but also of MOR. In contrast, pre-incubation with DAMGO only induced desensitization of MOR, but not that of ORL1, suggesting that MOR-ORL1 heteromerization selectively causes the cross-desensitization of MOR-mediated signal transduction pathways [99]. This suggests a potential role for heteromerization in the modulation of MOR and ORL-1 functions *in vivo*.

15.5.5 MOR-NK1

Substance P (SP) is released from nociceptive primary afferents in the spinal and trigeminal dorsal horn, where it activates spino-thalamic projection neurons by binding to its main receptor, NK1. The rationale for studying MOR-NK1 heteromerization comes from studies showing that NK1 and MOR coexist in trigeminal dorsal horn neurons and are highly expressed not only in brain regions implicated in depression, anxiety, and stress, but also in other regions such as the nucleus accumbens, which mediate the motivational properties of drugs of abuse including opioids [102, 103]. Furthermore, the rewarding effects of morphine are absent in mice lacking the NK1 receptor [104, 105]. Heteromerization between MOR and NK1 in HEK293 cells was demonstrated by co-immunoprecipitation of differentially tagged receptors and by BRET assays [106]. Heteromerization did not lead to changes in the pharmacological and signaling properties of the individual protomers. However, ligand-dependent receptor trafficking was affected in that SP (NK1 agonist) treatment promoted internalization of both NK1 and MOR, and reciprocally DAMGO (MOR agonist) treatment promoted internalization of both MOR and NK1. Interestingly, SP and DAMGO induced cointernalization of NK1 and MOR in a stable complex along with β -arrestin into the same endosomal compartment. Finally, receptor phosphorylation studies showed trans-phosphorylation of the protomers in the heteromeric complex in agreement with a cross-desensitization of the receptor complex [106]. Further studies are necessary to determine the role of MOR-NK1 heteromerization in the regulation nociception and the rewarding effects of opiates.

15.5.6 MOR-Somatostatin 2A Receptor (*sst2a*)

The heteromerization between MOR and *sst2a* was explored on the basis of their sequence homology: MOR and *sst2a* are closely related GPCRs that exhibit 38% homology within the amino acid sequence [107]. Heteromerization between MOR and *sst2a* was demonstrated by co-immunoprecipitation of differentially tagged recombinant receptors from HEK293 cells [108]. Ligand binding and receptor signaling (inhibition of adenylyl cyclase activity and MAP kinase phosphorylation)

were not significantly altered by receptor heteromerization. In contrast, heteromerization was found to alter ligand-mediated receptor trafficking in that treatment with L-779,976 (sst_{2a} agonist) promoted internalization of both sst_{2a} and MOR. However, treatment with DAMGO (MOR agonist) promoted internalization of MOR but not of sst_{2A} [107].

It is possible that the internalized receptor corresponds to MOR homomers, and that the MOR-sst_{2a} heteromers do not undergo internalization upon MOR agonist treatment. It is to be noted, that despite these differences in agonist-mediated internalization, both MOR and sst_{2a} agonists induced receptor cross-desensitization of the adenylyl cyclase activity and MAP kinase signaling [107]. Further studies are needed to examine the physiological consequences of MOR-sst_{2a} heteromerization.

15.5.7 MOR-Chemokine Receptor 5 (CCR5)

Exposure to opiates and opioids has been reported to inhibit cellular immune responses and induce chemotactic responses in immune cells [109]. These effects on the immune system may contribute to impaired immune function. Possible direct interaction between MOR and CCR5 that could contribute to opiate-chemokine receptor interaction was examined. Differentially tagged receptors expressed in CHO cells could be co-immunoprecipitated [110] only in cells co-expressing the two receptors, but not from a mixture of cells expressing individual receptors. Treatment with DAMGO or RANTES (CCR5 agonist) induced chemotaxis in CHO cells co-expressing both receptors, and preincubation with either DAMGO or RANTES inhibited chemotaxis caused by the other ligand. DAMGO treatment led to enhanced CCR5 receptor phosphorylation and reduced RANTES-promoted G protein coupling. Conversely, preincubation with RANTES slightly increased MOR phosphorylation and significantly reduced DAMGO-induced G protein coupling. These results indicate cross-desensitization of MOR and CCR5 within the heteromer complex that may play a role in the modulation of the immune system.

15.5.8 DOR- α_{2a} AR

Functional interactions between DOR and α_{2a} AR agonists were noted in studies comparing the effects of DOR and α_{2a} ligands in wild type and mice lacking MOR or functional α_{2a} AR [79, 111]. For example, a loss of synergy between selective MOR or DOR agonists and a α_{2a} AR agonist were observed in mice lacking a functional α_{2a} AR [79]. Immunohistochemical studies have demonstrated an extensive co-localization of DOR with adrenergic receptors in substance P containing neurons in the spinal cord [112–114].

The likelihood that the possible physical interaction between DOR and α_{2a} AR could contribute to the functional interaction between these receptors was demonstrated using recombinant receptors expressed in HEK293 cells by co-immunoprecipitation of differentially tagged receptors and BRET analysis [115]. The functional significance of this interaction was explored by examining the modulation of DOR-mediated neurite outgrowth in Neuro2A cells expressing recombinant receptors. Presence of α_{2a} AR was found to be sufficient to enhance DOR-mediated neurite outgrowth suggesting that the non-activated α_{2a} AR is able to modulate DOR signaling [115]. This suggests a potential physiological role for heteromerization in the regulation of DOR function and observed functional interactions.

15.5.9 DOR- β_2 AR

Association of DOR and β_2 AR was studied in an effort to examine the possibility of regulation of signaling and trafficking by heteromerization of receptors that are coupled to distinct G proteins; β_2 AR has classically been known to couple to stimulatory G α s whereas DOR has been known to couple to inhibitory G α i/o. DOR and β_2 AR were found to occur as heteromers at the cell surface by co-immunoprecipitation of differentially tagged plasma membrane receptors in HEK293 cells [116] and by BRET studies [38]. While the binding and coupling properties of DOR or β_2 AR were not found to be altered, ligand-mediated receptor trafficking properties were altered [116]. For example, treatment with DOR agonists led to the internalization of both DOR and β_2 AR in cells co-expressing both receptors indicating that heteromerization can regulate the function of receptors with different coupling properties.

15.5.10 DOR-Chemokine Receptor CXCR2

Functional interactions between DOR and the chemokine CXCR2 receptor were suggested by studies showing that a DOR agonist, dermankephalin, inhibited the ability of an agonist of the CXCR2 receptor, interleukin-8, to induce chemotaxis of human neutrophils [117]. Resonance energy transfer techniques such as BRET, FRET, and time-resolved FRET as well as co-immunoprecipitation studies show that DOR and CXCR2 receptors are in close proximity to form interacting complexes in HEK-293 cells co-expressing both receptors [118]. Co-expression of a DOR-G protein fusion construct, where the DOR agonist can bind the receptor but not transduce signal, with a CXCR2-G protein fusion construct, where the CXCR2 agonist can bind and transduce signal, resulted in DOR agonists being able to transduce signal further confirming that DOR-CXCR2 can form functional heteromers in heterologous systems [118]. Interestingly, the non-peptidic CXCR2 antagonist, SB225002, was able to potentiate DOR agonist mediated increases in [35 S]GTP γ S binding only when DOR and CXCR2 were co-expressed and not when each receptor was individually expressed [118].

15.5.11 DOR-Sensory Neuron Specific Receptor (SNSR)

SNSRs are exclusively expressed in dorsal root ganglia cells [119, 120], where SNSR-4 has pronociceptive properties [121]. The endogenous ligands for SNSRs originate from proenkephalin (that also gives rise to the antinociceptive peptides, Leu- and Met-enkephalins) by proteolytic cleavage mediated by prohormone convertases. Some of these peptides bind SNSRs with high affinity [120]. One of these peptides, bovine adrenal medulla peptide 22 (BAM22), has the ability to bind and activate both SNSR-4 and DOR; however, removal of the N-terminal tyrosine residue (BAM2-22) is sufficient to generate a selective SNSR-4 agonist that cannot bind DOR [120].

The shared localization of the receptors and ligands for DOR and SNSR-4, and their functional antagonism suggested that they could physically interact. This was examined in HEK293 cells using the BRET assay. Heteromerization of DOR-SNSR-4 did not affect the G protein coupling of individual receptors (activation of $\text{G}\alpha_q$ for SNSR and of $\text{G}\alpha_i/\text{o}$ for DOR) upon specific activation of either receptor [114]. However, simultaneous activation of the two receptors by the dual agonist BAM22 or by two receptor-specific agonists (DPDPE, Leu-enkephalin for DOR and BAM1-12 for SNSR-4) led to a selective activation of phospholipase C ($\text{G}\alpha_q$ -mediated) without inhibition of the adenylyl cyclase ($\text{G}\alpha_i/\text{o}$ -mediated) pathway and to an impaired DOR internalization [114]. Such a receptor-exclusive signaling indicates a dominant-negative effect of SNSR-4 on DOR signaling [122]. This was not due to a competitive antagonistic mechanism since the authors showed that each of the protomers could simultaneously bind their respective agonist within a heteromer complex; this was not due to heterologous cross-talk since PMA induced-inhibition of DOR signaling was much smaller than that observed by the co-activation with either the bivalent ligand, BAM22, or by the co-administration of DOR- and SNSR-selective agonists. The authors proposed that this dominant-negative effect of SNSR-4 on DOR signaling was caused by G protein competition and preferential coupling of the heteromer to $\text{G}\alpha_q$ [122]. Since similar heteromer properties were also observed in primary DRG neurons, these results suggest DOR-SNSR heteromerization could play an important role in the regulation of nociception.

15.5.12 DOR-CXCR4

DOR and CXCR4 have been found to be expressed in immune cells [123]; therefore their involvement in inflammation could be regulated by receptor heteromerization. Such an association was examined using FRET in HEK293 cells expressing CFP-CXCR4 and YFP-hDOR [124]. FRET efficiency demonstrated the presence of DOR-CXCR4 complexes, in the absence and presence of ligands. Association between DOR and CXCR4 was confirmed by co-immunoprecipitation experiments

in MM1 cells and human monocytes expressing endogenous receptors [124]. Treatment of cells expressing both receptors showed that stimulation of individual receptor with its cognate ligand led to a full response, whereas co-stimulation with ligands for the two receptors led to a decrease in the extent of signaling by the complex [124]. It is likely that DOR-CXCR4 plays an important role in the regulation of immune response.

15.5.13 KOR- β_2 AR

KOR and β_2 AR are co-expressed in the heart, and the existence of a functional cross-talk between the two receptors in the heart has been reported [125, 126]. Association of KOR and β_2 AR was examined by co-immunoprecipitation studies in HEK293 cells co-expressing recombinant KOR and β_2 AR [116]. Further analysis revealed that heteromerization of these two receptors did not affect the pharmacology of each individual receptor. In contrast, heteromerization between these two receptors led to changes in receptor trafficking and MAP kinase signaling properties. In cells expressing KOR- β_2 AR heteromers, treatment with a β_2 AR agonist did not induce internalization of either of the receptors, indicating that heteromerization with KOR drastically alters the internalization properties of β_2 AR [116]. This suggests a potential physiological role for heteromerization in the regulation of β_2 AR receptor function.

15.6 Opioid Receptor Heteromers as Drug Targets

Heteromers, by representing unique functional units with new pharmacological properties and physiological functions represent promising novel drug targets. As mentioned earlier, one strategy to specifically target heteromers is to develop bivalent ligands, composed of selective pharmacophores targeting individual protomers (within the heterodimer) bound by a molecular bridge. For instance, a bivalent ligand targeting the DOR-KOR heteromer was developed (KDAN18) that was a KOR agonist and a DOR antagonist [127]. This compound, when administered intrathecally led to antinociceptive activity (as determined by the tail flick assay) that was blocked by the DOR antagonist (NTB) and by the KOR antagonist (nor-BNI), demonstrating the binding of the bivalent ligand to DOR and KOR [127], and suggesting a role for DOR-KOR heteromer in antinociception.

While the “bivalent ligand” approach is both rational and intuitive, one potential obstacle for the use of such ligands for clinical purposes is that they do not fit Lipinski’s “rule-of-five” [128] and hence could make them unsuitable as potential drugs. An alternate idea, that heteromerization leads to an alteration of the heteromer binding pocket, was probed by Waldhoer et al. [129] using 6'-guanidinonal-trindole (6'-GNTI). This compound follows Lipinski’s rule and thus is likely to be

a “drug-like” molecule. This compound was found to bind and activate the DOR-KOR heteromer but not DOR or KOR homomers.

In vivo experiments demonstrated that 6'-GNTI elicited analgesia when administered directly into the spinal cord, but had virtually no effect when administered directly into the brain. Moreover, this spinal-selective analgesic effect was blocked by a selective bivalent DOR-KOR antagonist, confirming the DOR-KOR heteromer as a functional target for analgesia in vivo [129]. These findings are consistent with the idea that heteromerization potentially contributes to the formation of a novel ligand-binding pocket and this would allow the design of new heteromer-specific compounds. Such compounds will be useful to target heteromers whose levels may be modulated under pathological conditions.

15.7 Receptor Heteromerization and Pathophysiology

A role for MOR-DOR heterodimerization in the development of analgesia and tolerance has been proposed for a long time. Several lines of evidence indicate that MOR and DOR interact to influence each other’s properties [62]. Although morphine acts primarily via MOR [130], DOR is critical for the development of morphine tolerance. Studies with DOR knockout animals have shown that these animals do not develop morphine tolerance [65, 131]. In addition, animals with reduced cell surface DOR expression (as seen in the case of knockout mice for the preprotachykinin A gene) do not develop morphine tolerance [132]. Furthermore, chronic morphine treatment up-regulates DOR [133, 134], and this leads to changes in MOR function, such as increased morphine efficacy [132].

Taken together, these results indicate that MOR and DOR functionally interact and that tolerance to morphine requires the presence of functional DOR at the plasma membrane. This is supported by studies showing that DOR is involved in modulating MOR function at the pharmacological level. For example, mice treated with DOR antagonists exhibit diminished morphine tolerance and dependence [64, 65]. Treatment with a combination of morphine and a DOR-selective antagonist leads to an enhancement in the antinociceptive potential of morphine [39]. Finally, a combination ultra low doses of DOR antagonists with spinal morphine leads to enhancement of morphine’s analgesic potential and also blocks the induction of tolerance [66]. These results, taken with the finding that MOR-DOR constitutively recruits and signals through β -arrestin2 [71] and that animals lacking β -arrestin 2 fail to develop morphine tolerance [135], indicates that MOR-DOR heteromers represent an exciting new target for the development of an opiate analgesic without side-effects such as development of tolerance and dependence.

If the levels of opioid receptor heterodimers are modulated by chronic morphine use, development of tolerance and dependence and/or other disease states (neuropathic pain, inflammatory pain), then ligands selectively targeting the heteromers would prove to be novel drugs in the treatment of these chronic debilitating conditions. In this context, we recently generated antibodies that selectively recognize

MOR-DOR heteromers and showed that chronic morphine treatment leads to the up-regulation of MOR-DOR heteromer levels in areas of the brain involved in the modulation of pain transmission such as the rostral ventral medulla and the medial nucleus of the trapezoid body [136].

15.8 Conclusion

Opioid receptor heteromerization leads to the formation of receptor complexes with distinct properties; hence the heteromer can be considered as a novel functional unit. It is interesting to note that each of the heteromer pairs exhibits unique altered characteristics including changes in ligand binding, coupling, signaling, trafficking, and/or physiological properties. This suggests the possibility of the existence of an array of different functional units, depending on the GPCRs expressed (i.e., the cellular context) in areas of the brain expressing opioid receptors.

At the molecular level, the next challenges for opioid heteromer biology are, to unravel the interacting surfaces of heteromers by molecular modeling and site-directed mutagenesis (and perhaps by crystallography), to explore the mechanisms of protomer trans-activation and to elucidate the new ligand binding pockets. These studies would be useful in the design of novel heteromer-specific reagents (ligands, allosteric modulators and antibodies). At the physiological level, the challenges are to understand the role and regulation of heteromerization in health and disease states that would allow development of novel therapies for the treatment of a variety of disorders.

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Chapter 16

Molecular Modulation of In Vivo Tolerance

Charles E. Inturrisi and Ann M. Gregus

Abstract Opioid tolerance can limit the clinical utility of opioids for pain management. It is a neuroadaptive response to the repeated administration of an opioid. Opioid tolerance is predominantly of the pharmacological (nonassociative) and pharmacodynamic type. Possible contributions to tolerance at the receptor level include receptor desensitization, internalization, and recycling. Alterations of G proteins, other intracellular signaling cascades and receptor–receptor interactions also may be involved. At the systems level, opioid-induced hyperalgesia, *N*-methyl-D-aspartate receptor mechanisms, activation of the immune system, and genetic factors have all been implicated. Each of these potential contributors is discussed.

Establishing the mechanism(s) of tolerance remains an elusive as well as a continuing challenge. However, some of the mechanisms discussed in this chapter appear to have the potential to provide new approaches for reducing or eliminating the development of opioid tolerance.

Keywords Analgesic tolerance • Pharmacological tolerance • Cross-tolerance • Cellular tolerance • Receptor desensitization • Intracellular signaling • Opioid-induced hyperalgesia • *N*-methyl-D-aspartate receptor • Immune system • Genetic factors

16.1 Introduction

In this chapter we will discuss opioid tolerance. First we will examine the definitions and clinical consequences of opioid tolerance with the focus almost exclusively on tolerance to the antinociceptive or analgesic effects of mu opioids. Then we will examine some of the proposed mechanism of tolerance from the

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perspective of opioid receptor adaptations (within-systems mechanisms) and how other systems influence the development and expression of opioid tolerance (between-systems adaptations).

The clinical utility of opioids for pain management can be limited significantly by the development of analgesic tolerance that can occur in many patients receiving long-term therapy [1, 2]. In addition to analgesic tolerance, some meaningful degree of tolerance also develops to opioid-induced respiratory depression, sedation, and nausea with chronic administration. In contrast, significant tolerance does not appear to develop to the constipating effects of opioids. The cellular mechanisms responsible for the very limited tolerance to the constipating effects of opioids are not understood.

Electrophysiological studies found that excitatory myenteric neurons from morphine-tolerant animals require increased morphine concentrations to produce the same inhibition of firing compared to naive tissues. However, neither mu opioid receptor (MOP) (previously termed MOR) function nor signaling mechanisms are impaired in morphine-tolerant myenteric tissues [3]. In addition, persistent occupation of MOP causes a sustained hyperpolarization of myenteric neurons, which may explain the powerful contractions of gastrointestinal smooth muscle that are seen with opioid withdrawal [3].

Opioid tolerance is defined as a reduction in response to the same dose of an opioid after repeated exposure or when increasing larger doses must be administered to obtain the effects observed with the original dose. In pharmacological terms, tolerance is the reduction of analgesic potency, which may be illustrated quantitatively by a rightward shift in the dose-response curve for analgesia and an increase in the ED₅₀, or median effective dose [4, 5].

In a small controlled trial, Houde [5] found that in cancer patients who received morphine for pain each day as required for 1 week, the morphine dose-response curve was shifted to the right so that 15 mg of morphine provided the same pain relief as 10 mg on day 1. Thus, tolerance can be defined in terms of the magnitude of the change in response and the temporal onset of this change.

In more general terms, a decrease in the effectiveness of drug as a result of repeated administration may be the result of the development of associative tolerance or due to pharmacological (nonassociative) tolerance. Preclinical studies showing that the analgesic effects of morphine can be environmentally dependent have led to the conclusion that the tolerance observed in these paradigms is not entirely pharmacologically based but may include a conditioned or associative component.

For some classically conditioned behaviors, the conditioned responses are compensatory or opposite to the unconditioned responses, as is the case with associative tolerance (see [6] for a review). *Pharmacological* (nonassociative) tolerance can be the result of changes in drug disposition, usually as a consequence of the induction of drug biotransformation reactions that decrease the effective concentrations of the drug of interest. This type of tolerance is designated *pharmacokinetic* tolerance. Alternatively, tolerance can result from a neuronal adaptation that decreases the response to the presence of constant or increasing concentrations of drug, an

adaptation known as pharmacodynamic tolerance. Tolerance to opioids is predominantly of the pharmacodynamic type.

In the tolerant state, analgesia may be restored by increasing the dose and/or frequency of administration of the opioid, but this approach can result in the exacerbation of adverse effects typically observed with therapeutic doses (e.g., sedation and constipation) and may initiate adverse effects such as multifocal myoclonus that are manifest at higher doses [7]. Clinically, tolerance may be observed as an increase in opioid requirements so that consideration must be given to those factors in the clinical situation that can result in a decrease in opioid analgesia and/or dose escalation. In addition to pharmacodynamic tolerance resulting in a loss of opioid potency, progression of disease (e.g., malignancy, arthritic changes, nerve damage), the effects of treatment (e.g., chemotherapy or radiation), psychological factors (e.g., depression, anxiety), addiction and/or diversion and opioid-induced hyperalgesia (OIH) can contribute or be the primary cause of dose escalation [8].

In pain patients it is difficult to separate a change in the pain stimulus (worsening pain) from the development of tolerance per se. Clearly in those patients whose opioid dose must be rapidly escalated to provide analgesia, the contribution of progression of disease must be evaluated. Thus in some patients, tolerance may be a primary driving force for analgesic dose escalation, or it may develop following the escalation of the opioid to manage an increase in pain.

Often the first sign of the development of opioid analgesic tolerance is a decrease in the duration of pain relief so that while some analgesia occurs at the time of the peak response, the duration of effective analgesia is appreciably diminished. There are no prospective randomized controlled trials on the actual rate of development of tolerance with chronic opioid administration. Some observational studies report a pattern of relatively rapid dose escalation lasting several weeks to a few months followed by a period of up to 3 years in which doses increase at a slower rate. In advanced cancer patients, progression of metastatic disease with increasing severity of pain was the major factor in escalation of opioid dose [9]. However, many patients particularly with noncancer pain discontinue chronic opioid therapy due to adverse effects that clearly limits the generality of these observations [8].

It is essential that the term tolerance is distinguished from the terms opioid physical dependence and opioid addiction. Opioid physical dependence refers to an altered physiological state produced by repeated administration that necessitates the continued administration of the opioid to prevent the appearance of a stereotypical syndrome – the withdrawal or abstinence syndrome that is characteristic for the particular opioid. In contrast, opioid addiction is a behavioral pattern of use, characterized by overwhelming involvement with the use of an opioid (compulsive use), the securing of a supply, continued use despite harm, and a high tendency to relapse after withdrawal.

Cross-tolerance refers to whether tolerance to the effects of one opioid confers tolerance to another opioid. Cross-tolerance is readily demonstrated in animal studies [10]. In a small controlled study in cancer patients with pain, Houde et al. [4, 5] measured tolerance and cross-tolerance between morphine and metopon (a semisynthetic analogue of morphine, 5-methyldihydromorphine) (Fig. 16.1).

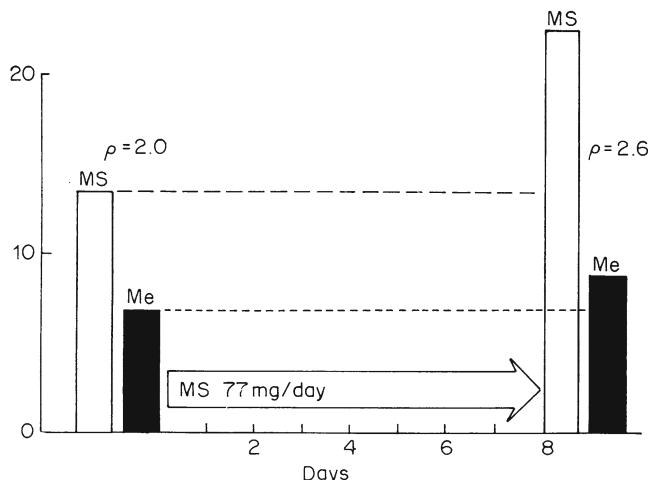


Fig. 16.1 Tolerance and apparent cross-tolerance to morphine plotted in terms of equianalgesic doses of morphine (open bars) and metopon (filled bars). Cancer patients were given morphine as required for pain in the interval between the first equianalgesic dose estimation (left plot) and after second determination (right plot). The patients received an average of daily dosage of 77 mg of morphine per day for 8 days (chronic administration). The relative potency estimates are indicated by rho (ρ). Initially metopon was twice as potent as morphine ($\rho=2.0$). By day 8, the relative potency of morphine had decreased so that metopon was now 2.6 times more potent than morphine ($\rho=2.6$) (from ref [5] with permission of the publisher)

He found that at the start of the study, the analgesic potency ratio of metopon to morphine was 2.0; i.e., metopon was two times more potent than morphine. Next, these patients received morphine on demand for the next week. On day 8 the analgesic potency ratio again was determined. The metopon to morphine ratio had increased to 2.6. Houde also did the converse experiment and found that if metopon was given on demand for 1 week instead of morphine, cross-tolerance to morphine was less than the analgesic tolerance to metopon [4]. Houde concluded that these results were observed because the tolerance to repeated morphine, which all the patients had been receiving, was greater than the cross-tolerance to “challenge” doses of metopon.

Further, he suggested that while some cross-tolerance was observed among mu opioids such as morphine and metopon, it was partial or “incomplete.” This observation, together with clinical anecdotal observations with other mu opioids is the basis for the use of opioid rotation in pain management. Thus, as tolerance or limiting adverse effects develop to the analgesic effects, a patient is switched from one mu opioid to another with the expectation that incomplete cross-tolerance will allow the use of a lower dose of the new opioid. Since the adverse effects of opioids are dose-related, the switch can allow more flexibility in dosing and open wider the therapeutic window between analgesia and adverse effects. However, this rotation from one opioid to another requires an estimate of the equianalgesic dose conversion ratio and this has led to confusion and controversy [11–13].

One pharmacological explanation for incomplete cross-tolerance suggests that the dose–response curves of mu opioids may not be parallel. The consequence of nonparallel analgesic dose–response curves is that the equianalgesic dose ratio will change as the dose of opioid is increased (i.e., as you move up the dose–response curve). Thus, for example, in Fig. 16.2 the dose ratio of drug A to B is 1:2 at the low end of the curve (10 mg of A is equianalgesic to 20 mg of B). If the dose–response curves remain parallel as the dose required to produce analgesia to A is increased (presumably due to the development of tolerance), then the 1:2 equianalgesic dose conversion ratio would be correct at all doses shown (compare curve A with dotted line curve for B). However, if the dose–response curve for B (dashed line) is not parallel to A (in this case as a

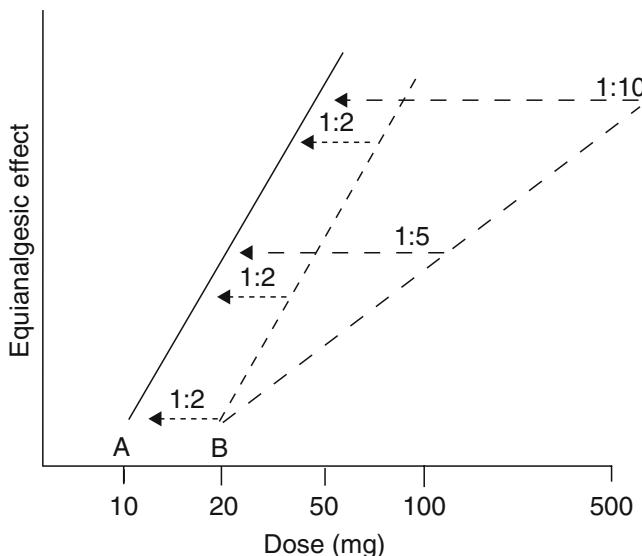


Fig. 16.2 The consequences of nonparallel analgesic dose–response curves for the estimation of the equianalgesic dose ratio as the dose is increased with the development of tolerance. The dose ratio of drug A to B is 1:2 at the low end of the curve (10 mg of A is equianalgesic to 20 mg of B). If the dose–response curves remain parallel as the dose required to produce analgesia to B is increased (presumably due to the development of tolerance) then the 1:2 equianalgesic dose conversion ratio would be correct at all doses shown (compare *curve A* with *dotted line curve* for B). However, if the dose–response curve for B (*dashed line*) is not parallel to A (in this case as a result of the more rapid development of tolerance to B than to A), then the initial equianalgesic dose conversion ratio of 1:2 is not correct as the dose of B is increased. In this example, the dose ratio of A to B is 1:5 in mid-range (20 mg of A is equianalgesic to 100 mg of B) and 1:10 at the higher end of the dose–response curve (compare the *curve* for A with the *dashed curve* for B). If the development of tolerance to drug B necessitated rotation to drug A, then the equianalgesic dose ratio would depend on where on the dose–response curve for B the rotation occurred. If it occurred at the high end of the dose–response curve for B, then use of the “standard” 1:2 ratio rather than the actual 1:10 ratio would result in the calculation of a rotation dose of B that is fivefold higher than is required.

result of the more rapid development of tolerance to B than to A), then the initial equianalgesic dose conversion ratio of 1:2 is not correct as the dose of B is increased.

In this example, the dose ratio of A to B is 1:5 in mid-range (20 mg of A is equianalgesic to 100 mg of B) and 1:10 at the higher end of the dose-response curve (compare the curve for A with the dashed curve for B). If the development of tolerance to drug B necessitated rotation to drug A, then the equianalgesic dose ratio would depend on where on the dose-response curve for B the rotation occurred. If it occurred at the high end of the dose-response curve for B, then use of the “standard” 1:2 ratio rather than the actual 1:10 ratio would result in the calculation of a rotation dose of A that is fivefold higher than is required. At the receptor level, these differences in dose-response may reflect the recently identified and characterized MOP receptor splice variants. Thus, incomplete cross-tolerance among MOP receptor ligands might reflect the differing selectivities of mu ligands for these MOP receptor subtypes [10].

16.2 Mechanisms of Opioid Tolerance

The mechanisms believed to underlie opioid tolerance may be classified into two categories: within-systems and between-systems [14]. These mechanisms of tolerance are not necessarily mutually exclusive, as there is abundant evidence in support of anatomical and functional overlap of both processes [14].

16.2.1 Cellular Tolerance: Within-Systems Adaptations

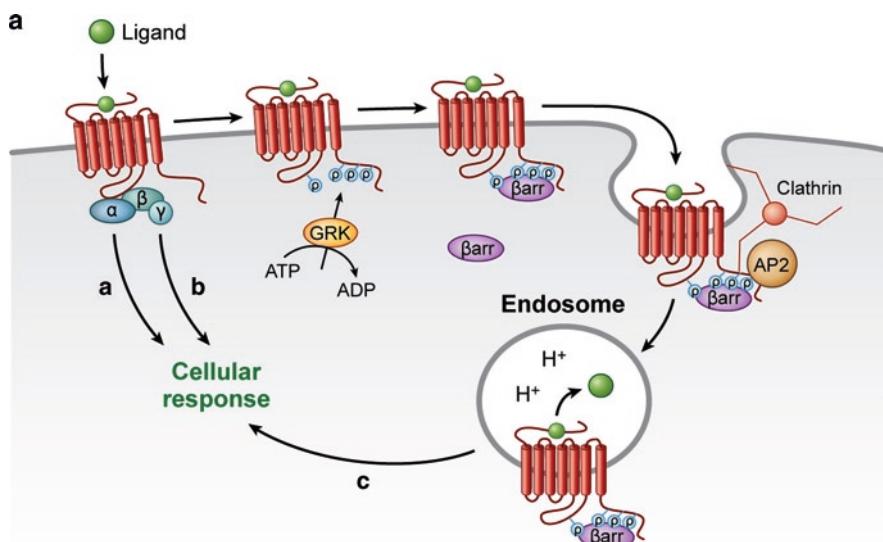
Within-systems processes of tolerance, often dubbed *cellular* or *homologous* tolerance, describe adaptations occurring at the level of the opioid receptors, which alter the number of functional receptors or their ability to signal to downstream effectors involved in analgesia. These adaptations can include modifications of the receptor itself, such as μ opioid agonist-induced phosphorylation of the MOP [15, 16], followed by desensitization that may include receptor internalization, endocytosis and downregulation [17–22].

16.2.1.1 Receptor Desensitization, Internalization, and Recycling

The MOP is believed to be desensitized by a mechanism that is similar to that described for the β -adrenergic receptor [23]. Figure 16.3a illustrates these trafficking steps. Ligand activation of the MOP promotes phosphorylation of the receptor by a G-protein-coupled receptor kinase (GRK), which allows the recruitment of β -arrestin (β arr) from the cytoplasm. This process desensitizes the receptor so that it can no longer couple to the heterotrimeric G proteins (α , β , γ), rendering it unable to signal downstream effects (cellular responses, a and b) and facilitating

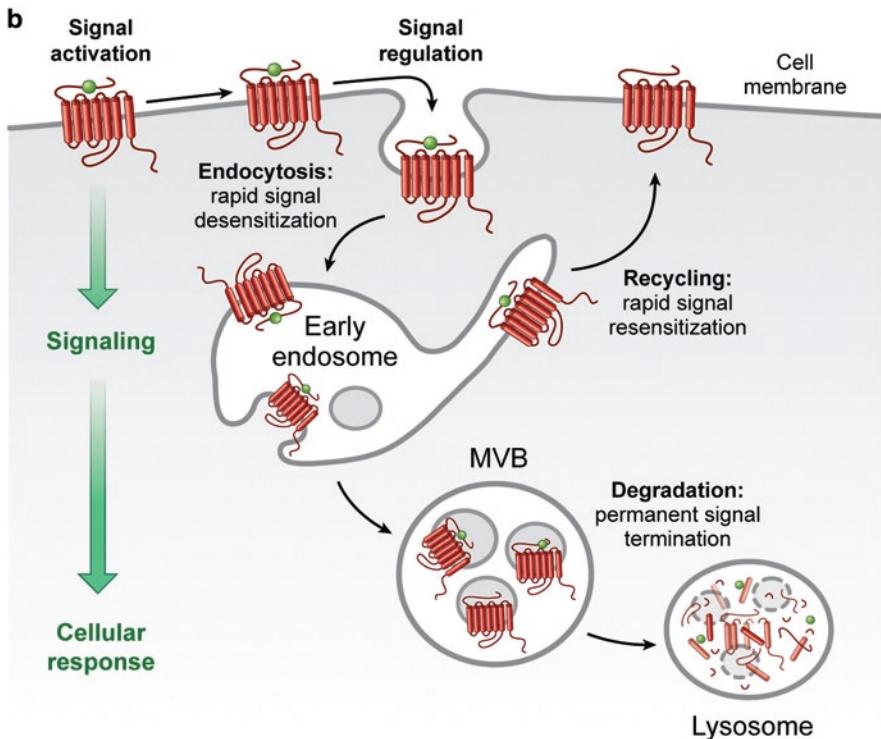
its endocytosis via clathrin-coated pits. Figure 16.3b shows that endocytosis results in trafficking of the MOP to early endosomes, where it can be recycled back to the cell membrane or degraded. The recycling pathway has been hypothesized to be a critical step in the recovery from desensitization in which phosphatase activity is necessary for the reinsertion of receptors in the membrane [23, 24].

Controversy on the role of internalization and endocytosis in the desensitization of the MOP and the development of tolerance is based in part on reports that morphine binding does not direct internalization of the MOP in some systems and that MOP mutations that facilitate endocytosis actually reduce the development of cellular tolerance [25]. Using a system that allowed the internalization of the MOP into neurons to be followed under physiological conditions in real time, Arttamangkul et al. [24] studied desensitization and MOP trafficking of selected opioid agonists. Three patterns of receptor trafficking and desensitization were observed. Met-enkephalin, etorphine and methadone produced both desensitization and receptor internalization; morphine and oxymorphone produced desensitization but not receptor internalization; and oxycodone was ineffective in both processes.



A Hanyaloglu AC, von Zastrow M. 2008.
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Fig. 16.3 Receptor desensitization and internalization of G protein-coupled receptors. (a) Ligand-bound phosphorylation of the receptor promotes the recruitment of arrestins (β arr) and receptor internalization (endosome). (b) Sorting of endocytosed receptor between divergent pathways produces recycling or degradation of the receptor. (c) Receptor phosphorylation promotes recruitment of arrestins from the cytoplasm, preventing subsequent activation of G proteins by receptors and promoting receptor endocytosis via clathrin-coated pits. There is emerging evidence that some GPCR-mediated signaling events may occur from the endosome membrane (from ref [23] with permission from the *Annual Review of Pharmacology and Toxicology*, www.annualreviews.org)



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Fig. 16.3 (continued)

These results support the hypothesis that ligand-specific regulation of opioid receptors occurs in these brain slice neurons. However, as the authors indicate, “Given that all opioid agonists result in tolerance, it is clear that no one process can completely account for the whole animal response to opioids” [24]. In addition, they suggest that mechanisms that produce rapid desensitization of the MOP are not the only adaptations that contribute to the reduction of opioid agonist potency that is manifest as opioid tolerance.

16.2.1.2 G Protein Signaling

Other cellular events that may contribute to tolerance include altered MOP-mediated signaling through G proteins [26]. These include the enhanced activity of G_s proteins [27, 28], the release of $G_{\beta\gamma}$ subunits [27], and/or the modulation of G proteins by regulators of G protein signaling (RGS) proteins [29]. During chronic opioid exposure a shift

occurs in MOP-coupled signaling from predominately G_{ia} , an inhibitory G protein, to G_{sa} , a stimulatory G protein resulting in the activation of adenylyl cyclase. Gintzler and Chakrabarti [30] have reviewed these within-systems post-receptor adaptations and proposed that this shift to G_s may account for the loss of analgesic (agonist) activity and the emergence of excitatory activity during chronic opioid administration.

Dual Receptor Targeting

The recognition that opioid receptors exist as dimers (both homodimers and heterodimers) has opened new areas of opioid pharmacophore investigation and helped to explain long-standing observations about the role of opioid receptor interactions in the modulation of tolerance [31, 32]. Functional blockade of delta opioid receptors (DOP) by selective antagonists [33], antisense [34], or gene deletion [35] attenuates the development of morphine tolerance and decreases other MOP-associated adverse effects. These observations suggested that a bivalent ligand that blocked the DOP while activating the MOP might result in a compound that produces analgesia while limiting the development of tolerance. Figure 16.4 shows an example of a bivalent ligand that is produced by linking oxymorphone (*right*, a MOP agonist) and naltrindole (*left*, a DOP antagonist), which can be used to simultaneously activate the MOP and block the DOP. A discussion of this approach is given by Dietis et al. [32].

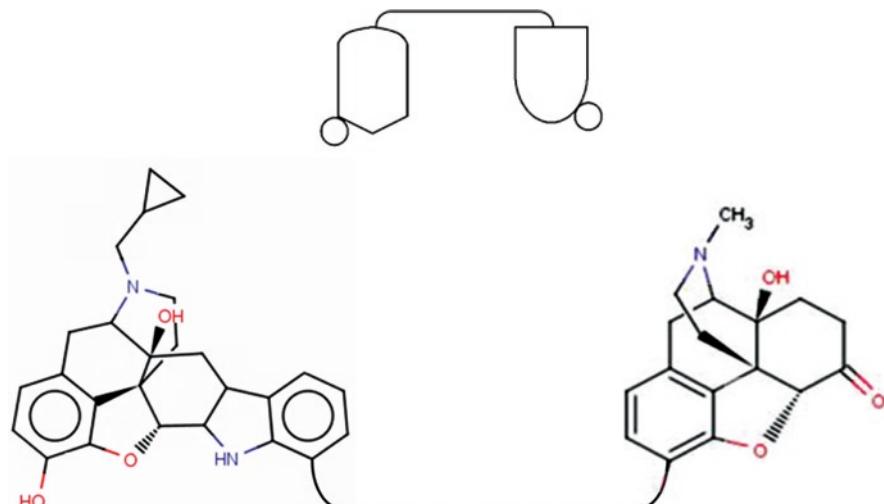


Fig. 16.4 Role of receptor heterodimers in the development of opioid tolerance. A bivalent ligand produced by linking oxymorphone (*right*, a MOP agonist) and naltrindole (*left*, a DOP antagonist) can be used to simultaneously activate the MOP and block the DOP (from ref [32] with permission of the publisher)

16.3 Intracellular Signaling and Opioid Tolerance

16.3.1 Protein Kinase C (PKC)

PKC is a family of second messenger-dependent protein kinases. In vitro and in vivo (preclinical) data demonstrate that selective inhibitors of PKC isoforms α , γ , and ϵ prevent or reverse the development of morphine tolerance [36]. In vitro studies indicate that mu opioid-mediated stimulation leads to translocation of PKC to the plasma membrane [37] and phosphorylation of the N-methyl-D-aspartate receptor (NMDAR). This process is believed to contribute significantly to tolerance development [38] and OIH (see below).

Constitutively active PKC also may phosphorylate MOP causing its desensitization, although this effect appears to vary with cell type and mu agonist potency [36]. The intrathecal (i.t.) administration of a PKC inhibitor [39] or antisense targeting PKC α [40] reduced i.t. morphine tolerance in rats. Finally, intracerebroventricular (i.c.v.) co-administration of selective inhibitors of receptor for activated C-kinase (RACK) proteins that bind α , γ , or ϵ isoforms of PKC reversed systemic morphine tolerance in mice [41].

16.3.1.1 Extracellular Signal-Regulated Kinases (ERK) and P38 Mitogen-Activated Protein (MAP) Kinases

The MAP kinases ERK1 and ERK2 (ERK) along with the α (neuronal) and β (glial) isoforms of P38 MAP kinases (P38) are expressed constitutively in brain and spinal cord [42, 43]. The activation of ERK by MOP occurs in a ras-dependent manner via the release of G $_{\beta\gamma}$ subunits [44] from G $_i$ /G $_o$ [45]. Phosphorylation of ERK and P38 as well as their subsequent regulation of gene expression is dependent on local increases in intracellular Ca $^{2+}$ levels, potentially via influx through NMDAR [46, 47]. Systemic morphine-induced phosphorylation of ERK has been observed in rat spinal cord dorsal horn [48, 49] in association with the development of morphine tolerance. Increased levels of phosphoP38 were observed in rat spinal microglia following repeated i.t. morphine treatment [50], while tolerance was attenuated by daily i.t. co-administration of a P38 inhibitor [50].

16.4 Mechanisms of Opioid Tolerance at the Systems Level

Between-systems processes of tolerance, on the other hand, are characterized by interactions that antagonize or compensate for the effects of the opioid usually via some direct anti-opioid mechanism. These anti-opioid factors may include non-opioid receptors, neurotransmitters, ion channels and second messengers [38]. One behavioral theory that is consistent with the observation that chronic exposure to opioids is

followed by a loss of analgesia and decreased pain thresholds (hyperalgesia) when opioid administration is discontinued is the opponent-process theory [51].

Applied to opioid administration/pain thresholds, this theory postulates that adaptive processes are recruited to counteract the analgesic effects of the opioid and return the elevated pain threshold towards baseline values (i.e., tolerance) [52, 53]. This model predicts that when opioid administration is abruptly interrupted (e.g., by an opioid antagonist) pain thresholds may drop transiently below baseline values and be manifest as hyperalgesia [53]. In rodents, this pain facilitatory process appears to be the result of activation of the NMDAR [52, 53] and neuroimmune factors [54] (see below). Furthermore, when opioid administration is stopped, a new equilibrium may develop between pronociceptive and endogenous antinociceptive systems [53].

16.4.1 Opioid Tolerance and Opioid-Induced Hyperalgesia

Preclinical studies suggest that opioid tolerance may result from excitatory central nervous system (CNS) changes that facilitate transmission of and increase sensitivity to pain [55]. This condition is termed *opioid-induced hyperalgesia* (OIH). The basic phenomenon appears to result from the up-regulation of pronociceptive systems. The neuroanatomical substrates and signaling pathways involved in OIH are emerging [55].

One model is derived from the ability of NMDAR activation to mediate excitatory activity and the ability of NMDAR antagonists to attenuate or reverse opioid tolerance [56, 57], OIH and some forms of neuropathic pain. This model (Fig. 16.5) suggests that these phenomena share some common mechanistic components [58]. Based on studies of the effects of morphine on glutamate transporters (GLU) in the spinal cord dorsal horn, some of the sequential steps in the development of morphine tolerance have emerged.

As depicted in Fig. 16.5, Morphine activates the MOP leading to inhibition of potassium (K^+) channels, one of the mechanisms responsible for the analgesic effects of morphine. With persistent morphine administration, there is a down-regulation of spinal GLU. Decreased surface expression of GLU results in increased synaptic glutamate, leading to activation of the NMDAR and causing an influx of calcium via the NMDAR channel into the postsynaptic cell [58, 59]. Stimulation of the MOP also may result in the activation of PKC and other calcium-sensitive intracellular signaling cascades, producing subsequent phosphorylation of the NR1 subunit of the NMDAR, thereby removing its voltage-dependent magnesium (Mg^{2+}) block. The resulting sustained activation of the NMDAR produces additional downstream changes including the activation of nitric oxide production [60]. These events ultimately lead to desensitization of the MOP. This process of sustained NMDAR activation, phosphorylation and downstream changes in gene expression is also characteristic of central sensitization [61, 62]. NMDAR-mediated central sensitization is involved in the hypersensitivity observed with inflammatory and neuropathic pain states [63].

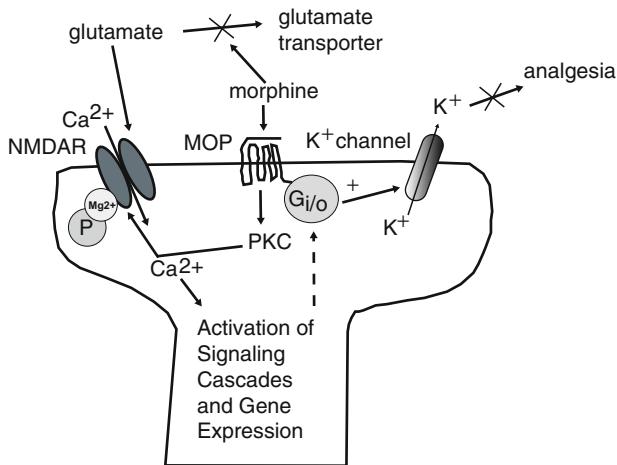


Fig. 16.5 The effects of morphine on spinal cord dorsal horn neurons may result in analgesia, hyperalgesia and tolerance. Morphine activates the MOP leading to inhibition of potassium (K^+) channels, one of the mechanisms responsible for the analgesic effects of morphine. With persistent morphine administration, there is a down-regulation of spinal glutamate transporters (GLU). Decreased surface expression of GLU results in increased synaptic glutamate, leading to activation of the NMDAR and causing an influx of calcium via the NMDAR channel into the postsynaptic cell [58, 59]. Stimulation of the MOP also may result in the activation of PKC and other calcium-sensitive intracellular signaling cascades, producing subsequent phosphorylation of the NR1 subunit of the NMDAR, thereby removing its voltage-dependent magnesium (Mg^{2+}) block. The resulting sustained activation of the NMDAR produces additional downstream changes in signaling cascades and gene expression (see above for some examples) that presumably result in desensitization of the MOP and tolerance

As shown in Fig. 16.6, although both tolerance and OIH reduce the potency of a given dose of the opioid (X), the dose-response characteristics of a loss of opioid potency due to tolerance are different from the change in potency that is seen with OIH. Tolerance results in a shift to the right of the dose-response curve (A to C), while OIH results in downward shift of the dose-response relationship (A to B) [55].

In addition, the magnitude of the contribution of OIH to clinical opioid tolerance and its consequences for continued opioid therapy remain controversial. There is general agreement that during opioid withdrawal, OIH may occur and contribute to an exacerbation of pain [64]. Therefore, acute withdrawal in opioid tolerant/dependent patients should be avoided. Switching from a morphine-like opioid to a mixed agonist-antagonist (pentazocine, nalbuphine, and butorphanol) or the partial agonist buprenorphine should be avoided because of these drugs' ability to induce abrupt opioid withdrawal and cause concomitant hyperalgesia in opioid-dependent individuals. [2] In contrast, employing a combination of an

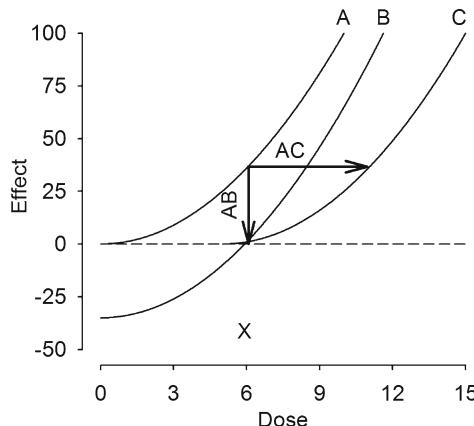


Fig. 16.6 Tolerance and OIH have different effects on the analgesic dose–response relationship. However, both result in an increase in analgesic dose requirements and therefore reduce the potency of a given dose of the opioid (X). The hypothetical dose–response curve for opioid-naïve patients is shown as (A). In opioid-induced hyperalgesia (OIH), the dose–response curve of the chronic opioid user is shifted downward (AB) and the patient experiences increased pain to noxious stimuli at baseline (shown as decreased analgesic response when analgesic dose is zero (B)). In analgesic tolerance, the slope of the dose–response curve of the chronic opioid user is shifted to the right (AC). In contrast to OIH, there is no significant change in pain sensitivity at baseline (C). This is shown as an identical analgesic response in opioid-naïve and chronic opioid users when analgesic dose is zero (*dashed line*) (from ref [55] with permission of the publisher)

opioid with a non-opioid can enhance analgesia and reduce the rate of tolerance development since tolerance does not develop to the non-opioid component of the mixture.

16.5 Activation of Glia and Cytokine Production

The role of the immune system in maintenance of inflammatory and neuropathic pain states is well established. Because of the pronociceptive actions of many cytokines, the interactions between chronic morphine administration in the presence and absence of injury states has been examined [65].

Chronic administration of morphine to rats has been shown to activate spinal glia and upregulate proinflammatory cytokines such as interleukin (IL)-1 β , IL-6, and tumor necrosis factor (TNF) alpha [65]. In addition, spinal inhibition of proinflammatory cytokines restored acute morphine antinociception and significantly reversed the development of morphine tolerance and withdrawal-induced hyperalgesia and allodynia in nerve-injured or sham-operated rats [54]. These results suggest that targeting glial activation and central cytokine production may

reduce the development of morphine tolerance as well as the expression of injury-induced hyperalgesic states [50, 54, 66].

16.6 Genetic Factors

Kest et al. [67] confirmed and extended a long-standing observation that morphine tolerance varied considerably among inbred rodent strains. In addition, these investigators found that the magnitude of OIH and analgesic tolerance was correlated significantly among these strains, suggesting a genetically-based association. Recently, Liang et al. [68] measured the susceptibility of fifteen strains of inbred mice to develop OIH. A genetic analysis resulted in the association of haplotypic blocks (conserved genomic regions) most strongly with sequences in the $\beta 2$ adrenergic receptor ($\beta 2$ -AR) gene. Using the selective $\beta 2$ -AR antagonist butoxamine, the authors observed a dose-dependent reversal of OIH. Furthermore, deletion of the $\beta 2$ -AR gene sharply reduced the mechanical allodynia present after morphine treatment in the wild-type mouse strain [68]. This is a developing area that holds much promise.

16.7 Summary

Opioid tolerance can limit the clinical utility of opioids for pain management. It is a neuroadaptive response to the repeated administration of an opioid. Opioid tolerance is predominantly of the pharmacological nonassociative and pharmacodynamic type. Possible contributions to tolerance at the receptor level include receptor desensitization, internalization, and recycling. Alterations of G proteins, other intracellular signaling cascades, and receptor–receptor interactions also may be involved. At the systems level, OIH, NMDAR mechanisms, activation of the immune system, and genetic factors have been implicated. Each of these potential contributors was discussed.

Establishing the mechanism(s) of tolerance remains an elusive as well as a continuing challenge. However, some of the mechanisms discussed above appear to have the potential to provide new approaches for reducing or eliminating the development of opioid tolerance.

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Chapter 17

Genetics of Opioid Actions

Jörn Lötsch and Jeffrey S. Mogil

Abstract The actions of opioids feature robust interindividual variability in their therapeutic and side-effects, complicating the clinical management of pain. Much of this variability is assumed to be due to genetic factors, and there is tremendous current interest in identifying the responsible genes and DNA polymorphisms. This review examines the current state of knowledge regarding opioid genetics in both rodents and humans, with special focus on the results of genetic linkage mapping studies in mice and genetic association studies in humans. At present, there is tentative evidence for the involvement of a handful of genes in modulating the potency and efficacy of opioid drugs, although much of this evidence is controversial. A fuller understanding of this field may lead to advances in the idiosyncratic management of pain and the treatment of opiate addiction, but the genetic complexity of the trait suggests that much work remains to be done.

Keywords Genetics • Opioid • Opiate • Analgesia • Pain • Association studies • Linkage mapping • Addiction • Side effects

17.1 Genetics of Opioid Analgesia

17.1.1 *Individual Differences in Opioid Analgesia in Humans*

Opioid drugs are among the most valuable medicinal compounds ever known to man, and this value derives from their high and reliable efficacy. Morphine – at some dose – inhibits pain of virtually any type, in virtually every context, and in virtually every person. Individual differences in the action of morphine and other

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opioids would not appear to be a major issue. However, as a practical matter, morphine features enough interindividual variability in its potency (if not necessarily in its maximum effect) to complicate its clinical use, even in the relatively straightforward case of the management of post-operative pain. Underdosing leaves the patient with unrelieved pain, a major risk factor for the development of chronic pain states. Overdosing leads to unwanted side-effects including sedation, constipation, mental clouding, and dependency. Thus, even for morphine, an understanding of those factors affecting the drug's potency (or its efficacy at the maximum tolerable dose) from one person to another would be immensely useful clinically.

The first systematic attempt to document the variability associated with clinical morphine analgesia was by Lasagna and Beecher [1], who examined the efficacy of 10 and 15 mg morphine against post-operative pain. Both doses featured high variability in efficacy, duration, and side-effects, and the authors concluded that one-third of patients given 10 mg and 20% of those receiving 15 mg were “not adequately relieved” [1].

Remarkably similar findings were obtained testing morphine’s effect against electrical pain in the laboratory, with approximately one-third of subjects displaying no statistically significant change in pain threshold or tolerance after 10 mg morphine [2]. Individual differences may be greater for morphine than for other opioids like alfentanil and fentanyl, for both pharmacokinetic and pharmacodynamic reasons [3]. There is also some evidence that variable response to opioids might be specific to the opioid used, such that an individual patient might simultaneously be resistant to one (at its maximally tolerated dose) but sensitive to another [4]. Variability is just as high when assessed via self-dosing rates in studies of patient-controlled analgesia [5].

Large modern studies have documented the impressive variability in morphine inhibition of post-operative pain [6], cancer pain [7], and chronic noncancer pain [8]. In the post-operative pain study of Aubrun and colleagues [6], intravenous morphine was titrated every 5 min in 3-mg increments, and pain was assessed every 5 min until pain relief (defined as a visual analog score <30 out of 100) was achieved. Based on the data collected from 3,170 patients, the effective dose of morphine ranged from 0.04 to 0.86 mg/kg [6]. The relationship between required morphine dose and initial pain score was significant, but only explained 12% of the variability in morphine potency.

17.1.2 What Is Responsible for Individual Differences in Opioid Potency?

Clearly then, there is important variability to explain. The next question is, to what extent is that variability produced by genetic or environmental factors, or their interaction? The “gold standard” technique for assessing heritability (i.e., degree of genetic determination) in humans is the twin study, but only one human twin study of opioid effects has been performed. Liston and colleagues [9] compared the

analgesic effect of 10 mg/70 kg morphine against ischemic pain in the cold pressor test in monozygotic twins compared to pairs of unrelated individuals. The results were admittedly nondefinitive, especially since no dizygotic twins were tested and the sample size was limited, but near-significant co-twin concordances were observed. The issue of the heritability of morphine analgesia in humans thus remains unresolved, but is now largely moot since positive human genetic association findings have been reported (see below).

One might also ask what mechanisms might underlie opioid analgesic variability? The obvious candidates are: (1) genetic or environmental effects on opioid pharmacokinetics, especially metabolism; (2) genetic or environmental effects on opioid receptor density, affinity, or coupling (i.e., opioid pharmacodynamics); and, (3) genetic or environmental effects on downstream processing of pain modulatory systems with an opioid receptor component. As will be described below, there is at least some evidence for all of these possibilities.

It is important to note, however, that opioid pharmacogenetics clearly involves more than simply the drug and its molecular targets. A hypothesis seems to be emerging in the literature that *endogenous* opioid analgesic systems – having evolved as part of a stress response [10] – are variably active and efficacious, and that their impairment might be associated with pathology requiring analgesic use.

It has been demonstrated that sustained muscle pain activates endogenous opioid release acting at μ -opioid receptors, and that the extent of this release in certain brain areas is strongly correlated to pain scores [11]. For genetic and/or environmental reasons, those with a family history of chronic pain appear to have impaired endogenous analgesia, which may in fact represent the true cause of their chronic pain [12].

Amanzio and colleagues [13] demonstrated convincingly that response variability to opioid analgesics including buprenorphine, tramadol, ketorolac, and metamizol is much higher when the injections are “open” compared to when they are “hidden,” suggesting that most of the drug variability is actually related to the placebo effect, which of course is mediated at least in part by the release of endogenous opioids [14].

17.1.3 Human Genetic Association Studies of Opioid Analgesia

Only two techniques are available in human-subject research for proving the involvement of individual genes in the mediation of biological variability: linkage mapping and genetic association [15]. In the former, the inheritance of a (usually qualitative) trait is followed in a pedigree, and compared to the inheritance of DNA variants (also called polymorphisms or alleles) spanning the genome.

To our knowledge, no linkage mapping study of opioid analgesia has ever been performed. A genetic association study involves the comparison of the frequency of DNA variants (and increasingly, blocks of nearby variants called haplotypes) in “cases” vs. “controls,” but one could just as easily compare drug responders to drug

nonresponders in this design. Until very recently, genetic association studies were focused on single candidate genes, it is now possible (although it remains extraordinarily expensive) to perform whole-genome association studies.

As of this writing, a handful of relevant genetic association studies in humans have been performed, in total considering only five genes (see [16, 17] for reviews). Studies focusing on opioid effects other than analgesia will be considered in Sect. 17.2 and 17.3 below. We focus here only on pain and pain inhibition.

17.1.3.1 OPRM1

The highest-priority candidate gene has been *OPRM1*, coding for the μ -opioid receptor, both because of the importance of the protein as the molecular binding site of virtually all clinically effective opioids but also because of the fairly common 118A>G variant. That is, at nucleotide position 118 of the *OPRM1* gene, 12% of the Caucasian population [18] possess at least one guanine (G) instead of the more common adenine (A), which results in the substitution of an asparagine instead of an aspartate as the 40th amino acid in the μ -opioid receptor protein (see Sect. 17.2.1 below).

Much of the interest in this particular *OPRM1* variant – 1 of over 100 known polymorphisms (see [19]) – stems from the observation that the affinity of β -endorphin for the μ -opioid receptor is enhanced in “mutant” (i.e., asparagine-containing) receptors [20], but subsequent studies have failed to replicate this finding for β -endorphin or any number of other opioid ligands [21, 22].

However, the variant may decrease opioid receptor signaling after agonist ligand binding in certain pain-relevant brain regions such as the secondary somatosensory area [23], and may be associated with decreased μ -opioid receptor expression in several human brain regions [24]. See Sect. 17.2.1 below for a more detailed discussion. As a clinical consequence, post-operative alfentanil requirements are increased in patients with the minor (G) allele [25], and morphine requirements in post-hysterectomy [26], post-arthroplasty [27] and cancer pain [28] patients are increased in those with a GG genotype.

The latter finding should be considered extremely tenuous as the sample size in the critical GG group was only $n=4$, and was not replicated in another study of cancer pain [29]. One other study of post-operative pain also yielded a trend towards higher morphine requirements in GG individuals, but here the $n=2$ [30]. The potency of morphine-6 β -glucuronide (M6G) against acute electrical pain in normal volunteers may be reduced in carriers of the G allele [31], but a similarly designed study failed to see an effect for either M6G or morphine [32]. Finally, a very recent study has demonstrated a significantly lower frequency of G alleles in patients with chronic pain compared to a group of acute (i.e., post-operative) pain patients, but no significant differences among genotypes were observed in pain scores or morphine requirements [33]. The complex *OPRM1* association study literature is discussed in a recent focused review [34].

Of interest is a recent paper describing a mouse mutant harboring the equivalent SNP to the human A118>G, the A112>G mouse [35]. Homozygous 112G/112G

mutants display decreased μ -opioid receptor protein expression, blunted morphine-induced hyperlocomotion, and impaired conditioned place preference and withdrawal-induced place aversion in females only. Measured on the hot-plate test, mutant mice displayed reduced morphine potency and efficacy, but no alterations in tolerance development [35]. It should be noted that a monkey orthologue, C77>G, is also known [36], but no pain-relevant studies have been conducted.

17.1.3.2 Other Genes

Besides *OPRM1*, five other genes have thus far been tested for genetic association with opioid analgesia: *COMT* (catechol-*O*-methyltransferase), *MC1R* (melanocortin-1 receptor), *KCNJ6* (potassium inwardly rectifying channels, subfamily J, member 6), *CYP2D6* (cytochrome P450 2D6), and *ABCB1* (P-glycoprotein). The latter two genes are involved in opioid pharmacokinetics, and will be discussed in Sect. 17.3 below.

COMT degrades catecholamine neurotransmitters such as norepinephrine and dopamine. Increased dopamine concentrations have been shown to suppress the production of endogenous opioid peptides [37]. Opioid receptor expression would be expected to be upregulated in compensation; for the V158M variant of *COMT* coded by the 472G>A SNP of the *COMT* gene, this has been demonstrated both in human postmortem brain tissue [38] and *in vivo* by assessing the binding of the μ -opioid receptor selective radiolabeled ^{11}C -carfentanil using positron emission tomography [39].

The variant leads to a low-functioning *COMT* enzyme, which fails to degrade dopamine, which may thus cause a depletion of enkephalin. *COMT* has been associated with both experimental and clinical pain [39–42], but here too other studies have found conflicting or no association [43–47]. With respect to morphine analgesia only one study has been performed, examining morphine requirements in cancer pain patients. It was observed that patients with the Met/Met genotype required less morphine than those with the Val/Val genotype [47]. A mouse *COMT* mutant has been tested for morphine and stress-induced analgesia (SIA), displaying a subtle and complex phenotype that depended on the nociceptive assay used [48].

MC1R is also a well-studied gene, in that it has been known for some time as the gene for red hair [49]. After the analogous mouse gene was implicated in the mediation of both κ - and μ -opioid analgesia using spontaneous mutant mice [50, 51], people with nonfunctional MC1Rs (carriers of two or more inactivating variants; all redheads) were compared to those with functional receptors (carriers of one or less inactivating variants; mostly non-redheads); increased pentazocine [50] and M6G [51] analgesia against experimental pain in redheads was observed, along with decreased sensitivity to electrical pain in the latter study.

KCNJ6 codes for potassium inwardly rectifying channels, subfamily J, member 6, (Kir3.2, GIRK2), which is a G protein-coupled channel important for opioid receptor transmission. It is involved in opioid effects on postsynaptic inhibition [52]

and mediates a significant component of analgesia [53, 54]. Genetic variants in *KCNJ6* have recently been shown to increase opioid requirements in Japanese patients after abdominal surgery [55], chronic pain, and opioid substitution therapy, but not pupil size [56].

17.1.3.3 Complicating Factors

Why are the results of these genetic association studies so contradictory? Part of the problem no doubt relates to the technique itself, which has been criticized on a number of grounds (see [57, 58]). It is also likely that the role of particular genes in mediating opioid analgesia is more subtle and complex than we might suspect. The *OPRM1* 118A>G SNP has been shown to significantly affect experimental pain sensitivity in humans, but in patterns entirely dependent on subject sex [59].

Sex-dependence was also demonstrated for *MCIR*, as pentazocine analgesia was higher in female redheads compared to non-redheads, but males of all hair colors were equianalgesic [50]. Complicating the *MCIR* story further is the fact that μ -opioid analgesia, in both mice and humans, was dependent on *MCIR* in both sexes [51].

Bruehl and colleagues [60] demonstrated a significant interaction between *OPRM1* A118G status and anger regulation style (“anger-out”) in the prediction of post-operative opioid analgesic use. Finally, in a reanalysis of two cancer pain data sets described above [28, 47], Reyes-Gibby and colleagues [61] observed a significant interaction between *OPRM1* and *COMT* genotypes in determining morphine dose requirements. As our hypotheses become more focused and sophisticated, reproducibility of genetic association study findings may improve.

17.1.4 Genetic Mediation of Opioid Analgesia: Lessons from Animal Models

The question asked by human linkage mapping and genetic association studies – what genes are responsible for inherited variability in opioid responses? – is in fact a restricted one. A larger and equally (or more) important question is: what genes are responsible for opioid actions?

This question is better answered by biological techniques using animal models, where the powerful genetic techniques of transgenesis and microarray-based gene expression profiling can be applied (see [62–64]). We have conducted thorough searches of the existing transgenic knockout literature, and have recently developed an interactive web-based browser containing results of every published study in which a transgenic knockout mouse has been tested for pain or analgesia [65]. A search of this browser (on February 9, 2010) for knockouts having been tested for opioid analgesic sensitivity revealed that 64 null mutants had shown a significant phenotypic difference in either direction compared to wildtypes. A summary of this search is presented in Table 17.1.

Table 17.1 Genes involved in mediating opioid analgesia, defined by a statistically significant null mutant phenotype (in at least one study) and identified via the *Pain Genes Database* (search conducted on February 9, 2010)

Gene	Protein	Opioid(s) ^a	Direction ^b
<i>Abcb1a</i>	ATP-binding cassette, sub-family B (MDR/TAP), member 1A	Morphine	▲
		DPDPE	▲
		M6G	▲
		Fentanyl	▲
		Methadone	▲
		Alfentanil	▲
<i>Abcc3</i>	ATP-binding cassette, sub-family C (CFTR/MRP), member 3	M6G	▼
<i>Adcy5</i>	Adenylate cyclase 5	Morphine	▼
<i>Adora2a</i>	Adenosine A2a receptor	Deltorphin	▼
		CI-977	▲
		N ₂ O	▼
<i>Adra2a</i>	Adrenergic receptor, α2a	Deltorphin	▼
		Buprenorphine	▲
		Tramadol	▲
		Morphine	▼
		U50,488	▼
<i>Alox12</i>	Arachidonate 12-lipoxygenase	Morphine	▲
<i>Aqp4</i>	Aquaporin 4	Morphine	▲
<i>Arrb2</i>	Arrestin, β2	Morphine	▲
<i>Cacna1e</i>	Calcium channel, voltage-dependent, R type, α1E subunit	Morphine	▲
<i>Calca</i>	Calcitonin/calcitonin-related polypeptide, alpha	Morphine	▼
		Morphine	▲/▼
<i>Cckbr</i>	Cholecystokinin B receptor	RB-101	▼
<i>Chrm1</i>	Cholinergic receptor, muscarinic 1, CNS	Morphine	▲
		Morphine	▼
<i>Dbh</i>	Dopamine β-hydroxylase	Morphine	▲
<i>Drd1a</i>	Dopamine receptor 1A	Morphine	▲
		Morphine	▲
<i>Drd2</i>	Dopamine receptor 2	M6G	▲
		NalBzoH	▲
		U50,488	▲
		Morphine	▲
<i>Fosb</i>	FBF osteosarcoma oncogene B	Morphine	▲
<i>Foxn1</i>	Forkhead box N1	Morphine	▼
<i>Gja1</i>	Gap junction protein, α1	Acupuncture	▼
<i>Gnaz</i>	Guanine nucleotide binding protein, α z subunit	Morphine	▼
<i>Grasp</i>	GRP1 (general receptor for phosphoinositides 1)-associated scaffold protein	Morphine	▼
<i>Grin2a</i>	Glutamate receptor, ionotropic, NMDA2A (epsilon 1)	Morphine	▲
<i>Hrh1</i>	Histamine receptor H ₁	Morphine	▲
<i>Hrh2</i>	Histamine receptor H ₂	Morphine	▲
<i>Hrh3</i>	Histamine receptor H ₃	Morphine	▲
<i>Il1</i>	Interleukin 1 complex	Morphine	▲
		Swim SIA	▼

(continued)

Table 17.1 (continued)

Gene	Protein	Opioid(s) ^a	Direction ^b
<i>Il1rap</i>	Interleukin 1 receptor accessory protein	Morphine	▲
<i>Il6</i>	Interleukin 6	Morphine	▼
<i>Itpr1</i>	Inositol 1,4,5-triphosphate receptor 1	Morphine	▼
<i>Kcnal1</i>	Potassium voltage-gated channel, shaker-related subfamily, member 1	Morphine	▼
<i>Kcnj3</i>	Potassium inwardly-rectifying channel, subfamily J, member 3	Morphine DAMGO Deltorphin	▼ ▼ ▼
<i>Kcnj6</i>	Potassium inwardly-rectifying channel, subfamily J, member 6	Morphine DAMGO Deltorphin	▼ ▼ ▼
<i>Kcnj9</i>	Potassium inwardly-rectifying channel, subfamily J, member 9	Morphine	▼
<i>Lmx</i>	LIM homeobox transcription factor 1 β	Morphine U50,488 DPDPE	▼ ▼ ▼
<i>Lyst</i>	Lysosomal trafficking regulator	Morphine DAMGO U50,488 Tfluadom	▼ ▼ ▲ ▼
<i>Mclr</i>	Melanocortin 1 receptor	Morphine M6G U50,488	▲ ▼ ▲
<i>Mme</i>	Membrane metallo-endopeptidase	Thiorphan	▼
<i>Nfkb1</i>	Nuclear factor of κ light chain gene enhancer in B-cells 1, p105	Acupuncture	▲
<i>Nos1</i>	Nitric oxide synthase 1, neuronal	Morphine DPDPE	▼/— ▼
<i>Nos2</i>	Nitric oxide synthase 2, inducible, macrophage	Acupuncture	▼
<i>Ntf5</i>	Neurotrophin 4	Morphine	▼
<i>Ntsr2</i>	Neurotensin receptor 2 (low affinity)	Swim SIA	▼
<i>Opdr1</i>	Opioid receptor, δ1	DPDPE Deltorphin BW363U86	▼/— ▼/— ▲
<i>Oprk1</i>	Opioid receptor, κ1	U50,488 Salvinorin A	▼ ▼
<i>Oprl</i>	Opioid receptor-like	Morphine Deltorphin II NalBzoH Dynorphin Buprenorphine	▼/— ▼ ▼ ▼ ▲

(continued)

Table 17.1 (continued)

Gene	Protein	Opioid(s) ^a	Direction ^b
<i>Oprm1</i>	Opioid receptor, μ	Morphine DPDPE M6G Endomorphin-1 Endomorphin-2 SNC80 Deltorphin Deltorphin II Buprenorphine RB-101 Tramadol U50,488	▼ ▼/▲ ▼ ▼ ▼ ▼ ▲/— ▼/— ▼ ▲ ▼ ▼ ▼/—
<i>Oxt</i>	Oxytocin	Swim SIA Restraint SIA	▼ ▼
<i>Pcsk2</i>	Proprotein convertase subtilisin/kexin type 2	Swim SIA	▲
<i>Pdyn</i>	Prodynorphin	U50,488	▼/—
<i>Plcb1</i>	Phospholipase C, β 1	Morphine	▼
<i>Plcb3</i>	Phospholipase C, β 3	Morphine	▲
<i>Pldn</i>	Pallidin	Morphine	▲
<i>Pnoc</i>	Prepronociceptin	N_2O	▼
<i>Prkcc</i>	Protein kinase C, γ	Fentanyl Naloxone	▲ ▲
<i>Prkce</i>	Protein kinase C, ϵ	Morphine	▲
<i>Prlhr</i>	Prolactin releasing hormone receptor	Morphine	▲
<i>Rabggtta</i>	Rab geranylgeranyl transferase, α subunit	Morphine	▲
<i>Rgs9</i>	Regulator of G-protein signaling 9	Morphine	▲
<i>Slc6a2</i>	Solute carrier family 6 (neurotransmitter transporter, noradrenalin), member 2	Morphine	▲
<i>Slc6a4</i>	Solute carrier family 6 (neurotransmitter transporter, noradrenalin), member 4	Morphine Meperidine Tramadol	▼ ▼ ▼
<i>Tacr1</i>	Tachykinin receptor 1	Morphine	▼
<i>Th</i>	Tyrosine hydroxylase	Morphine	▼
<i>Tlr4</i>	Toll-like receptor 4	Morphine	▲

^aIncluded are opioid receptor agonists/antagonists, opioid synthesis/degradation inhibitors, and manipulations known to involve endogenous opioid release. *DAMGO* [D-Ala², N-Me-Phe⁴-Gly-ol] enkephalin; *DPDPE* [D-Pen², D-Pen⁵]enkephalin; *M6G* morphine-6 β -glucuronide; *N₂O* nitrous oxide; *NalBzoH* naloxone benzoylhydrazone; *SIA* stress-induced analgesia

^bFilled triangles, knockout mice displayed more analgesia than wild-type mice; filled inverted triangles, knockout mice displayed less analgesia than wildtype mice; filled dashes, no significant difference between wildtype and knockout mice. Where more than one symbol is displayed, there is a contradiction in the literature. See the *Pain Genes Database* (http://paingenetictslab.ca/4105/06_02_pain_genetics_database.asp) for details and references

A full discussion of these findings is outside the scope of this chapter, but the sheer number of genes contained in Table 17.1 should cause one to reflect on the comprehensiveness of our current understanding in humans. If 64 genes have already been shown to play direct or indirect roles in opioid analgesic magnitude in mice (and of course, in the fullness of time this number will undoubtedly grow considerably), is it reasonable to assume that *OPRM1*, *COMT*, *MC1R*, *CYP2D6*, and *ABCB1* are the sole genetic players in humans? Human genetic studies are obviously in their infancy, and should continue to be guided by findings from animal genetic studies, which will now be described.

17.1.5 *Mouse and Rat Strain Differences in Opioid Analgesia*

Due to the capture and almost 100 years of mating of wild rats and mice by breeders catering to mouse and rat “fanciers,” there exist today large numbers of inbred strains with well-annotated pedigrees [66, 67]. The comparison of phenotypes among panels of inbred strains is the standard first step in the genetic dissection of behavioral traits.

It should be noted, however, that many other genetic models have been employed in the study of opioid analgesia, including spontaneous mutants and selectively bred mouse lines. These have been thoroughly reviewed previously (see [19, 68–72]), and will not be considered here.

17.1.5.1 *Mouse Strain Differences in Opioid Analgesic Magnitude*

Mouse strain differences in opioid analgesia have been appreciated since 1966 [73]. The robust difference between the C57BL/6 and DBA/2 mouse strains in their responses to morphine was studied intensely in the 1970s and 1980s [74–83]. Of interest from these studies was the observation that genetic sensitivity to one effect of morphine (e.g., analgesia; DBA/2 sensitive, C57BL/6 resistant) might show an inverse correlation, or even no correlation with genetic sensitivity to another effect of morphine (e.g., locomotor activation; C57BL/6 sensitive, DBA/2 resistant) (e.g., [75, 84]). As of today, robust differences among multiple inbred mouse strains have now been documented for the acute analgesic effects of the following compounds acting on the opioid system: morphine (e.g., [85–87]), acetorphan [88], D-amino acids [82, 89], [Dmt^1]DALDA [90], DAMGO [91], DPDPE [91], ethylketazocine [83], heroin [92], MR 2096 [93], MR 2266 [94], naloxone [95], naloxone benzoylhydrazone [96], nitrous oxide [97, 98], and U50,488H [85, 96].

Attempts have been made to explain such analgesic differences in terms of strain-specific opioid receptor binding [99–102], but no consensus has emerged. Many strain differences have also been observed with respect to chronic effects (e.g., tolerance, dependence) of these same drugs (e.g., [103–105]), but a full discussion of these is outside the scope of the present chapter.

Inbred mouse strain differences have also been observed in the action of endogenous analgesic systems involving the release of opioid peptides [106–113]. There is nothing extraordinary about the variability seen among strains with respect to opioid analgesia, of course, since strain differences in non-opioid analgesics such as acetaminophen [114], alprazolam [115], clonidine [85], epibatidine [85, 116], indomethacin [114], lysine acetylsalicylic acid [114], nicotine [117] orphanin FQ/nociceptin [118], oxotremorine [119, 120], and WIN55,212-2 [85] have been documented as well. Genotype-dependent opioid analgesia represents a confounding factor of virtually all studies in the field, since outbred (randomly bred) strain differences (e.g., [121, 122]) and so-called “vendor effects” [123, 124] have been demonstrated as well.

17.1.5.2 Rat Strain Differences in Opioid Analgesic Magnitude

Many fewer inbred rat strains are in common use than inbred mouse strains, but there are many examples as well of rat strain differences in exogenous and endogenous opioid analgesia [125–134]. The most systematic work in this field has been performed by Mitchell Picker and colleagues, who have studied the interaction between genotype, sex, stimulus intensity and intrinsic efficacy at the μ -opioid receptor of various opioid drugs in determining analgesia. They demonstrate that strain differences are more apparent when high-intensity stimuli and lower efficacy opioids (e.g., butorphanol, nalbuphine) are used [135, 136].

One very intriguing study performed in inbred rat strains suggest that the reason for differences in opioid analgesia may not be explainable entirely by direct genetic influences on pain-processing mechanisms. Sudakov and colleagues compared Fischer-344 (morphine sensitive) and WAG/G (morphine resistant) rats reared by dams of the same or opposite strain. The results of this cross-fostering study revealed that all rats raised by Fischer-344 mothers were sensitive to morphine, independent of genotype [137]. Unfortunately, this result has never been replicated or extended, so it is difficult to know how generalizable it may be.

17.1.5.3 Qualitative Differences in Opioid Analgesia

It is abundantly clear, and no longer a surprise, that the variability and potency and efficacy of opioid analgesics should have a genetic basis, at least in part. A number of findings in the existing literature suggest something far more interesting, that opioid analgesic mechanisms might operate in qualitatively different ways in different rodent strains.

For example, a number of forms of endogenous SIA have been found to be opioid-mediated (i.e., naloxone- or naltrexone-reversible) in some strains but non-opioid (i.e., resistant to naloxone or naltrexone blockade) in others [112, 113,

[138–141]. Strain-dependent opioid/non-opioid mediation has also been observed for nitrous oxide analgesia [97] and clonidine analgesia [142]. Other findings suggest that neural circuitries underlying drug analgesia may differ between strains.

A very early finding suggested that serotonin depletion using *p*-chlorophenylalanine decreased morphine analgesia in Sprague Dawley but not Fischer strain rats [130]. Plesan and colleagues [128] have observed that the *N*-methyl-D-aspartate (NMDA) antagonist, dextromethorphan, robustly potentiates morphine analgesia in three rat strains but not the Dark-Agouti strain. Terner and colleagues [143] demonstrate that low doses of naltrexone (in the ng/kg range) significantly potentiate morphine analgesia in Sprague Dawley and Long Evans rats, but not Fischer-344 or Lewis strain rats.

In perhaps the most striking example, Rady and colleagues show that inbred and outbred mouse strains fall into three separate neurochemical categories with respect to their opioid receptor mediation of heroin analgesia. Using receptor subtype-specific antagonists, they conclude that in outbred Swiss Webster and inbred C567BL/6J, heroin analgesia is primarily mediated by δ -opioid receptors [92, 144].

In BALB/cByJ and AKR/J mice, however, heroin analgesia seems to be a κ -opioid phenomenon; in ICR, DBA/2J, CBA/J, and C3H/HeJ strains the μ -opioid receptor appears to be the major player [92, 144]. The groupings are in fact consistent with the known ancestry of the strains [66]. Supporting the contention that these sorts of pharmacological strain differences may actually be due to anatomical differences in pain-processing mechanisms are the observations of Proudfit's laboratory [145, 146] that the noradrenergic innervation of the spinal cord is qualitatively distinct in two substrains of Sprague Dawley rat, projecting unilaterally and terminating in the deep dorsal horn in rats from the now defunct Sasco, Inc., but projecting bilaterally and terminating in the superficial dorsal horn in rats from Harlan Sprague Dawley, Inc.

17.1.6 What Have We Learned from Strain Comparisons?

There are a number of reasons to engage in the sorts of studies described in the section above. Of course, the very demonstration of strain differences can be taken as strong evidence of the heritability of the phenomena studied, at least in that particular rodent species. Furthermore, strain surveys can identify extreme-responding strains that one could then use as the progenitors of a segregating population (e.g., F_2 hybrids, backcross) for gene mapping by linkage [15].

As of very recently, such segregating populations are no longer required for gene mapping; one can find genomic regions correlated with strain variation *in silico*, simply by comparing phenotypic data with databased SNP haplotypes (see [147–149]). However, there is much that can be learned even prior to the identification of the genes responsible for inherited variability. Most notably, the demonstration of genetic correlation or genetic independence of traits has proven considerably heuristic (see [150, 151]).

17.1.6.1 Genetic Correlation Between Basal Nociception and Opioid Analgesia

Anecdotal clinical observations spurred Sergienko and colleagues [152] to investigate the relationship between baseline nociception and morphine inhibition of the same noxious stimulus in outbred mice; they found a significant negative correlation for electrical but not thermal or mechanical stimuli. Selective breeding studies also supported such a relationship, since mouse lines selected for high and low opioid analgesia demonstrated altered baselines [153, 154].

Many modern studies have also observed this negative correlation (resistant to nociception, sensitive to morphine analgesia, and vice versa), regardless of the noxious stimulus modality [85, 86, 155, 156]. Such a correlation between nociception and analgesia could be explained either by a “parallel” or “serial” mechanism. That is, either a common (i.e., pleiotropic) genetic and physiological substrate is relevant to both phenotypes, or genetic influences on the first (nociception) subsequently affect the second (antinociception).

Elmer and colleagues [86] argue for a serial mechanism, and point to studies suggesting that a direct relationship exists between stimulus intensity and the fractional receptor occupancy (i.e., the proportion of receptors currently bound by ligand) required to produce analgesia [157]. That is, mouse strains more sensitive to pain effectively experience a higher stimulus intensity from a given noxious stimulus, which would thus necessitate a higher dose of analgesic. Note that to the extent that the serial explanation of the genetic correlation between basal nociception and drug analgesia is true, then there are *no* “analgesia genes,” only nociception genes.

This conclusion is strengthened by the fact that in several studies, using both opioid and non-opioid drugs, it was demonstrated that the correlation between nociception and analgesia persists even when a different nociceptive assay is used [85, 86, 158]. A particular strain can simultaneously, therefore, be *sensitive* to morphine inhibition of chemical nociception and *resistant* to morphine inhibition of thermal nociception (e.g., C3H/He mice in [86]). As we have argued previously, this renders it unlikely that a strain’s sensitivity to morphine analgesia can be fully explained by μ -opioid receptor gene (*Oprm1*) polymorphisms. If a particular strain had more or more active μ -opioid receptors, shouldn’t it be more sensitive to *all* of morphine’s actions? Obviously, the true explanation is considerably more complicated.

Given the reliability of the correlation between nociception and analgesia in mice, human studies have evaluated whether this correlation can be used to predict clinical opioid analgesia. Indeed, it has been shown that post-operative pain as measured by visual analog scale (VAS) scores can be predicted by preoperative experimental pain assessment using either heat or pressure stimuli, with negative correlations ranging from $r=-0.31$ to -0.65 [159–161]. A similar relationship holds for clinical pain, as basal heat pain thresholds were shown to predict opioid analgesia in post-herpetic neuralgia patients [162].

17.1.6.2 Genetic Correlation Between Analgesics

A surprising finding from our study of the heritability of analgesia is that multiple, neurochemically distinct analgesic drugs – the μ -opioid receptor agonist, morphine; the κ -opioid receptor agonist, U50,488H; the α_2 -adrenergic receptor agonist, clonidine, the CB-1 (cannabinoid) receptor agonist, WIN55,212-2; and the nicotinic receptor agonist, epibatidine – all show high genetic correlation with one another [85]. That is, the same strains were generally sensitive (or resistant) to *all* these analgesics, despite the fact that each binds to a different receptor protein and feature independent metabolic pathways.

Again, this would seem to argue against a simple-minded notion of genetic variability in drug response being explained by polymorphisms in the gene coding for each drug's molecular receptor. Instead, the genetic variability might be found in pain modulatory circuits well distal to each drug's binding site, where multiple analgesic mechanisms have converged into a final common pathway. We have recently provided evidence that one such “master analgesia gene” may be *Kcnj9*, encoding the GIRQ3 (Kir3.3) G protein-gated inwardly rectifying potassium channel subunit [163]. Note, of course, that the genetic correlation among these disparate analgesics may simply follow from each of their correlations with the same baseline nociception measure.

17.1.6.3 Interactions Between Sex and Genotype

As a matter of course, our laboratory routinely tests equal numbers of mice from both sexes in all experiments [164], including genetic studies. We are thus able to evaluate whether sex differences depend on genotype, and whether strain differences can be specific to sex. These interactions have been reviewed recently (see [165]); suffice it to say that the evidence for such interactions is overwhelming. In addition to evidence from strain surveys, linkage mapping studies of opioid analgesia have uncovered evidence for single-sex linkages, such that genetic variability in a gene contributes to phenotypic variability between strains in one sex but not the other [50, 166, 167]. As mentioned in Sect. 17.1.3.3 above, interactions between sex and genotype have been noted in human studies as well.

17.1.7 Linkage Mapping of Opioid Analgesia

Linkage mapping, as mentioned above, is one of two available techniques for establishing causal relationships between DNA variation and phenotypic variation [15]. It cannot easily be used in humans (except for the simplest, monogenic traits), because even the largest available pedigrees do not confer sufficient statistical power. However, in laboratory animals, extremely large (and simply organized)

“pedigrees” (i.e., segregating populations) can be formed, and sufficient statistical power harnessed to find genes contributing to complex, quantitative traits. As a result, linkage mapping in animals is often referred to as quantitative trait locus (QTL) mapping.

Two major QTL mapping projects have been performed examining opioid analgesia, one for morphine and one for the κ -opioid agonist, U50,488H. A more recent study used haplotype mapping to examine morphine-induced hyperalgesia. Note that although QTL mapping is a considerable undertaking, it features a major advantage over other approaches to identifying trait-relevant genes (see [63]). Because in QTL mapping one is associating DNA variants with the phenotype as opposed to mRNA levels, one can be sure that the DNA variants are in fact the *cause* of the trait variability. Using transgenic knockout mice, one can never quite distinguish direct from indirect effects, and in microarray studies, gene expression changes could cause the trait variability or instead, be caused by the trait variability.

17.1.7.1 QTL Mapping of Morphine Analgesia

This study, performed in the laboratory of John Belknap at Oregon Health Sciences University in Portland, OR, utilized two segregating populations: the BXD/Ty panel of recombinant inbred (RI) strains, and a confirmatory ($C57BL/6 \times DBA/2$) F_2 hybrid cross. The BXD/Ty RI panel is itself a set of strains re-inbred from a ($C57BL/6 \times DBA/2$) F_2 cross [168], and as these strains have already been genotyped at DNA markers (simple sequence length polymorphisms; “microsatellites”) spanning the mouse genome [169], one can perform preliminary QTL mapping without having to genotype.

The statistical power is limited, though [170], and so a confirmatory F_2 cross is usually required to achieve statistical significance. Initially, 20 RI strains were tested for their analgesic response to 16 mg/kg morphine on the 52.5°C hot-plate test [171]. Robust variability among the strains was noted, with two strains (BXD-12 and BXD-22) displaying even less analgesia than $C57BL/6$ mice, and one strain (BXD-24) displaying virtually equivalent analgesia to $DBA/2$ mice. [3H]-Naloxone whole-brain binding experiments were also performed on these strains, and a significant correlation ($r=0.52$) was observed between binding and analgesia.

Suggestive linkage was obtained with microsatellite markers on the proximal end of mouse chromosome 10, and data from the F_2 intercross confirmed this, with maximal evidence for linkage at approximately 8 centiMorgans (cM; note that at the time, the precise physical locations of genes and markers were not known; the cM is a measure of genetic distance based on recombination frequency). As the mouse *Oprm1* gene was located at 8 cM as well (now known to be at 3.3–3.6 Mb from the proximal end of mouse chromosome 10), and since morphine drinking had previously been shown to map to the same location [172], the authors concluded (but did not prove) that the QTL was likely *Oprm1* [171].

A follow-up study [167] considered both these data and new data obtained using short-term selective breeding [173] and a marker-assisted congenic strategy [174]. They report statistical evidence for three additional QTLs, two on mouse chromosome 9 and one on mouse chromosome 1 [167]. One of the chromosome 9 QTLs is very likely *Htr1b*, encoding the serotonin 5-HT_{1B} receptor [175]. The others have yet to be identified, but are of interest in that they are sex-specific QTLs, contributing to morphine analgesic variance in female but not male mice [167]. Note that although the chromosome 10 QTL (presumably, *Oprm1*) is the largest of the four, it only accounts for 9% of the trait variance (and in females, only 2%). Even in mice, therefore, *Oprm1* is far from a complete explanation of morphine analgesic variability. Note also that an unpublished QTL study examining morphine analgesia on another nociceptive assay (the acetic acid abdominal constriction test) failed to identify any linkages on mouse chromosome 10 (Hain et al., unpublished data).

17.1.7.2 QTL Mapping of U50,488H Analgesia

A previous QTL mapping study of naloxone-insensitive (i.e., non-opioid) swim SIA [166], using the same RI/F₂ strategy as employed for morphine analgesia, revealed the presence of a robust, female-specific QTL on mouse chromosome 8 (52–84 cM). Although this would seem to have little relevance to opioid drug analgesia, existing data suggested that this particular swim SIA paradigm shared much in common with κ-opioid drug analgesia, in that both could be reversed by blockade of the NMDA receptor in male but not female mice [176, 177]. Since it had also been reported that clinical κ-opioid drug analgesia was more efficacious in women compared to men [178], we became very interested in the possibility of identifying the female “equivalent” of the NMDA receptor. A (C57BL/6 × DBA/2)F₂ cross confirmed a wholly female-specific linkage of distal mouse chromosome 8 to U50,488H (50 mg/kg, i.p., using the 49°C tail-withdrawal test) analgesia [50].

An intriguing candidate gene was identified within the linked region: *MC1R*, coding for the MC1R. A series of pharmacological and genetic studies confirmed the female-specific role of the MC1R in U50,488H analgesia, and as mentioned previously, a human genetic association study confirmed the female-specific role of *MC1R* in pentazocine analgesia in humans [50]. It’s important to point out that *MC1R* would not have been on anyone’s priority list of genes to examine for pain or analgesia in general, never mind pentazocine analgesia in particular. The human finding was solely inspired by the mouse QTL study.

A follow-up study, again using mutant “recessive yellow” (C57BL/6-*MC1R*^{e/e}) mice and redhead women, but this time testing for pain sensitivity and μ-opioid analgesia (morphine and M6G analgesia in mice; M6G analgesia in humans), revealed that the *MC1R/MC1R* genes were involved in these traits as well [51]. However, in this case the effects were *not* female-specific, but were rather seen equally in both sexes, for reasons that as yet defy clear explanation.

17.1.7.3 *In Silico* QTL Mapping of Morphine-Induced Hyperalgesia

The paradoxical hyperalgesia (i.e., increased pain sensitivity) accompanying chronic administration of opioids is the subject of increasing study (see [179]). Liang and colleagues [180] took advantage of the recent ability to perform QTL mapping completely *in silico*, by simply comparing the phenotypes of a large number of strains to SNP haplotypes that have already been identified following sequencing of those strains [147]. This analysis revealed *Adrb2*, encoding the β_2 -adrenergic receptor, as a likely causal gene, and the hypothesis was backed up with pharmacological and transgenic knockout data [180].

A follow-up study revealed the influence of another gene, *Abcb1b*, encoding the P-glycoprotein drug transporter [181]. Illustrative of the complexity of this enterprise is the fact that *Adrb2* showed association only with morphine-induced hyperalgesia as assessed by changes in *mechanical* thresholds, whereas *Abcb1b* showed association only with morphine-induced hyperalgesia as assessed by changes in *thermal* thresholds.

17.2 Genetics of μ -Opioid Receptor Expression and Signaling

The individual drug response is known to be under genetic control, either via modulation of the drug effects or their modulation of the drug concentrations at the target site (see [182–185] for reviews).

17.2.1 Polymorphisms of the μ -Opioid Receptor Gene

The *OPRM1* gene is highly polymorphic, with 2,253 human single nucleotide polymorphisms (SNPs) currently listed in the NCBI SNP database (www.ncbi.nlm.nih.gov/SNP/; accessed on February 7, 2010). However, of the few more frequently found (>5%) SNPs (e.g., -172G>T, 17C>T, 118A>G, IVS2-31G>A and IVS2-691G>A; with “IVS2” denoting SNPs in intron 2), substantial evidence for a functional consequence for the effects of endogenous or exogenous opioids is available only for one: 118A>G [186].

The 118A>G SNP is a coding variant causing an amino acid exchange of the aspartate into an asparagine at position 40 of the receptor protein deleting a putative extracellular glycosylation site. The β -endorphin binding affinity at N40D μ -opioid receptors was increased in variant receptors expressed in Syrian hamster adenovirus-12-induced tumor cells (AV-12) [20]. This might explain the decreased pain responsiveness observed in carriers of the variant 118G allele [59, 187] by an increased endogenous opioid tone. However, the increased β -endorphin affinity at variant N40D receptors was not reproduced in subsequent experiments employing human N40D receptors expressed in *Cercopithecus*

aethiops (COS) cells, human 293 embryonic kidney (HEK293) cells, and AV-12 cells [21, 22, 188]. Moreover, all in vitro assessments in cell lines transfected with the variant 118G cDNA failed to reveal any changes in the affinity or receptor signaling related to exogenous opioids [20–22]. An alternative to altered ligand binding was suggested to be a decrease in μ -opioid receptor expression caused by the *OPRM1* 118G variant, which has been demonstrated in transfection experiments with HEK293 [21] and Chinese hamster ovary (CHO) cells [24].

However, a prior publication contradicted this by demonstrating unchanged receptor expression when the N40D variant receptors had been expressed in HEK293 cells [22]. Adding to the inconsistency of molecular findings related to the *OPRM1* 118A>G SNP, a recent report demonstrated either decreased or unchanged receptor expression in HEK293 and AV-12 cells, respectively, depending on the transfection method [188]. More recently, assessment of *OPRM1* mRNA expression in post-mortem brain tissue samples also suggested a reduced N40D variant μ -opioid receptor protein expression [24]. In addition, the variant decreases opioid receptor signaling after agonist ligand binding in certain pain-relevant brain regions such as the secondary somatosensory area [23].

Among several other *OPRM1* mutations having been described [189–191], coding mutations affecting the third intracellular loop of the μ -opioid receptor (e.g., 779G>A, 794G>A, 802T>C) have been shown to result in altered G protein coupling, altered receptor signaling, and in modified receptor desensitization [22, 192, 193]. The 802T>C SNP resulting in a Ser268Pro μ -opioid receptor (intracellular receptor portion) produced altered receptor desensitization and receptor signaling with decreased G protein coupling [193]. The affinity of morphine, diprenorphine, DAMGO, β -endorphin, met-enkephalin and dynorphin was not changed, but the potency and efficacy of DAMGO, β -endorphin and morphine were greatly diminished [22], though not always [192]. Since the 802T>C SNP is very rare (allele frequency <1%; see [22]), an investigation of the clinical consequences of the mutation is difficult and its clinical relevance is limited.

17.2.1.1 Consequences of μ -Opioid Receptor Polymorphisms for Respiratory Depression

Even under controlled clinical conditions opioid administration can result in fatal respiratory depression [194]. The decreased effects of opioids on pain in carriers of the 118A>G SNP (see Sect. 17.1.3.1) raise the question of the consequences of this particular variant for opioid-induced respiratory depression. However, at least in heterozygous carriers (however, with only $n=4$), neither the respiratory depression induced by M6G [195] nor that induced by alfentanil [196] differed between carriers or noncarriers of the 118G variant allele. In contrast, the *OPRM1* 118A>G SNP resulted in a broadened therapeutic range of alfentanil in healthy homozygous carriers. Two to four times higher alfentanil concentrations are sufficient to produce

the same degree of analgesia in subjects with a homozygous *OPRM1* 118G genotype as in subjects not carrying this particular genotype, whereas 10–12 times higher alfentanil concentrations are needed to produce the same degree of respiratory depression [196].

17.2.1.2 Consequences of μ -Opioid Receptor Polymorphisms for Other Opioid Side Effects

Opioid-induced nausea and vomiting have not yet investigated systematically for their modulation by genetic polymorphisms. Nevertheless, as a side observation in a study focused on the effects of M6G on pupil size and experimental pain, significantly less frequent vomiting was observed in carriers of the variant *OPRM1* 118G allele, despite tendentially higher M6G doses required to produce similar analgesia in carriers of the variant as in noncarriers [32]. This suggested, as with the respiratory depression [196], an increased therapeutic range of opioids in carriers of the 118G allele. Further support for this hypothesis came from an observation that of two men with renal failure under oral morphine analgesic therapy: the homozygous carrier of the 118G allele tolerated high accumulated M6G concentrations well, whereas the wild-type (A118) patient experienced severe sedation (including lack of response when addressed verbally), requiring a switch to another opioid, which does not have an active renally eliminated metabolite and is therefore better suited for patients with renal failure. In the present case, tramadol was chosen, which although producing less efficacious analgesia than morphine proofed to provide sufficient pain treatment in that particular patient [197].

17.2.1.3 Consequences of μ -Opioid Receptor Polymorphisms for Opioid Addiction

Opioid receptor polymorphisms have been screened for their importance regarding opiate addiction liability. The 17C>T SNP was found to be more frequent in drug addicts than in non-addicts [20, 198], and an association between the 118A>G and the 691C>G (intron 2) SNPs and opioid dependence was reported for Chinese heroin addicts [199]. The G-alleles of these SNPs were more frequent in addicts (39.5 and 30.8% for 118A>G and 691C>G, respectively) than in nonaddicts (29.4 and 21.1% for 118A>G and 691C>G respectively).

Indian controls carried the 118G allele less frequently than Indian heroin addicts [200]. Furthermore, a pattern of mutations (-1793T>A, -1699insT, -1320A>G, -111C>T, 17C>T) was associated with heroin/cocaine dependence [191]. Addicted subjects carrying both the IVS2 31G>A and the 118A>G SNP consumed comparatively higher doses of heroin than noncarriers of those mutations [201]. Alcoholics carrying the mutated 118G allele treated with naltrexone had significantly lower

rates of relapse and a longer time to return to heavy drinking than those homozygous for the 118A allele [202]. However, associations of substance dependence with the 17C>T or 118A>G SNPs have not always been found [201, 203–206]. Probably, alterations in opioid neuropeptide systems might underlie enhanced opiate abuse vulnerability apparent in 118G individuals [207]. That is, while in heroin users the preproenkephalin (*PENK1*) and preprodynorphin (*PDYN*) genes are down-regulated, this down-regulation may be more pronounced in carriers of the 118G variant allele [207].

17.2.1.4 Consequences of μ -Opioid Receptor Polymorphisms for Pupil Constriction

As a measure of central opioid effects, changes in pupil size [208] have been repeatedly employed for opioids such as alfentanil, tramadol, morphine or methadone [32, 209–211]. This biomarker especially appears to provide a reliable estimate of the delay at which central nervous system (CNS) effects of opioids follow the time course of their plasma concentrations. The consequence of the 118A>G SNP has consistently been observed to be a decrease in opioid potency for pupil constriction, which resulted in a right-shift of the pupil size vs. opioid concentration. Evidence for this is available for various opioids such as morphine [32], M6G [32, 212] and methadone [211].

Thus, despite reproducible pharmacogenetic effects of human μ -opioid receptor variants, especially 118A>G, the picture of clinical consequences is not consistent. This is not unexpected due to the complexity of the MOR receptor itself including several splice variants that confer distinct functions and could be differently affected by genetic variants. Mutations may affect only one splice variant when located in a DNA portion that is not translated in another splice variant, or they can affect splicing itself. Moreover, additional targets may exist for opioids such as the proposal of a particular morphine-6-glucuronide receptor [213] or non-opioid receptor mediated effects [214]. Genetics might serve as a tool to identify or verify those targets when it can be shown that the effects of certain but not all opioids (i.e., M6G) are genetically modulated via variants in independent genes other than *OPRM1*.

17.3 Genetics of Clinical Opioid Effects via Altered Opioid Pharmacokinetics

Opioids are subject to metabolic clearance and to active transport across biological barriers. Pharmacogenetics of drug metabolism refers to increased or decreased activity of drug metabolizing enzymes due to genetic polymorphisms. The consequences for drug effects depend upon whether an active drug or an inactive prodrug are administered (see Fig. 17.1). In the case that the

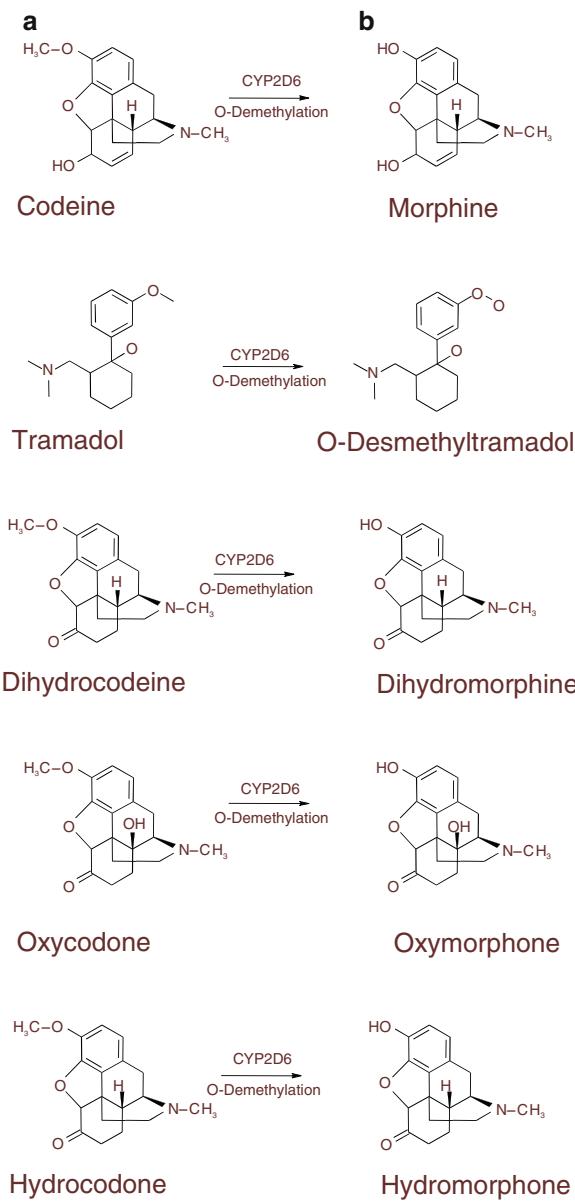


Fig. 17.1 Altered drug metabolism results in opposite consequences for the clinical effects of an active drug (*on the right*) and a prodrug (*on the left*)

administered compound is the active principal of opioid treatment, increased metabolism would lead to decreased effects due to faster elimination. On the other hand, decreased metabolism due to diminished enzyme expression or function will lead to increased effects when the dosing schedule is not adapted

to the lower elimination rate. In contrast, when a prodrug is administered, the consequences of altered drug metabolism are the opposite of those for an administered active drug.

That is, increased enzyme function will cause increased opioid effects due to enhanced production of the active metabolite, whereas decreased enzyme function will decrease the effects of an opioid (perhaps rendering them clinically ineffective), because the active compound is not produced anymore.

Dual consequences (see Fig. 17.2) are also to be expected from changes in the function of transmembrane transporters, which are expressed, for example, in the intestine, the blood–brain barrier (BBB), and the kidney. In the case of outward transport, decreased transporter function results in increased opioid concentrations at the place from where the opioid is outwardly transported. In the intestine, the bioavailability will be increased when outward transporter function is decreased, and at the BBB, CNS opioid concentrations and thus effects will increase when outward transporter function is decreased. The opposite consequences are expected when the function of an inward or uptake transporter is decreased, and the consequences of increased function of outward or inward transporters can be easily anticipated from the principles stated above.

The general principles apply to all opioids that are metabolized or transported. However, despite a great many possible candidate structures for such a modulation, clinical relevance has been demonstrated only for a few opioids, enzymes and transmembrane transporters. The available evidence for a modulation of opioid effects by genetic polymorphisms in drug metabolizing enzymes or drug transporters will be summarized in the following sections.

17.3.1 Cytochrome P450 2D6

CYP2D6 is involved in the metabolism of several opioids such as codeine, oxycodone, hydrocodone, tramadol, ethylmorphine, methadone, and dihydrocodeine (see Fig. 17.3). About 1–3% of Middle-Europeans, but up to 29% of Ethiopians, display gene duplications [215–217], which are associated with increased CYP2D6 enzyme function forming the so-called ultra-rapid metabolizer (UM) phenotype. However, UMs are more frequent than suggested by the frequency of *CYP2D6* gene amplification. Of the up to 7% UMs among Caucasian populations [218], only 10–30% are accounted for by gene amplification [219]. On the other hand, about 7–10% of Caucasians lack any CYP2D6 activity due to deletions and frame-shift or splice-site mutations of the gene [215, 216]. The nonfunctional CYP2D6 variants are coded by the *CYP2D6* alleles *3, *4, *5, *6, *7, *8 [220]. Other alleles code for CYP2D6 with decreased, but not absent function (*9, *10, *41).

Several other nonfunctional *CYP2D6* variants have been described. However, usually they are rare and genetic screening includes only the alleles more frequent in the population. In the case of their absence, the normal functional allele *1 is assigned to the respective subject. At least in Caucasians, a stratification of decreased

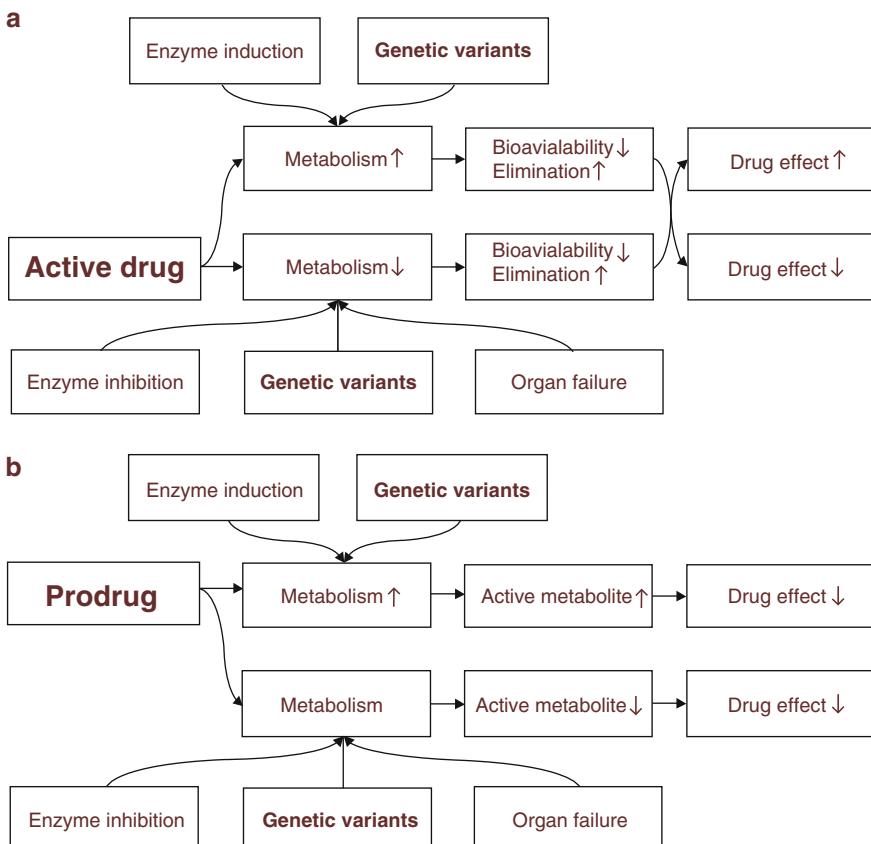


Fig. 17.2 Opioid analgesics and their CYP2D6-dependent active metabolites

CYP2D6 function can be based on the numbers of *CYP2D6* allele *1 and *41, whereas in Asians, allele *10 is the most important decreased function allele. That is, carriers of two copies of allele *1 are assigned to the extensive metabolizer (EM) phenotype, which denotes normal CYP2D6 function. Carriers of allele *1 together with a nonfunctional allele are assigned to the heterozygous EM phenotype, displaying a slightly decreased CYP2D6 function. Carriers of allele *41 together with a nonfunctional allele are assigned to the intermediate metabolizer (IM) phenotype displaying substantially decreased but not absent CYP2D6 function [221].

17.3.1.1 Codeine

Codeine has a 200-times lower affinity at μ -opioid receptors than its metabolite, morphine [222], and therefore its clinical effects largely depend upon its O-demethylation to morphine [223] by cytochrome P450 2D6 [224], although some of its clinical effects appear to persist independently of morphine formation

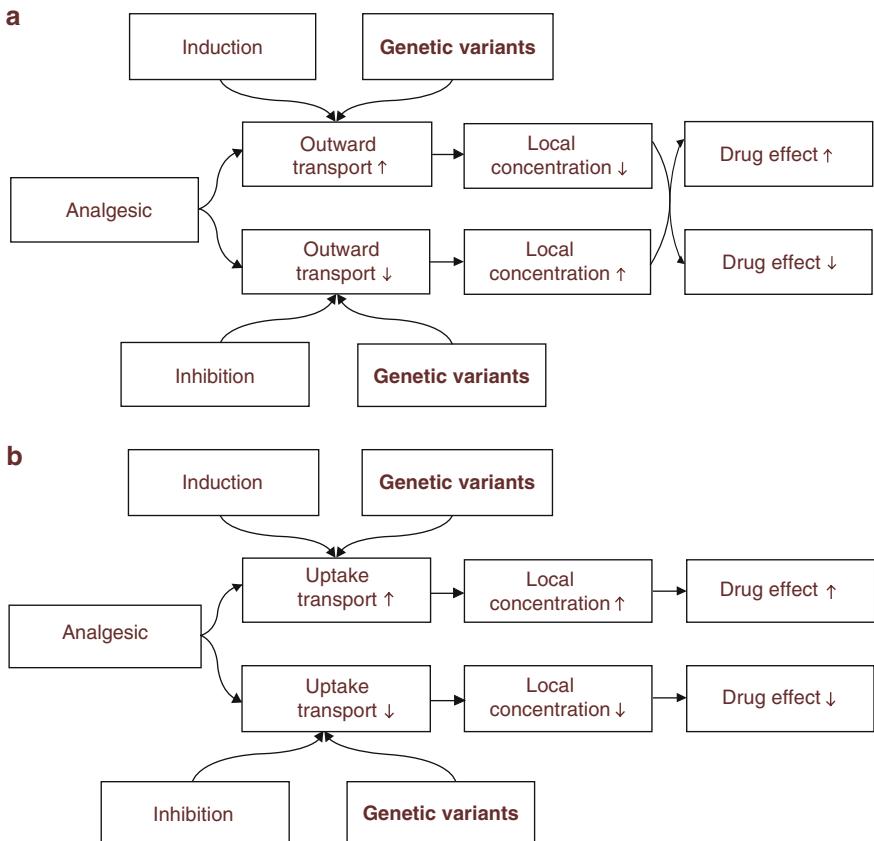


Fig. 17.3 Altered transmembrane drug transport results in opposite consequences for the clinical effects of an active drug, depending on whether the function of outward (a) or uptake (b) transporters is modified

[223, 225]. When CYP2D6 activity is increased in subjects with a UM phenotype, a higher amount of morphine is formed from codeine leading to unexpectedly high opioid effects after codeine administration. For example, life-threatening opioid intoxication developed in a 62-year-old man after he was given 25 mg oral codeine three times a day for 4 days. Twelve hours after the last dose had been administered the patient's level of consciousness deteriorated and he became unresponsive [226]. A 33-year old woman took 60 mg codeine prophylactically to avoid pain in connection with tooth extraction. Within 30 min she experienced euphoria, dizziness, and severe epigastric pain [227].

A 29-month-old child experienced apnea resulting in brain injury following codeine administration for pain relief after tonsillectomy [228]. A newborn died from morphine poisoning when his mother used codeine while breastfeeding [229]. Another newborn also died on day 13 after his mother had been prescribed codeine for post-episiotomy pain; the mother was diagnosed as a UM [230]. Thus, codeine

has increased effects in subjects carrying CYP2D6 gene amplifications and probably in subjects not carrying gene amplifications but nevertheless displaying a UM phenotype. Evidence is available for codeine-associated side effects, whereas evidence for the probably also enhanced analgesic effects has not been reported yet. On the other hand, codeine is ineffective in producing analgesia in patients with absent CYP2D6 function [231].

17.3.1.2 Tramadol

Tramadol has a lower affinity at μ -opioid receptors than its metabolite *O*-desmethyltramadol (M1). Specifically, the K_i values for the racemic mixture of tramadol, its (+) and (-) enantiomers, the racemic mixture of M1, and its (+) and (-) enantiomers at μ -opioid receptors expressed in transfected HN9.10 neuroblastoma cells were 17,000, 15,700, 28,800, 3,190, 153, and 9,680 nM, respectively, compared to 7.1 nM for morphine [232]. Therefore, the CYP2D6 activity is a major determinant of the analgesia elicited after (+)-tramadol administration [233].

Indeed, due to the lack of formation of the active metabolite *O*-desmethyltramadol in CYP2D6 poor metabolizers (PM), the analgesic effects of tramadol on experimental pain are decreased [234]. However, tramadol is not completely devoid of analgesic effects in persons without functional CYP2D6 [235] because it possesses opioid activity itself and acts also through non-opioid-dependent mechanisms [236]. In patients, the percentage of non-responders to post-operative tramadol administration was higher in PMs than in patients with functional CYP2D6 [237], which corresponded with the formation of *O*-desmethyltramadol stratified according to the CYP2D6 genotype and phenotype [238]. Moreover, the pupillary response to systemic tramadol administration differed between EMs and PMs [209]. From this modulation of the extent of the clinical effects of tramadol in the presence of differently active CYP2D6, it is reasonable to expect increased effects of tramadol in UM subjects.

17.3.1.3 Dihydrocodeine

The active, CYP2D6-dependent metabolite, dihydromorphine, and its 6-glucuronide both have an approximately 100-fold higher affinity at μ -opioid receptors than the parent compound dihydrocodeine [239], which differs in its affinity to μ -opioid receptors little from codeine (K_i values for [3 H]-DAMGO replacement from μ -receptors of 0.24 and 0.35 μ M for dihydrocodeine and codeine, respectively; see [240]). The plasma concentrations of dihydromorphine were reduced in PMs of debrisoquine/sparteine [241, 242]. However, the CYP2D6 phenotype did not influence overall pharmacokinetics of dihydrocodeine [241], possibly owing to the fact that only a small fraction of the dihydrocodeine molecules are transformed by CYP2D6, the majority being subjected to glucuronidation via UGT2B7 or N-demethylation via CYP3A [222, 241, 243].

In contrast to codeine, the effects of dihydrocodeine do not appear to depend on the activity of CYP2D6. That is, pupil constriction after dihydrocodeine administration was similar in CYP2D6 extensive ($n=5$) and poor metabolizers ($n=4$) [244]. Sufficient pain control was reported to be similarly achieved in CYP2D6 poor and extensive metabolizers; that is, in 9 of 14 extensive metabolizers and in four of eight poor metabolizers [244]. Dihydromorphine was not found to significantly contribute to dihydrocodeine analgesia [245]. Finally, the effects of dihydrocodeine on electrical, heat and rectal distension pain tolerance were not altered by quinidine inhibition of CYP2D6 [242].

17.3.1.4 Oxycodone and Hydrocodone

The response to hydrocodone, codeine and oxycodone in CYP2D6 poor metabolizers persists to be subject of discussion and the lack of CYP2D6-dependent metabolites plays a role as a candidate cause for therapeutic failure to this opioids [246]. Nevertheless, quinidine pre-treatment did not change the psychomotor, subjective, miotic and side effects of oxycodone despite the absence of its active metabolite oxymorphone from plasma [247]. This is consistent with the report that the central opioid effects of oxycodone are governed by the parent drug, with a negligible contribution from its circulating oxidative and reductive metabolites [248]. However, a recent study came to opposite conclusions, showing that its analgesic effects are decreased when the formation of oxymorphone was impossible due to CYP2D6 inactivity [249, 250]. Finally, inhibition of CYP2D6 or a PM status did not decrease the effects of hydrocodone despite the lower formation of hydromorphone [251].

17.3.2 Other Drug-Metabolizing Enzymes

17.3.2.1 UGT2B7

M6G and codeine-6-glucuronide (C6G) have been identified as active metabolites [252, 253], with K_i values for [3 H]-DAMGO replacement from μ -receptors of 0.0035 and 0.79 μ M, respectively [240]. The glucuronidation of morphine and codeine is mainly mediated by the UDP glucuronosyl transferase (UGT) 2B7 [254] with some contribution of UGT1A3 [255].

For the *UGT2B7* gene [256] a couple of genetic polymorphisms have been described [257–259]. However, the UGT2B7-mediated glucuronidation in liver microsomes of morphine [257], codeine, naloxone, nalorphine, nalmefene, levorphanol, oxymorphone, nalbuphine, or naltrexone [260] was not different for enzymes with either a histidine or a tyrosine at position 268 [257], coded by *UGT2B7*1* and *2 alleles, respectively [261]. These alleles are related to a 802C>T SNP of the gene [261], occurring at an allele frequency of 51.1 and 48.9% for the *1 and *2 alleles, respectively [257]. In patients, the morphine-glucuronide to morphine plasma concentration ratios were unaffected by the *UGT2B7* H268Y poly-

morphism [262, 263]. Morphine glucuronidation was also found to be unaffected by numerous other *UGT2B7* mutations [259].

The only hint at a genetic modulation of opioid glucuronidation is the observation that the Tyr268 *UGT2B7* is able to glucuronidate the drug buprenorphine with a tenfold higher efficiency *in vitro* than the His268 isoform [260]. Whether a –161C>T promoter variant linked to the 802C>T – found to be more frequent in low glucuronidators [263] and shown to be associated with a trend for reduced M6G/morphine ratios in patients with T/T, C/T, and C/C genotypes (T/T>C/T>C/C) – will gain clinical relevance remains to be evaluated.

17.3.3 Transmembrane Transporters

17.3.3.1 P-Glycoprotein

P-glycoprotein (P-gp; multidrug resistance-1), coded by the *ABCB1* gene, is mainly located in organs with excretory function such as liver, kidney and the gastrointestinal tract [264], and has also been found at the BBB where it forms an outward transporter [265]. Therefore, functional impairment of P-gp transport may be expected to result in increased bioavailability of orally administered drug, or in increased brain concentrations of its substrates. Both mechanisms give rise to the expectation of decreased dose requirements or increased clinical effects of analgesics that are substrates of P-gp. For example, fentanyl is a substrate of P-gp [210, 266].

A diplotype consisting of three SNP positions in the *ABCB1* gene (1236TT, 2677TT, and 3435TT) was found to be associated with increased susceptibility to clinical fentanyl effects [267]. Moreover, the opioid loperamide, clinically prescribed as anti-diarrheic because it does not produce effective CNS concentrations due to its rapid excretion from the CNS by P-gp [266], produced CNS opioid effects associated with an *ABCB1* 3435TT genotype [32]. In addition, the homozygous *ABCB1* diplotype (GG-CC) with respect to *ABCB1* positions 2,677 and 3,435 conferred an odds ratio of 0.12 (95% confidence interval, 0.01–0.98) with regard to the use of ondansetron for post-operative nausea or vomiting in the context of post-operative morphine analgesic therapy [30].

A mouse with a null mutation of the *Abcb1* gene has been developed and used to show *in vivo* that P-gp is an outward transporter of opioids from the CNS, the likely reason why P-gp knockout mice were more sensitive to systemic morphine and less sensitive to morphine given centrally [268]. With the use of *in situ* brain perfusion, the brain uptake clearance of morphine was found to be increased by a factor of 1.3–1.4 in these knockout mice as compared with wild-type mice [269, 270]. Consistent with the elevated brain concentrations of morphine, the analgesic effects of morphine were increased by a factor of 4 in knockout mice [271]. However, knocking out *Abcb1* genes does not lead to enhanced CNS effects of all opioids. For example, in knockout mice enhanced M6G effects in the CNS were

absent [271, 272] despite the fact that M6G is a P-gp substrate [273]. An explanation is that M6G is a substrate of other transporters that can substitute for the absent P-gp [274, 275].

17.4 Conclusions

Consequences of genetic polymorphisms for the effects of opioid analgesics have been shown to apply to the underlying disease – pain – to the pharmacodynamic effects of the analgesic at its target – opioid receptors – and to the pharmacokinetics of particular opioids. Whereas for pain and opioid pharmacodynamics the relevant polymorphisms are widely shared because the main target of opioids are opioid receptors that are part of the endogenous pain-defensive opioid system, genetic polymorphisms affecting drug-metabolizing enzymes and transporters are distinct. For all three levels, clinical consequences have been reported to modulate the responses to opioid administration and may serve as a basis for personalized approaches to analgesic treatment, either for dose adjustments, or for rational genetics-based selection of analgesics. Given the complexity of the subject, however, meaningful accomplishment of these goals probably remains a long way off.

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