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REVIEW ARTICLE

The Presence of Endogenous Morphine Signaling in Animals

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Abstract Recent empirical findings have contributed valuable mechanistic information in support of a regulated *de novo* biosynthetic pathway for chemically authentic morphine and related morphinan alkaloids within animal cells. Importantly, we and others have established that endogenously expressed morphine represents a key regulatory molecule effecting local circuit autocrine/paracrine cellular signaling via a novel μ_3 opiate receptor coupled to constitutive nitric oxide production and release. The present report provides an integrated review of the biochemical, pharmacological, and molecular demonstration of μ_3 opiate receptors in historical linkage to the elucidation of mechanisms of endogenous morphine production by animal cells and organ systems. Ongoing research in this exciting area provides a rare window of opportunity to firmly establish essential biochemical linkages between dopamine, a morphine precursor, and animal biosynthetic pathways involved in morphine biosynthesis that have been conserved throughout evolution.

Keywords Morphine · Opiate receptors · Nitric oxide · μ_3 Opiate receptor

Demonstration and Characterization of Novel μ_3 Opiate Receptors

The first demonstration by Kosterlitz and coworkers [1] that exogenous morphine can bind to receptors in the mammalian brain indicated that morphine binds to the same sites as those used by the endogenous opioid peptides (e.g., enkephalins). Since then, demonstration of the multiplicity of receptor types has led to the understanding that, depending on their site of action, opioid peptides as well as opiate alkaloids may bind to more than one opiate receptor subtype [2–5]. Different degrees of selectivity have been recognized for the various ligands by comparing affinity constants as well as relative strength in competitive binding assays. For example, Pasternak and Snyder (see [6]) reported both high and low affinity binding sites for [^{3}H]-dihydromorphine (^{3}DHM) and [^{3}H]-naloxone in the rat brain. The higher affinity type was designated μ_1 and the lower affinity morphine-selective type was designated μ_2 [7, 8].

Our laboratory was instrumental in the biochemical identification and characterization of a novel μ opiate receptor subtype, designated μ_3, located on immunocytes and neural tissues of invertebrates, such as *Mytilus edulis*, as well as human monocytes, granulocytes, vascular endothelial cells and other neuronal and non-neuronal cell types (Table 1) [9, 18, 19]. The μ_3 receptor differed from previously described neuronal opioid receptor subtypes in that it exhibited non-detectable or exceedingly low affinities for naturally occurring endogenous opioid peptides, peptide analogues, μ_1 opioid-selective endomorphins 1 and 2, and synthetic opioid alkaloids of the benzomorphan and phenylpiperidine classes [9, 16–19, 29]. In contrast, the μ_3 opiate receptor displayed high affinity binding for morphine-related morphinan alkaloids such as dihydromorphine and the clinically established antagonists naloxone and naltrexone as well as the active metabolite morphine 6-glucuronide [30]. Finally, it was also established that the μ_3 receptor is coupled to G protein isoforms, based on guanine nucleotide effects on agonist binding [19].

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Table 1 μ_3 Receptor binding and expression

μ3 Opiate receptor binding
Tissues or cell lines
Mytilus edulis: immunocytes [9], ganglia [10–14]
Cat: astrocytes and microglia [15]
Rat: Kupffler cells [15]
Murine: cell lines (J774.2, RAW 264.7, BAC1.2F5) [16], mouse neuroblastoma [17]
Human tissues: vascular [18], white blood cells [9, 19], neural [20]
Receptor expression
Mytilus edulis: ganglia [21, 22]
Human tissues: Sh-Sy5y, [23], heart [24, 25], vascular [23, 24, 26], white blood cells [23, 27], neural [20], stem cells [28]

Functional Coupling of μ_3 Opiate Receptors to Constitutive Nitric Oxide Production and Release

The neurochemical literature has documented a functional association of morphine action with stimulated nitric oxide (NO) production and release. Peripheral morphine analgesia involves NO-stimulated increases in intracellular cGMP [31]. Nitric oxide has been associated with antinociception [32] as well as tolerance and dependence [33]. In addition, the morphine-induced suppression of splenic lymphocyte proliferation has been shown to involve NO [34]. Morphine and NO have been linked in gastrointestinal regulation [35, 36]. Furthermore, morphine, not opioid peptides, stimulate constitutive NO release in macrophages, granulocytes, various types of human and rat endothelial cells, invertebrate neurons and immunocytes and in rat median eminence fragments, all in a naloxone antagonizable manner [29, 30, 37–44]. These data suggest that the μ_3 receptor is coupled to constitutive NO release in these cells [39, 45, 46].

Furthermore, morphine's actions in these diverse tissues complements what is known about NO mediating immune and vascular functions, namely that it can down regulate them from an excitatory state or prevent the excitatory state from occurring [30, 42, 44, 47]. Additional information on opiate alkaloid signaling substances can be summarized as follows: Injection of vertebrate animals with morphine results in deficient macrophage function [48] and an alteration of T-cell activity [49]. Morphine also antagonizes interleukin-1alpha or tumor necrosis factor-alpha-induced chemotaxis in human granulocytes and monocytes [50, 51].

Molecular Cloning, Structural Elucidation, and Functional Cellular Expression of the μ_3 Opiate Receptor

Molecular cloning of a μ_3 receptor encoding cDNA employed a screening probe derived from a conserved region of the human μ_1 receptor and a human testis cDNA library. A full-length 1,338 base pair cDNA was cloned and subsequently sequenced (Fig. 1 and Table 1) [23]. NCBI Blast analysis indicated that the clone exhibited 100% identity to the μ_1 opioid receptor subtype in its central conserved region with most of exon 1 spliced out at

Fig. 1 (**a**) μ_3 cDNA sequence (1,338 bp). The *underlined* sequence represents the novel 263 bp segment, and the *bold* letters represents *Homo sapiens* Oprm 3′UTR DNA sequence (nucleotide position 1,625–1,829 of Oprm). (**b**) The diagram shows the μ_3 cDNA sequence starting at position 503 of Oprm. The dotted lines represent the novel 263 bp μ_3 sequence (between nucleotides 1,376 and 1,625 of Oprm), and position 1,625–1,829 is the Oprm 3′UTR DNA sequence that represents the 3′-end of μ_3 [23]

A

Mu3 Opiate Receptor Transcript

5'atacaccaagatgaagactgccaccaacatctacattttcaaccttgctctggcagatgccttagccaccagtaccctgccctc
cagagtgtgaattacctaatgggaacatggccatttggaaccatcctttgcaagatagtgatctccatagattactataacatgttca
ccagcatattcaccctctgcaccatgagtgttgatcgatacattgcagtctgccaccctgtcaaggccttagatttccgtactccccg
aaatgccaaaattatcaatgtctgcaactggatcctctcttcagccattggtcttcctgtaatgttcatggctacaacaaaatacaggc
aaggttccatagattgtacactaacattctctcatccaacctggtactgggaaaacctgctgaagatctgtgttttcatcttcgccttca
ttatgccagtgctcatcattaccgtgtgctatggactgatgatcttgcgcctcaagagtgtccgcatgctctctggctccaaagaaa
aggacaggaatcttcgaaggatcaccaggatggtgctggtggtggtggctgtgttcatcgtctgctggactcccattcacatttac
gtcatcattaaagccttggttacaatcccagaaactacgttccagactgtttcttggcacttctgcattgctctaggttacacaaacag
ctgcctcaacccagtcctttatgcatttctggatgaaaacttcaaacgatgcttcagagagttctgtatcccaacctcttccaacattg
agcaacaaaactccactcgaattcgtcagaacactagagaccacccctccacggccaatacagtggatagaactaatcatcaga
attattatataattcatagatgttgctgcaataccctcttatttctcaaaagccagtcttgctctggttctgtgattaaagagagagggt
gagtgccttgcccactgtggtcatggatgcaagatattcacagaaaattagcatcatagaaaaaaaannnaaaaaaaaaaaaaa
aaaancatgtcggccgcctcggccaaacatcgggtcgagcatgcatctagggcggccaattccgcccctctccccccccngcn
ntttc**cacaccgaggagtccagtttgtgcaagacacccagcggaaccaaaacccatcgtggtatgtgaatcgaagtcatc
ataaaaggtgacccttctgtctgtaagattttaatttaagcatatatttatgacctcaacaaagacgaaccatcttttgttaa
ttcaccgtagtaacacataaagttatgctacctctgatcaaag-3'**

B

MOR-1: 1_____-2162

MOR-3: 1 _____ _ _ _ _ _____1338

its 5′-end (Fig. 1). The 3′-end of the μ_3 receptor cDNA contained the 3′-end of the μ_1 receptor protein coding sequence with a spliced insert of 263 bases containing a stop codon and terminated by a μ_1-specific 202 base untranslated region [23].

RT-PCR and subsequent sequence analysis revealed the presence of this opiate receptor subtype in human vascular endothelial cells, mononuclear cells and polymorphic nuclear cells. To determine if the cDNA clone we isolated was functional and had the biochemical properties expected of the μ_3 receptor, the cDNA was expressed in a heterologous system (Cos-1 cells). Following exposure to morphine, the transfected Cos-1 cells released NO in a naloxone-reversible manner [23]. Untransfected Cos-1 cells failed to produce any detectable NO upon addition of morphine [23]. The addition of Met-enkephalin, DPDPE, or Leu-enkephalin did not stimulate NO release in the controls or transfected cells [23]. In sum, heterologous cellular expression of the cloned μ_3 opiate receptor cDNA by Cos-1 cells resulted in morphine-selective stimulation of NO release, consistent with previous biochemical data presented above [23, 52, 53].

Endogenous Morphine

Prior demonstration of low concentrations of morphine [47, 54–59] and its precursor molecules tetrahydropapaveroline (THP), reticuline, salutaridine, thebaine, and codeine in vertebrate tissues [58, 60–62] provides strong presumptive evidence for the physiological relevance of novel μ_3 opiate receptors selectively activated by endogenously expressed morphinan alkaloids. In invertebrates, specifically *Mytilus edulis*, the presence of morphine, morphine 6-glucuronide, morphine 3-glucuronide, codeine, THP and reticuline also have been reported [9, 53, 63–65]. Endogenous opiate levels can be induced to change following stimulation [53, 66–70]. Morphine has also been found in human plasma [71, 72], suggesting a hormonal action with immune, vascular and gut tissues as targets [36, 73]. Additionally, immunocytochemical localization of a morphine-like material was reported in neural and immune tissues [74–77] as well as in invertebrate tissues [9]. Taken together, these reports suggest that animals appear to have the ability to synthesize opiate alkaloids (Fig. 2 and Table 2).

Recent studies from our group employing well established ex vivo invertebrate nervous tissue preparations and primary cultures of human white blood cells [78, 86, 100] and those by Zenk and coworkers using human tumor-derived cell lines [84, 101] have markedly facilitated the formulation of an evidence-based model of *de novo* formation of endogenous morphine in animal cells with remarkable similarities to the well-characterized enzymatic pathway described in *Papaver somniferum* (Fig. 2) [102]. Key observations from these studies indicate that L-tyrosine, its monoamine homolog tyramine, and their respective catechol derivatives, L-DOPA and DA serve as substrates for *de novo* morphine production and that pharmacological characterization of tyramine utilization as a morphine precursor indicates one or more catalytic steps mediated by microsomal CYP 2D6 (Fig. 1) [78, 86]. The significance of tyramine as a biosynthetic intermediate is validated by in vitro enzyme kinetic studies demonstrating dopamine formation via CYP 2D6-catalyzed ring hydroxylation of tyramine [103–106] which in turn lends support to the existence of a potentially important tyrosine hydroxylase-independent pool of cytosolic DA that is available for endogenous morphine expression [78, 86, 100]. These data are complemented by metabolic labeling/isotope enrichment studies employing SH-SY5Y neuroblastoma cells [84, 101], indicating asymmetric isotopic labeling of the benzyl and isoquinoline chemical domains of newly formed morphine that is operationally determined by the type of L-tyrosine-derived precursor molecule that is employed: L-tyrosine and L-DOPA are incorporated in both the benzyl and isoquinoline chemical domains of morphine, whereas dopamine and tyramine are only incorporated into the isoquinoline domain.

Taken together, we have formulated a hypothesis, which states that endogenous morphine, used as either a hormone or neurotransmitter down regulates immune, vascular, neural and gut tissues under normal and following trauma situations [30, 36, 47, 53, 77]. In this regard, as noted in these reports, tolerance is viewed as a mechanism to ensure that morphine's continued presence does not permanently limit a needed tissue excitatory state, since excitation may be required to overcome a traumatic event, which morphine would continue to down regulate. Finally, our laboratory has recently demonstrated the putative role of endogenous morphine in HANS stimulation of invertebrate neural tissues via constitutive NO generation [107].

Future Directions

Ongoing studies from our group support the existence and biological importance of a distinct morphinergic signaling pathway utilizing endogenously synthesized, chemically authentic, L-morphine and its cognate μ_3 opiate receptor subtype. Recent data support a novel regulatory role of tonically released NO to maintain various classes of stem cells in metabolically viable, undifferentiated states of biological readiness via activation of a μ_3-like receptor found on human stem cells [28].

Sequence analysis indicates striking structural similarities between μ_3 and μ_3-like receptors, most notably the lack

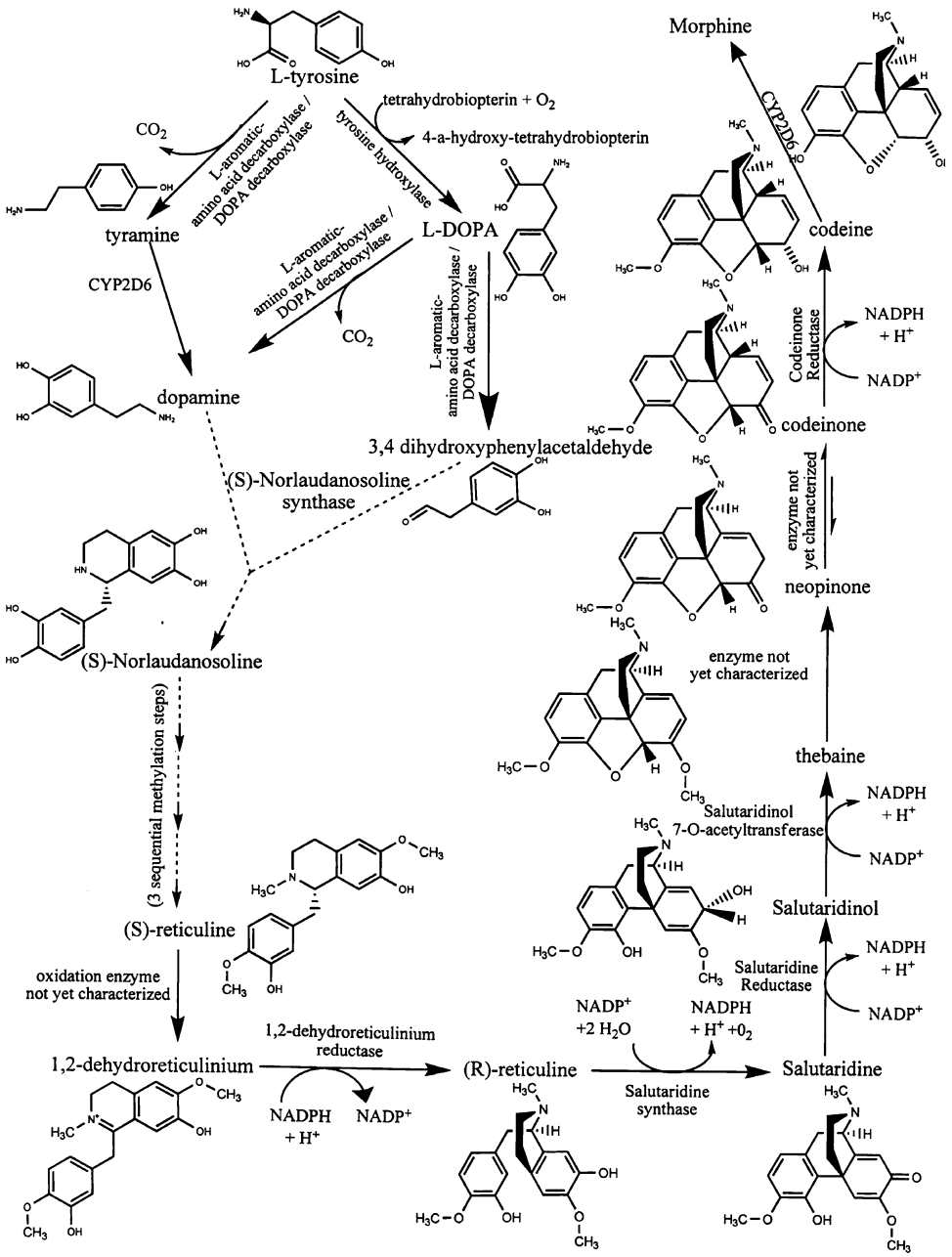

Fig. 2 Detailed schematic indicating relevant enzymes and chemical intermediates in the morphine biosynthetic pathway

Table 2 Morphine presence in animal tissues

Invertebrates
Mytilus edulis: immunocytes [9], ganglia [62, 64, 65, 78, 79], hemolymph [9]
Modiolus deminensus: ganglia [63]
Ascaris suum: ganglia [80], muscle [81], uterus [81]
Dracunculus medinensis: whole body extraction [82]
Schistosoma mansoni: whole body extraction [82]
Homarus americanus: ganglia [83]
Vertebrates
Human: cell line SH-SY-5Y [84], heart [85], white blood cells [86], plasma [71, 76], cerebrospinal fluid [56]
Other mammals (rat, mice, cattle): cell line PC-12 [87, 88], neural [89, 90], adrenal [55, 91–93], brain/neural [54, 55, 57–59, 61, 74, 75, 94–99]

of an extended N-terminal glycosylated domain and transmembrane helical domain expressed from exon 1 of the μ opioid receptor gene and unique intracellular C-terminal coupling domains [108]. Future structure–function studies will be designed to elucidate the biochemical basis of the selective molecular recognition profiles of these receptors for morphine and morphinan alkaloids. In conclusion, the demonstration of μ_3-like receptors on human stem cells provides compelling evidence in support of both the evolutionary primacy and primordial regulatory role of morphine/NO-coupling in embryogenesis. Taken together with the presence of morphine in human immune, vascular and neuronal tissues demonstrates that the endogenous morphine hypothesis extends beyond analgesic functions. Thus, this discovery opens up a new field of investigation with a very old and familiar signaling molecule.

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