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Mouse Models and the Genetics of Nicotine Dependence

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Detailed studies of inbred mouse strains have provided remarkable insights into how genetics shape complex processes, ranging from cancer susceptibility to immunity. The mouse models of response to addictive substances such as nicotine are now showing similar promise for revealing the underlying complex genetics and physiological mechanisms contributing to dependence. This chapter examines key issues in using mouse models for nicotine dependence, including

- *The molecular biology of the nicotinic acetylcholine receptors and how these receptors contribute to tissue-specific responses within the context of strain-specific genetic background*
- *The interaction of nicotine with physiological systems through oral, intravenous, and subcutaneous administration and how experimental results from these routes of administration in mice may relate to the physiology of human smoking*
- *The way mouse models recapitulate many basic features of nicotine dependence in humans, including behavioral reinforcement, self-administration, development of tolerance, and altered reward-related behavior*

On the basis of available evidence, and given its receptiveness to genetic manipulation, the mouse model appears to hold promise as a powerful tool for understanding how genetics and behavioral measures combine to individualize the response to nicotine.

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Introduction

This chapter provides an evidence-based review of issues in using mouse models for genetic research in nicotine dependence, including the biology of neuronal nicotinic acetylcholine receptors (nAChRs), issues in the administration and metabolism of nicotine, experimental design, and strain selection considerations, and aspects of behavioral responses to nicotine in mice. These factors all contribute to a knowledge base for the design of effective mouse model research that, in turn, may contribute to further understanding of the genetic basis of nicotine dependency in humans.

Experimentally defining the genetics that shape the brain—and ultimately the behaviors it controls, such as those leading to the complex outcomes of dependence—is a challenging but promising endeavor. Humans and mice share a close genetic and physiological relationship; comparisons of the human and mouse genomes indicate 85% identity. These genomes compare favorably in their susceptibility to many simple and complex genetic diseases including those related to addictive drugs such as nicotine. Intensive inbreeding has provided many hundreds of genetically isogenic strains with phenotypically distinct features that have been very successfully exploited to identify and often define the genetics of well over 100 models of human disease.[1] Some mouse strains also display responses that closely parallel responses and behaviors seen in humans. These strain-specific genetic characteristics are stable over decades of inbreeding,[2] providing considerable stability in gene-phenotype relations. Mice have additional features conducive to long-term developmental research—for example, relatively small size, economical maintenance, and rapid development.

Factors such as these, together with the species' amenability to genetic manipulation, allow for the study of complex genetic contributions to behaviors that occur in a nexus of physical maturation and environmental exposure. Consistent with these virtues, mouse strains were recognized more than four decades ago[3] as a resource for examining the distinct and often highly varied responses to nicotine on behavior. Subsequent studies have extended these early observations to provide considerable insight into how the genetics of this animal model can be exploited to examine a broad range of mechanisms through which nicotine imparts its effects, including possible physiological substrata of nicotine dependence.[4–8]

At the same time, caution is needed when embarking upon experiments using the mouse model system. Among the most important is the consideration that behavioral-genetic relations are fine-tuned over the natural history of this species. Thus, mice may—or may not—be physically able or behaviorally motivated to perform tasks that would be appropriate for closely related species such as rats.[2] Therefore, each experiment and finding must be evaluated as to species-specific response to stressors (e.g., noise, time of day, handling); appropriateness of experimental manipulations, equipment, and assessment strategy (e.g., platform height, visual lines); and strain and species limitations in behavioral and adaptive repertoires (e.g., congenital retinal degeneration in C3H mice).

Animal models of dependence not only involve inferences and generalizations across species (e.g., mouse to human), but they also involve inferences and generalizations across behavioral and physiological phenomena. Investigations are predicated on the assumption that the behaviors (e.g., conditioned place preferences) and physiological responses (e.g., receptor

upregulation) observed have relevance to human dependence. Thus, experimental procedures must be appropriate for both the organism and for transspecific inferences regarding dependence processes. Investigators must not only consider species and strain differences, and the validity of their dependence assays, but they must also consider other issues, such as developmental processes and how these may affect the biological and behavioral processes relevant to dependence, as well as render behavioral assays that are more or less appropriate.

Of course, dependence phenomena are, no doubt, affected by multiple gene-phenotype relations. This means that the considerations and caveats listed above may be conditional upon the particular genetic variants targeted. Different variants will exert different influences on biological and behavioral processes, and these will show different patterns as a function of development, strain and species, and dependence assay. Thus, a significant goal of genetic mapping of nicotine dependence in the mouse is the strategic selection of experimental strategies that (1) are appropriate for the behavioral repertoire of the organism, (2) target behavioral and biological processes of relevance to clinical dependence phenomena, (3) are developmentally appropriate both in terms of the animal's repertoire and in terms of targeted dependence processes, and, perhaps most important, (4) cosegregate with the targeted genetic variants.

Within this context, an overview is provided of what is known of how gene function, within the context of mouse-strain-specific anatomical architecture and physiology, can shape the varied behavioral responses to nicotine. This overview is intended to permit a more meaningful interpretation of past research and foster improved experimental strategies to model homologous processes that contribute to nicotine dependence between mice and humans.

Nicotinic Receptor Functional Diversity

The family of neuronal nAChRs are excellent and obvious "candidate genes" for examining the genetics contributing to the physiological process of nicotine dependence because they are a defined target of this agent's action. However, these receptors do not act alone; their function in the broader genetic context of multiple genes and biological cascades must also be considered. This complexity is reflected in dramatic differences among mouse strains in response to acute and/or chronic administration of nicotine. In the brain, the sustained presence of nicotine alters neurotransmission at the level of the synapse because, unlike the endogenous neurotransmitter acetylcholine, it is neither rapidly degraded, nor is it actively removed from the synapse. This sustained presence can lead to both persistent activation of some nAChRs as well as induction of a desensitized or "nonactive" state that reverses slowly, or possibly in some cases, not at all.[9–12] Chronic nicotine exposure may also induce the curious phenomenon of "upregulation"; that is, the number of high-affinity nicotine-binding sites in the brain actually increases.[13,14] However, not all nAChRs are of high affinity, nor do they all upregulate. Further, other mouse strain differences that have not traditionally been assumed to be directly influenced by nicotine, such as strain differences in pro-inflammatory status or metabolic rates, may partly account for strain differences in nicotine sensitivity and behavioral response.[8,15,16] Therefore, the considerable genetic variability across mouse strains is likely to summate across all of these processes. It is important to consider the complex interplay of regionally specific nAChR expression, the nature of the specific behavioral tests employed, and physiological responses to identify genetic contributions to the effects of nicotine, especially those contributing to the development of dependence.

Molecular Biology of the nAChR Gene Family

Acetylcholine receptors, like other ligand-activated neurotransmitter receptors, consist of two major subtypes: the metabotropic muscarinic receptors and fast-ionotropic nicotinic receptors.[9,12,17] Both share the property of being activated by the endogenous neurotransmitter acetylcholine, and they are both expressed in neuronal and nonneuronal cells throughout the body. The metabotropic receptors are second-messenger, G-coupled, seven-transmembrane proteins classically defined as activated by muscarine and inhibited by atropine. The other subtype of acetylcholine receptors comprises the microsecond-fast ionotropic cationic channel acetylcholine receptors that are distinguished by their sensitivity to nicotine (figures 4.1 and 4.2).

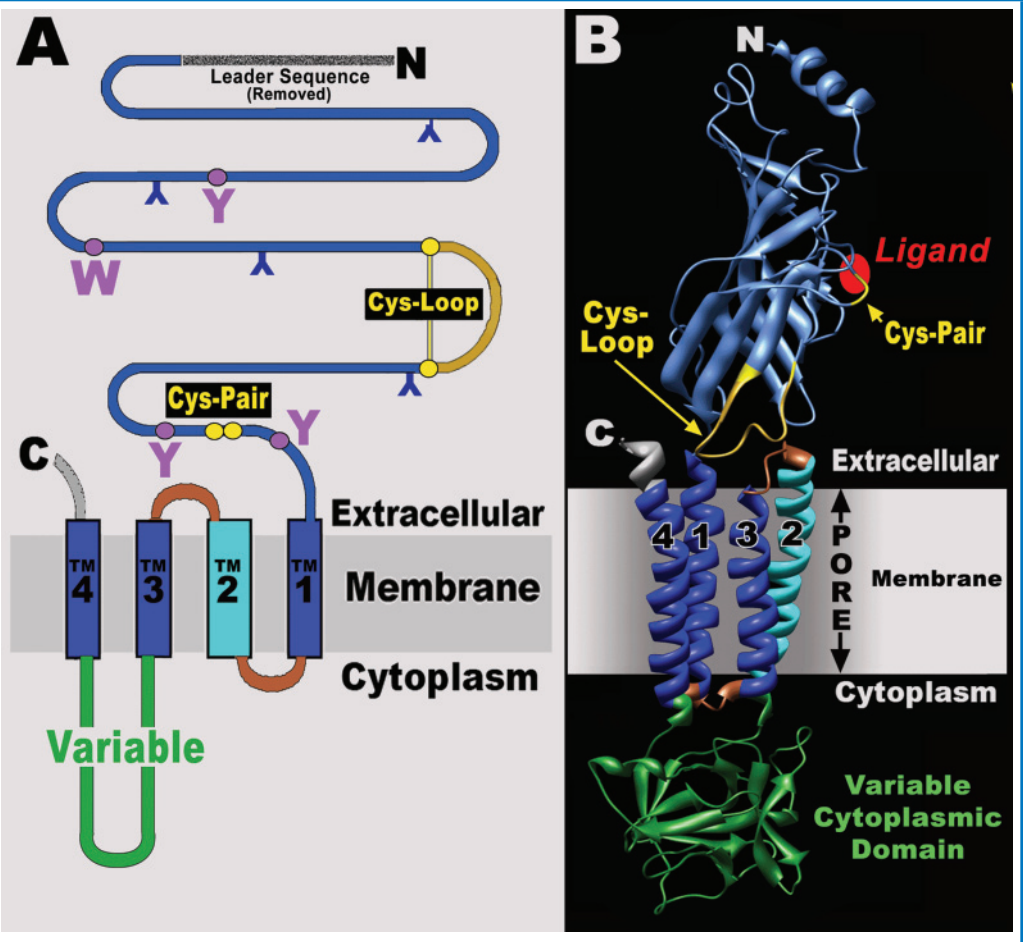
Although all receptor channels are permeable to sodium ions, which are the major agent of depolarization, there is also variable permeability to calcium. Because calcium is an important mediator of second-messenger and posttranslational processes such as gene expression and proteolysis, the regulation of local calcium concentrations imparted by various nAChRs is an important element in how these receptors contribute to establishing physiological microdomains and impact on overall metabolic tone. All subunits (figure 4.1) also share a conserved structure of a large extracellular N-terminal domain and four transmembrane domains, as well as a cytoplasmic domain of variable size and sequence that resides between the third and fourth domain (also referred to as the 3+1 configuration). Each subunit also harbors a cysteine (Cys) loop in the extracellular domain that is defined by two cysteines that, in the mammalian subunits, are separated by 13 intervening amino acids (figure 4.1). The 3+1 transmembrane domain arrangement in combination with the Cys-loop defines an extended family of ligand-activated ion channels that, in addition to nAChRs, includes $GABA_A$, glycine, and 5HT3 (serotonin) receptors.

All mammals examined so far share a similar nAChR genetic composition of 17 homologous subunits.[17,20] These are classified into alpha and nonalpha subunits on the basis of the presence of a Cys-Cys pair in the major extracellular domain near the entrance of the first transmembrane crossing (figure 4.1). A Cys-Cys pair is required (but not necessarily sufficient) for agonist binding to form the ligand-binding site for receptor activation, and it imparts the "alpha" designation. Subunits without this primary structural feature receive the nonalpha designation.[20] This leads to the subdivision of nAChRs into the muscle or neuronal nAChR subtypes. The muscle receptors consist of five subunits (α1 and nonalpha subunits named β1, delta, gamma, and epsilon). The neuronal nAChR subunits consist of the alpha-like subunits termed α2, α3, α4, α5, α6, α7, α9, α10 (α8 is an avian-specific subunit) and three nonalpha subunits termed β2, β3, and β4, respectively. The term *neuronal* was applied to these subunits on the basis of their cloning from the neuronal-like PC-12 pheochromocytoma cell line and brain-derived complementary DNA (cDNA) libraries.[21] In general, the number assigned reflects the order of discovery. Although the present review focuses on nicotine and its effects on functional states of the central nervous system (CNS), ample evidence indicates that most "neuronal" nAChR subunits are also expressed by neuronal and nonneuronal cell types throughout the body, where they influence multiple physiological and metabolic processes.[22–24]

Assembly and Functional Diversity of nAChRs

The mature nAChR is a pentamer assembled from varied combinations of the starting

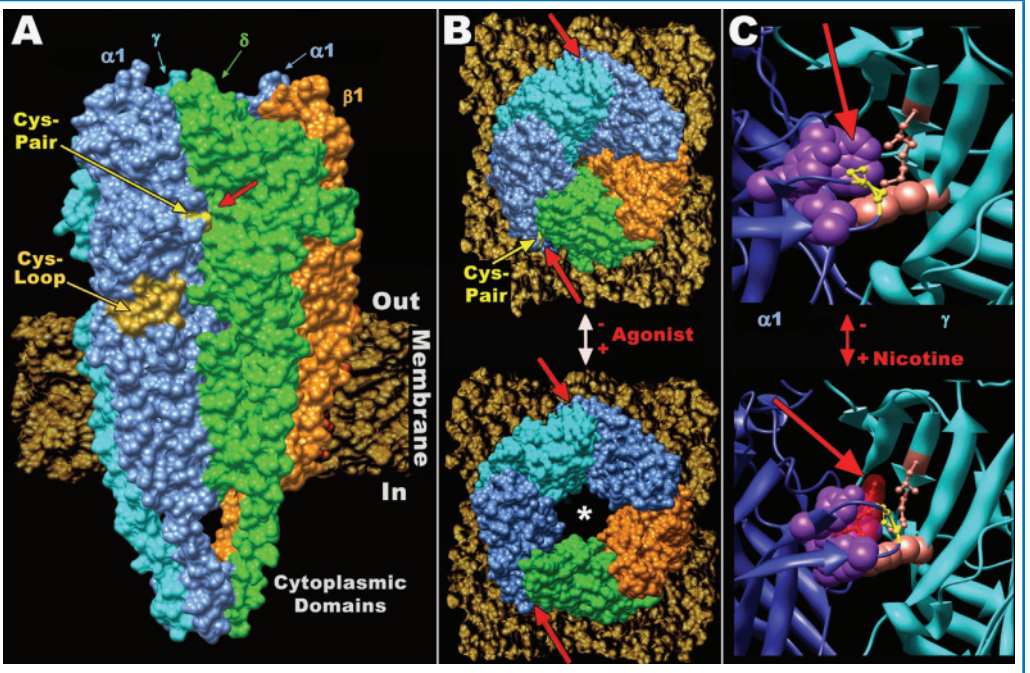
Figure 4.1 Nicotinic Receptor Subunit Structure



Note. Panel A. A linear presentation is shown of the basic structure shared by all neuronal nicotinic acetylcholine receptors (nAChRs). This includes an extracellular domain, four transmembrane domains (TMs), and a cytoplasmic domain that is located between TM3 and TM4 and varies considerably in size and amino acid sequence between subunits. TM2 lines the ion channel. Short connecting sequences between TM1 and TM2 (cytoplasmic) and TM2 and TM3 (extracellular) are shown in brown and contribute to channel gating and receptor flexibility. The highly conserved Cys-loop structural motif (extracellular domain) places nAChRs in the superfamily of ligand-gated ion channels (see text). All alpha subunits by definition contain a Cys-pair that is important for binding ligand, which is absent in nonalpha subunits. The extracellular domain is initially translated with a leader sequence that is prototypically removed. The extracellular domain also includes glycosylation sites (blue "Y"), and amino acids that in addition to the Cys-pair are important to ligand binding (in purple; tyrosines (Y) and a tryptophan (W)). Panel B. The 3-dimensional folded structure of the nAChR subunit in panel A is depicted, as reported by Unwin.[18] Molecular graphics images were produced using the Chimera package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco[19] (NIH P41 RR-01081). The color coding is matched between panels, although glycosylation is omitted. Also not shown in this depiction, but returned to in figure 4.2, are the tyrosine and tryptophan amino acids that, with the loop harboring the Cys-pair, form the ligand-binding site (red circle). Sequence differences among nAChR subunits in these domains contribute to the unique ligand selectivity and functional properties of the assembled receptors (see text).

subunit pool (figure 4.2). In the muscle, this is a developmentally regulated process in which receptors develop such that they comprise two α1 subunits separated by an intervening subunit that is either a gamma (immature muscle), or epsilon (mature muscle) along with two additional subunits including one β1 and delta (figure 4.2).

Figure 4.2 Three-Dimensional Structure of the Nicotinic Acetylcholine Receptor (nAChR)

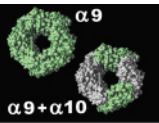
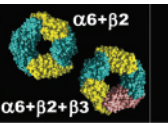


Note. Panel A. A side view is shown of the Torpedo (musclelike) completely assembled nicotinic receptor as resolved by Unwin.[18] Five subunits coassemble to form a tubelike structure through the membrane. The alpha subunits (α1) are paired with either a delta or gamma subunit. The beta subunit (β1) fills the fifth position and does not directly participate in ligand binding, although it does influence receptor pharmacology and function. In this receptor, the cytoplasmic domains are depicted as single alpha helices that form a loosely associated structure within the cell. Note how the Cys-loop approaches the extracellular membrane surface. Panel B. The image in panel A is rotated 90° to look down on the receptor from the extracellular face. In this image, the organization of subunits around a central pore is apparent. When two agonist molecules (e.g., acetylcholine or nicotine) bind in the ligand-binding pocket between the α1 subunits and their respective adjacent subunits (red arrows), there is a conformational change to increase the pore size (gate the channel) and permit ion passage, as shown by an asterisk). Upon removal of the agonist, the receptor closes. The receptor can, however, close if the agonist remains associated with the ligand-binding site, which is termed desensitization. Panel C. A closer view of the ligand-binding site (red arrow) between the α1 and adjacent γ subunit shows how the Cys-pair (yellow), tyrosines, and the tryptophan (depicted by purple and shown in figure 4.1, panel A) converge in the 3-dimensional structure to form a "pocket" within the structure of the receptor. Also contributing to this pocket are amino acids from the adjacent subunit (pink). When a ligand occupies the pocket, as shown in the lower panel for nicotine (red), the receptor closes around it to induce a conformational change that gates the channel. Through varying subunit assembly, the contributions by unique amino acid sequences to this pocket and the activation mechanisms for pore opening customize the function of the various nAChRs to their physiological function.

The rules of neuronal receptor assembly are less well defined (figure 4.3). Some nAChRs are homomeric, including those assembled exclusively from α7 subunits[25] or possibly α9 subunits,[26] respectively. Others are heteromeric and are, in general, formed from at least two alpha subunits (including α2, α3, α4, α6, α7, and α9) and structural subunits including α5, α10, β2, β3, and β4. In this case, it is notable that the alpha designation applies to α5 and α10 because of the presence of the Cys-Cys pair, but neither can form a ligand-binding site or functional receptor without coassembly with other alpha subunits.

Examples of how various subunits alter receptor function and subcellular localization are abundant. Receptors constructed from various combinations

Figure 4.3 Influence of Subunit Composition on Nicotinic Receptors

PROMINENT RECEPTORS	 α1+β1+δ +(γ or ε)	 α9 α9+α10	 α7	 α3+β4	 α6+β2 α6+β2+β3	 α4+β2
MAJOR LOCATION	Muscle	Sensory Epithelium, Peripherhal Cells	Peripheral Cells, Interneurons	Autonomic Ganglia	Basal Ganglia	CNS Neurons
FUNCTION	Muscle Contraction, Development	Vesicle Release	High Calcium, Neurotransm., Intracel. Signals	Symp. & Parasymp. Control	Modulate Dopamine Release	Upregulation, Modulate GABA Release
DISEASE	Myasthenia gravis	Hearing, Autoimmune, Heart?	Schizophrenia, AD, Inflammation	Autonomic Dysfunction, Cardiovascular?	Parkinsons Disease, Addiction	AD, Epilepsy, Aging, Addiction

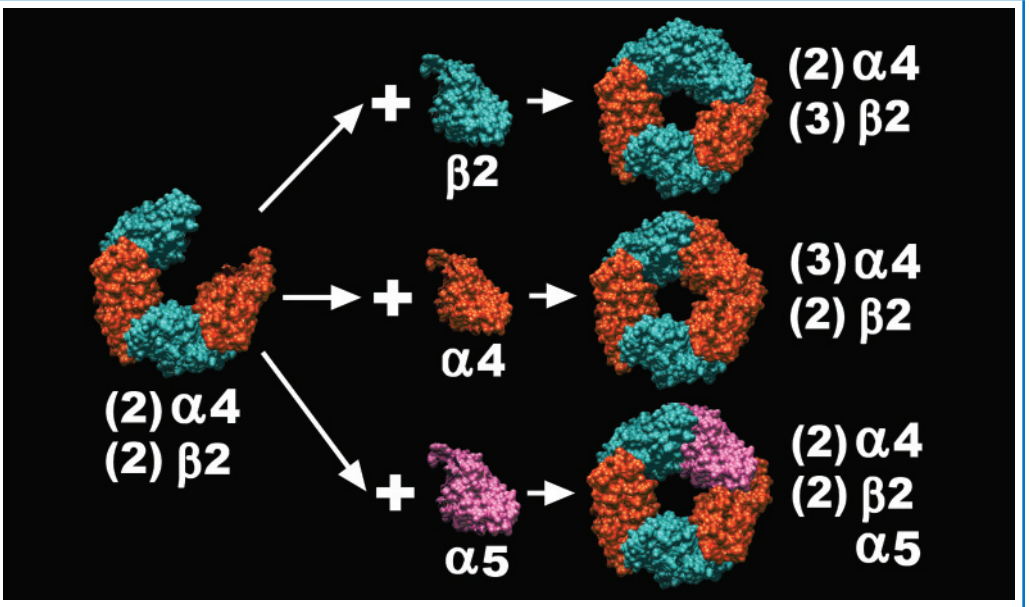
Note. Examples are given of the local expression of nicotinic receptors of different subunit composition where they contribute to both tissue-specific physiological and disease processes (see text). Major receptor subtypes (viewed from the top) are depicted in proposed subunit stoichiometries, which, except for the muscle receptor, are not known and may vary (see text). Also absent are receptors harboring α2 or other possible combinations (e.g., α4β4) whose physiological functions are not well defined. The use of "peripheral cells" refers to both neuronal and nonneuronal cells located outside of the central nervous system. CNS = central nervous system; Symp. = sympathetic; Neurotransm. = neurotransmission; Parasymp. = parasympathetic; Intracel = intracellular; GABA = γ-aminobutyric acid; AD = Alzheimer's disease.

of alpha and structural subunits exhibit dramatic differences in ligand affinity, agonist and antagonist efficacy, rates of desensitization, and response to modulators (figure 4.3). Although alpha subunits control much of the determinants of selectivity for ligand binding, the nonalpha subunits have a significant impact on function. One of the earliest examples[27,28] of this was the finding that when nAChRs composed of α3β4 subunits were exposed to an agonist, bursts of activity followed that were often clustered and of relatively long duration. In contrast the α3β2 receptors exhibit frequent and rapid bursting.

Customizing these properties is consistent with the need to adjust their function within the context of local physiological demands or neurotransmission specifications (figure 4.3). Hence, receptors harboring the β4 subunit tend to be expressed during development in autonomic ganglia where they provide longer and more sustained bursts to enhance their functional impact. Receptors with the β2 subunit are more often involved in modulating neurotransmission in which rapid and precise bursting is favored.[12,29–31] Subsequent investigations have shown that β4 also imparts novel pharmacological properties involving altered agonist and antagonist efficacy[32–34] and altered sensitivity to other compounds including sensitivity to zinc,[35] mercury,[36] and cocaine.[37,38]

Another example of how receptor heterogeneity can be generated from a limited array of subunits is through altering either the stoichiometry of α4β2-containing receptors (figure 4.4), or whether a subunit such as α5 is included in the structural fifth position to close the receptor (figure 4.4). Of note is that receptors with considerably different pharmacological, physical, and ion permeability can be generated from these receptors of varied, but similar, subunit composition.[39,40] In a similar context, the homomeric α7 nAChR provides another example of how local regulation of the expression of a relatively few subunits can dramatically influence the diversity of how the overall system response will be affected by nAChRs. This receptor desensitizes rapidly, but while the channel is open, it is highly calcium permeable.[12] This receptor also tends to localize away from the synaptic junction and has been reported to aggregate in lipid rafts,[41,42] indicating that local increases

Figure 4.4 Nicotinic Receptors of Closely Related Subunit Composition Differing in Function through Variation in Subunit Stoichiometry



Note. Measuring or predicting the contribution of different subunits to neuronal nicotinic receptors is complicated by the possibility that receptors of similar (if not identical) subunit composition, but different relative stoichiometries, can be assembled in different cells or brain regions. This is depicted for α4β2 receptors where the fifth position of the receptor can be filled by an additional α4, β2, or α5 subunit. Depending upon which receptor is assembled, there are notable differences in their expression, affinity for ligand,[46] and function, including susceptibility to magnitude and the rate at which desensitization occurs, as well as degree of calcium permeability.[39,40] The possibility that inflammatory cytokines can influence this process[47] further emphasizes how genetic strain background can influence nicotinic receptor expression and function and reveals that much remains to be determined about how receptor assembly influences the effects of nicotine in mouse strains and different pathologies.

in calcium can impart signaling though calcium-activated second-messenger systems. This further distinguishes the α7 nAChR from other nAChRs and even other ligand-activated ion channels. However, calcium permeability and sensitivity to agonists and antagonists can be altered by the coexpression of additional nAChR subunits. Finally, the structural subunits such as α5 nAChR also exert an effect on function and subcellular localization.[43] When this subunit is coassembled with α7 nAChR in heterologous expression systems, the receptors have similar but distinct properties, including altered rates of desensitization relative to the homomeric channel. Similarly, if α5 incorporates into receptors containing α3β2 nAChR subunits,[44,45] the resulting receptor exhibits distinguishing functional characteristics, but the differences tend to be small. In contrast, if this subunit incorporates into receptors with α3β4 nAChRs, as in the peripheral nervous system,[45] the burst duration of the resulting receptor channel is increased almost threefold.

Another practical concern is how subunit diversity affects interpretation of experiments that use ligand binding or limited pharmacological methods to infer identity or changes in nAChRs during a treatment regime. For example, muscarinic versus nicotinic receptor contribution may be blurred in some instances because nAChRs comprising either homomeric α9 or heteromeric α9α10 subunits are sensitive to blockade by the traditionally

muscarinic antagonist atropine.[48,49] Similarly, the function of α3-containing receptors[50] and α4β4 nAChRs[51] can be modulated in a dose-dependent manner by relatively high concentrations of this "muscarinic" receptor antagonist, although such concentrations are commonly used in buffers by electrophysiologists to ensure that only nAChRs are recorded in response to acetylcholine administration. Consequently, investigations may yield confusing or possibly misleading results when only a single assay of nAChR function is used. Certain other nAChRs of diverse subunit composition may also have overlapping pharmacology, or they are simply not detected by available methods. This could be true of α9 and α9α10 nAChRs, which might be mistaken for α7 subtype receptors because they also exhibit exquisite sensitivity to α-bungarotoxin.[26] Although overlap of these respective receptor subtypes appears to be very small in central systems, there is substantial α9-subtype expression in peripheral systems,[52–55] and consequently, the identity of the nAChR subtype being measured must be carefully assigned.

Finally, traditional ligand-binding determination methods (e.g., high-affinity nicotine or α-bungarotoxin binding) may be inadequate to infer the finer aspects of nAChR involvement in local circuitry, especially in regions such as the hippocampus. It is now well established that the nAChR systems in this limbic region affect both inhibitory and excitatory tone through modulating inhibitory interneuron activity.[56–58] In particular, differing combinations of α7, α4β2, and α3β4 nAChRs, respectively, have been implicated in collectively establishing theta-wave synchronization[59–61] and mechanisms of long-term potentiation.[11,58,59,62–64] Therefore, mixed combinations of receptors on restricted numbers of inhibitory interneurons whose location is strategically placed within the circuit will contribute significantly to establishing the hippocampal activity imparting a behavior. However, methods to distinguish among the expression of these various receptors can be very challenging technically. In some cases, they are missed entirely when high-affinity nicotine or α-bungarotoxin binding methods are used, or if their overall abundance is too low to be detectable over the background from the entire cellular milieu. However, the addition of new high-affinity ligands (e.g., the frog toxin epibatidine) or some with varied, but defined, nAChR subtype selectivity (e.g., the α-conotoxin MII) are proving to be of exceptional value for identifying the coexpression of these receptors.[65] Nevertheless, the limitations of such assays should be borne in mind when designing new studies or when evaluating extant data. The lack of sensitivity and specificity of such assays may be responsible for some of the inconsistent results yielded by early studies in this area.

Customizing Local nAChR Function through Limiting Subunit Expression

Among the first discoveries following the discovery of the cDNA family of nAChR subunits was that their expression in the brain was restricted to subunit-specific patterns that overlapped in various brain regions.[12,66–68] This manifests in considerable overlap: α4 and β2 are widely and coincidently expressed.[65,69] Together, they form the majority of high-affinity nicotine-binding sites in the CNS and are the primary receptor to undergo upregulation in response to nicotine.[70] Another receptor, α7, is also expressed throughout the CNS, but not in all regions. Its relatively high permeability to calcium and rapid desensitization to nicotine make it a particularly important subunit for regulating second-messenger and transcriptional mechanisms (see below

and figure 4.3). However, there is incomplete understanding of the contribution of each subunit to proposed regional specialization of nAChR structure and function (figures 4.3 and 4.4). This is due, in part, to measurements that rely solely upon RNA analysis. Such analyses can vary in sensitivity, and do not provide spatial resolution of the final receptor product, which can be located very distantly from the site of synthesis because of processes such as axonal transport. One example of this is the expression of the β4 subunit in the adult CNS. The expression of this subunit was originally reported to be highly restricted to only a very few brain regions, most notably the medial habenula,[71,72] or in the peripheral nervous system with α3.[73] When other studies were conducted that used assays of increased sensitivity and resolution,[74] including single-cell polymerase chain reaction (PCR),[75] β4 was found to be more widely expressed in the CNS.[76,77] Similarly, immunohistochemical measurement of β4 reveals that this subunit may be expressed at sites very distal to the cell body, as in axon terminal fields of the barrel cortex whose cell bodies originate in the ventral thalamus, or in terminal fields of the lateral lemniscus within the inferior colliculus.[78] Therefore, the site of the nAChR contribution to regulating local circuitry may be distal from its site of synthesis.

In the many brain regions, electrophysiological recordings and immunolocalization reveal a more complicated story.[11,64,79–81] Nicotinic receptors can be located presynaptically, postsynaptically, and nonsynaptically (e.g., aggregates of α7 nAChR) on both pyramidal and nonpyramidal interneurons. Differential subcellular localization can, in turn, lead to at least three different, and often complementary, outcomes on cell response.[9,12,64] First, when located presynaptically, depolarization through these nAChRs can add to or sustain the activation of voltage-gated calcium channels to enhance neurotransmitter (either excitatory or inhibitory) release. Second, when nAChRs are localized to the postsynaptic face of the synapse, they can participate directly in promoting fast-excitatory neurotransmission. Finally, as when located in lipid rafts, the collective activation of these receptors can directly affect the intensity of local intracellular ion concentrations to influence downstream pathways, leading to changes in gene expression, metabolic and physiological stasis, and even proteolytic mechanisms.

One example of when different nAChRs are coexpressed to combine to modulate the overall tone in a local circuit involves the GABAergic interneurons of the hippocampus. In fact, no fewer than three subclasses of these inhibitory interneurons can be distinguished on the basis of the unique expression of different nAChR subtypes[64,79] such as α7, α4β2, and α3β4, respectively, although some interneuron subtypes also express combinations of these receptor types and possibly others.[75] These interneurons collectively play an important role in the magnitude of GABAergic inhibition exerted by nAChR activation in the CA1 region. For example, activation of interneurons harboring the α7 nAChR in the stratum lacunosum moleculare is strongly inhibited to produce a selective disinhibition of the dendritic segments of pyramidal neurons innervated by axon terminals of the perforant path. In contrast, activation of α4β2 nAChRs inhibit interneurons in the stratum radiatum and stratum lacunosum molecular to produce disinhibition of dendritic areas innervated by both neuron types. Moreover, nAChR immunoreactivity has been localized to astrocytes in this brain region.[69,81] Given that these astrocytes release agents that interact with glutamate receptors to maintain excitatory "tone,"[82] it is not surprising that the nAChRs have been implicated in neurological diseases ranging from schizophrenia to Tourette

syndrome to neurodegeneration, as seen in Alzheimer's disease (figure 4.3). Therefore, when assessing the impact of nAChRs with respect to nAChR subunit composition, the magnitude of expression, the site of final receptor localization, and the anatomical context in which nAChRs are expressed are important considerations in unraveling the strain-specific effects of nicotine responses.

Nicotine's Function as Agonist and Antagonist

Ligand-activated ion channels, by necessity, are transmembrane proteins that when fully assembled create a hydrated receptor channel permeable to selected ions that are regulated by conditions of the extracellular, intracellular, and transmembrane environment (figure 4.2). Notably, ion-channel receptors reside in a constant equilibrium between open and closed states, and their tendency to open is carefully regulated through the presence of neurotransmitters or other agents that broadly fall under the functionally defined categories of agonist (activator) or antagonist (inhibitor). Nicotine is most often viewed as an agonist of nAChRs. However, this compound is not rapidly degraded or transported away from the receptor (as are normal endogenous transmitters), and in the absence of compensating mechanisms, the sustained presence of an agonist would lead to increased receptor opening and, in turn, cell death (figure 4.2). The compensatory mechanism in this case is the process of receptor desensitization, which permits the receptor to close in the presence of sustained agonist exposure. When acetylcholine is the neurotransmitter, desensitization is brief, because this endogenous transmitter is rapidly degraded by acetylcholine esterases. In contrast, nicotine may accumulate in the receptor vicinity; this has the effect of actually favoring the desensitized or the nonfunctional state. Notably, persistent, elevated concentrations of an agonist can even result in a state of deep desensitization that can lead to complete receptor inactivation or degradation.[12] In practical terms, this produces cases in which nicotine becomes a potent inhibitor of receptor function that can actually exceed the antagonism accomplished by many pharmacological agents designed for this purpose. Consequently, the effect of nicotine on a system may, in some cases, be more accurately ascribed to sustained receptor inactivation rather than to activation.

Whether activation or desensitization dominates the effect of nicotine is, in part, determined by the receptor subtype(s) expressed. For example, some receptors are activated by very low concentrations of nicotine but become desensitized as the concentration increases (e.g., α7 subtypes), while others (α3β4 subtypes) may be fully activated only at concentrations at the high end of physiological relevancy.[12,64] In some cases, both conditions may occur, as in the nucleus accumbens, where most nAChRs desensitize (or even inactivate) rapidly to nicotine,[83] yet dopamine overflow related to nAChR activation persists well after exposure.[84–86] Other mechanisms associated with nicotine's actions may actually be imparted through indirect or conditional mechanisms. These include the production of nicotine metabolites or mediators of stress responses such as salt imbalance (especially if nicotine tartrate is used) or local pH (also note that nicotine is most stable when acidic).[87] Further, the differential expression of nAChRs by multiple cell types can collectively influence the activation of additional signaling pathways such as those that are downstream of cyclic adenosine monophosphate–response element binding (CREB) activation (see below and Brunzell and colleagues[88]) or the enhancement of nitric oxide release.[89,90] So, while the focus of this review is on the impact of this drug on immediate nicotinic cholinergic mechanisms and

responses, a full causal account could be highly complex because other signaling and neurotransmission systems are, no doubt, also involved. While this has produced some confusion, it emphasizes the need to view the nAChR system as a modulator of physiological "tone." This also includes the influence of the rate of nicotine administration, its absolute dose, and its local persistence. This suggests that the route of administration is a vital element of experimental design and is discussed later in this chapter.

Nicotinic Receptor Upregulation

When a tissue receives sustained exposure to nAChR ligands such as nicotine, the curious phenomenon of upregulation occurs. This was first recognized when quantitation of high-affinity nicotine-binding sites from brain tissue taken from rats[13] or DBA/2 mice exposed to chronic nicotine,[14] revealed that such sites were increased by as much as fourfold over nonnicotine-treated controls. Further, this was similar to the increased number of high-affinity nicotine-binding sites measured in brain tissues from smokers.[91,92] Subsequently, upregulation has been measured directly using brain imaging methods such as quantitative dynamic single-photon-emission computed tomography of the living baboon brain[93] and in human smokers.[94] Immunoprecipitation studies of the high-affinity nicotine-binding sites in rats first demonstrated that the α4β2 subtype of nAChR was essential to both high-affinity binding and coincident upregulation in response to nicotine.[70] This property is intrinsic to this receptor-subunit composition because upregulation of this receptor occurs in all animals so far examined and in receptors expressed in heterologous expression systems including transfected human embryonic kidney cells.[95] The importance of subunit composition is critical to the relative degree of upregulation, and in fact, not all nAChRs exhibit this property (e.g., all receptors harboring α4β2 appear to upregulate, while receptors composed of α4β4 show reduced upregulation, and those harboring α3 do not upregulate[95]). Mice deficient for β2 through subunit knockout exhibit essentially no high-affinity binding sites and do not upregulate receptors.[96,97] Therefore, the identity of the alpha and beta subunits contributes qualitatively and quantitatively to upregulation. This phenomenon, which has been related to the development of reinforcement (see Picciotto and colleagues[98] and below), produces a situation in which there are more receptors but the overall function is reduced.[12,64] Also, because upregulation influences receptor subtypes preferentially, the importance of this process to optimizing the performance of local circuitry is likely to be equally specialized (figure 4.3), as indicated by the dominance of α4β2 in central systems such as the basal ganglia and hippocampus versus the autonomic nervous system, where the majority of receptors are composed of α3β4 subunits.[17,73,99]

The cellular mechanisms underlying this important cellular phenomenon are not yet resolved, and the published explanations can differ, often from the same laboratory.[100–102] What these studies and others seem to suggest is that multiple mechanisms underlie this effect, including increased assembly efficiency,[103] altered receptor stoichiometry,[46,102] increased export from the endoplasmic reticulum and increased surface trafficking,[101,104] altered affinity for ligand,[105] and decreased degradation.[100,106] It is likely that upregulation reflects the summation of several processes whose regulation and relative contribution depend upon the cell type, the subunits expressed by a cell, and conditions in the surrounding environment (figure 4.4).

Finally, some reports suggest that some receptors may downregulate in response

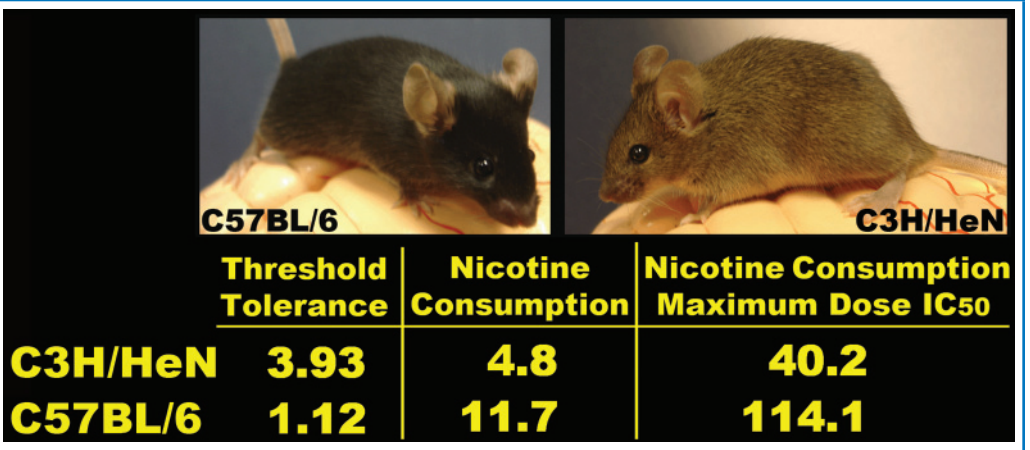
to nicotine. For example, chronic exposure to nicotine downregulates the expression of α6-containing receptors in mice and rats;[107,108] however, those composed of α6β3 are unregulated by chronic nicotine in transfected cells.[106] Therefore, while the understanding of upregulation, and possibly downregulation, remains cloudy, the importance of this process to the outcome of nicotine's effects on behavior are certain and are revisited below. Thus, the issue of receptor down- or upregulation is just one more consideration that investigators must ponder when attempting to link genetic variants and physiological substrata with dependence-related phenotypes.

Routes of Nicotine Administration: Interaction of the Drug with Physiological Systems

Just as age, gender, and general health affect nicotine metabolism in people,[15,16,109] the same is true of mice (figure 4.5). Also, how nicotine enters the body can affect nicotine effects in ways that must be carefully considered when attempting to draw inferences about nicotine actions and their genetic bases. Although plasma levels of nicotine are easily determined, such levels may not accurately reflect functional exposure of critical, or targeted, tissue. In fact, tissue levels of nicotine can vary dramatically from plasma levels. Researchers have characterized the effects of 24 hours of constant intravenous (IV) infusion of nicotine on tissue distribution and concentration of nicotine in rabbits.[8,15,16,109] In such studies, the brain, heart, liver, and gastrointestinal tract contained three- to fourfold more nicotine than did the plasma, whereas the increment in muscle and lung was approximately twofold. The major site of excretion, the kidneys, can exceed a 21-fold increment. One curiosity is that adipose tissue exhibits relatively poor nicotine retention (approximately one-half the concentration of plasma). Nicotine also crosses the placenta[16] and is concentrated in breast milk in which concentrations can reach threefold that of plasma.[8] Nicotine also concentrates in the brain or lungs following direct infusion and may achieve concentrations tenfold greater than those of the plasma. Finally, nicotine delivered directly to the rat lung becomes concentrated in that tissue and is slow to enter into circulation.[110] These data show highly variable concentrations of nicotine in different tissues or body compartments and that specific concentrations reflect route of administration.

An often overlooked concern is that some methods of administration may introduce undesirable contaminants, such as lipopolysaccharides (LPSs), or induce local inflammatory events. Using saline as the control in these experiments is inadequate in that the contaminants in the saline are generally nonpyrogenic (LPS-free), whereas nicotine from the shelf or other commercial sources is likely to have been contaminated at some time with small amounts of bacteria. Several researchers have discussed this issue.[111–113] It is possible that conditions related to chronic inflammation such as fatigue and cachexia could complicate, and even be confused with, the drug effect. Consequently, the duration of exposure, route of administration, and the tissue being examined are important variables when assessing the effects of nicotine. Therefore, since no single route of administration models all aspects of the behavioral components of nicotine dependence, it is important to carefully define the behavior or motivational phenomenon of interest and to employ a route of administration that is both

Figure 4.5 Genetic Influences on Nicotine Tolerance and Self-Administration



Note. A comparison showing the often dramatic difference for key responses to nicotine administration by different mouse strains are shown for C3H/HeN and C57BL/6 mice. These data are taken directly from Table 6 of a study by Crawley and colleagues[7] in which similar values for additional effects of nicotine that include as many as 16 additional strains can be found. As reported there, the threshold tolerance dose is reported in milligrams per kilogram (mg/kg) of nicotine infused per hour and reflects the minimal infusion dose that increased the effective dose for nicotine to reliably produce tolerance on activity and temperature thresholds. The maximal dose (IC_{50}) for nicotine consumption (mg/kg/day) and the nicotine concentration (microgram per milliliter) that decreases preference ratios to 50 percent are shown.

theoretically relevant and does not create artifacts that mask or distort target effects. A review by Matta and colleagues[8] provides an outstanding and comprehensive resource for questions concerning routes of nicotine administration for the mouse.

Intravenous Nicotine Administration

IV administration is a commonly used delivery system to study the effects of nicotine. The tendency for a behavior to become routine or automatic may contribute to high levels of drug use.[114,115] Similarly, cues associated with nicotine self-administration may also elicit strong dopaminergic neurotransmission and instigate increased self-dosing.[83] If these elements are not represented in the phenotype, important genetic bases of nicotine dependence and vulnerability may go undetected. One way this has been dealt with, especially in rats, is through establishing regimes that allow for nicotine self-administration through control of IV injection of nicotine.[116–120] This introduces a rapid rise in plasma nicotine during active cycles that resembles the pulselike use of nicotine seen in humans. One disadvantage is that active cycles of infusion may produce nicotine concentrations that exceed plasma concentrations achieved by normal physiological routes by possibly as much as 10-fold.[121] This exceedingly high concentration of nicotine can produce unanticipated and possibly nonphysiological effects, including rapid and generalized receptor desensitization (or even receptor inactivation). Also, the drug can readily exceed 100 microns in the vicinity of the injection to produce undesirable side effects, such as neuronal death by excitotoxicity.[122–124] Therefore, the control of nonspecific effects (possibly via a receptor knockout mouse as described below) is important if an experimental goal is to distinguish the behavioral outcome as being related to a rapid increase in receptor activation or possibly reduced receptor function.

Subcutaneous Nicotine Administration

The osmotic minipump,[125–127] an efficient subcutaneous method for administration of nicotine into the periphery or brain, has been used with success for many years (for a review, see Marks and colleagues[14]). It permits relatively prolonged periods of exposure (perhaps as long as six weeks) and affords control over the rate and timing of infusion. In addition, removal of the minipumps allows investigators to match the specific duration of withdrawal with changes in cellular events. Thus, for experiments in which withdrawal-induced changes in cell signaling are assessed, use of the minipump may be more advantageous than methods that do not allow for precise timing of cessation of nicotine administration, such as oral self-administration.

As with every method of chronic nicotine administration, some disadvantages exist, but they are often specific to experimental design and focus. For instance, implantation and removal of the pump requires minor surgery; although the surgery lasts fewer than five minutes, the use of anesthesia or introduction of other confounding effects such as animal handling may be problematic for some studies. Also, a potential problem is that weight gain could affect dosage;[8] however, this depends on the length of the study and may be a greater concern for studies in rats, which may show greater weight gain than do mice. Finally, any discussion of methods of chronic nicotine administration must consider how the model relates to features of human nicotine dependence that may be important to elucidating its genetic substrata. For instance, the minipump system does not involve a self-administration ritual. Studies have shown that self-administration ritual and response to environmental cues may influence behaviors related to nicotine dependence.[128–130] In addition, the minipump continuously administers nicotine in comparison to episodic administration. There is evidence that rate of rise time of nicotine receipt in critical brain regions determines certain hedonic reactions to the drug such as elation and euphoria, "buzz," "rush," and "high."[131–133] However, smokers may exhibit different patterns of smoking, with some smokers seeking boosts in plasma nicotine levels and other smokers attempting to maintain steady-state plasma nicotine levels.[50,134]

As a result, it is not clear how best to model human nicotine consumption, and it is not clear if different genetics are involved in the different consumption patterns. The use of different methods of chronic nicotine administration in mice would allow for this genetic question to be investigated. Finally, no matter what method of delivery is chosen, it must be remembered that the half-life of nicotine in the mouse is approximately 7–10 minutes,[135] compared to approximately 60 minutes in rats[136] and approximately 120 minutes in humans.[137] Thus, intermittent administration of nicotine that will produce similar plasma steady-state levels of nicotine as seen in smokers may be difficult to achieve because of the short half-life in mice.

Oral Administration

Oral administration of nicotine (e.g., via drinking water) has become increasingly popular as a method to achieve chronic or long-term nicotine exposure in primates and rodents.[8] This route of administration also has some physiological and sensory relevance to humans who self-administer tobacco or nicotine orally. For instance, smokeless tobacco and nicotine aerosols involve oral use, and much of the available nicotine ultimately is swallowed.[138] The main advantage of using drinking water as a vehicle is that it is relatively easy, inexpensive, and reduces considerably the handling and manipulation of the

animal. In addition, it yields plasma nicotine concentrations that are similar to those observed in smokers, and because most drinking occurs in the evening hours, this method reproduces the cyclic (episodic) increase and decrease of nicotine administration that occurs in smokers.[8] For example, drinking-water administration yields plasma nicotine levels that range from 10–20 nanograms per milliliter (ng/mL);[8,88,139,140] these levels are similar to the lower ranges observed in smokers.

Further, oral nicotine produces a broad range of effects associated with chronic nicotine exposure and dependence, effects that were originally obtained in animals with IV-injection methods—for example, receptor upregulation, tolerance, neuroprotection,[77,141–145] and mouse-strain-specific responses reflective of physical dependence.[146] However, nicotine in drinking water, like injection, does not replicate the prolonged exposure of the oral mucosa to nicotine, which is an important determinant of nicotine absorption with some methods of human self-administration.[8]

Other problems encountered with this method of administration include the bitter taste of nicotine (which can limit consumption in a strain-specific manner[8]) and the failure of some mice to tolerate the amount of nicotine consumed, possibly because of toxicity and occasionally irritation of the gastrointestinal tract. The bitter taste is usually overcome by supplementing the drinking water with 1%–2% saccharin. Animals receiving only saccharin water are routinely used in studies to ensure control for nonspecific effects.[8,88,139,140]

For oral administration, it is important to use nicotine in the free-base form to avoid the complications related to tartrate salt. The authors of this chapter found that C57BL/6, CBA/HeN, and C3H strains can all be administered oral nicotine for time periods ranging from several weeks to years.[81,139,147] Finally, while taste may constrain dependence development in some mouse strains, this may not be a problem to the extent that taste sensitivity plays a significant role in affecting dependence vulnerability in humans.[148]

In terms of how route of administration affects intake and dose, reports examining the biological activity of cotinine—the major metabolite of nicotine—raise interesting questions about the impact of nicotine and the suitability of different dosing paradigms. Administration of cotinine to rhesus monkeys and rats can recapitulate many of nicotine's effects, including protective effects to differentiated PC-12 cell survival, but it fails to induce receptor upregulation.[149] However, cotinine is generally much less efficacious than is nicotine,[143] although it occurs at greater concentrations in the plasma. This must be kept in mind when evaluating nicotine effects yielded by systems such as direct nicotine infusion into the brain. This strategy provides little opportunity for nicotine metabolism and might obviate the effects of cotinine in a manner inconsistent with human nicotine use and dependence. At the very least, such pathways must also be considered when assessing the genetic contribution to the addictive process, especially when extrapolating to humans.

Finally, the route of administration may have additional unintended consequences on the outcome of experiments and possibly mask important aspects of nicotine biology. For example, different routes of nicotine administration (e.g., injection versus oral) may enhance or abrogate nicotine's anti-inflammatory effects in ways not yet entirely defined.[24,150] Curiously, to the extent that nicotine administration is anti-inflammatory, it would allow some mouse strains to tolerate experimental

The Metabolic Fate of Nicotine

The extent to which the features of nicotine use and dependence are related to metabolite levels and actions requires further investigation. What is clear is that catabolism of nicotine promotes differential responses to nicotine and that both route of administration and strain-specific genetics contribute to this effect. There is evidence that metabolism is an important influence on nicotine self-administration and magnitude of drug effect. For example, studies of rats, mice, and humans[a] show significant intraspecific variability in nicotine metabolism, including differences in plasma levels of several major catabolites such as cotinine.[b,c,d] Among humans, disparities in nicotine intake have been directly related to differences among individuals in their respective rates of nicotine catabolism.[e] Further, altered oxidation of nicotine,[c,f] and conversion to cotinine in some individuals, corresponds with an allelic form of the principal enzyme of nicotine metabolism, CYP2A6.[a,c] In mice, the CYP2A6 homologue is Cyp2a5; this, too, appears to contribute to differential nicotine consumption behaviors between strains, as witnessed in male F2 mice that exhibit increased Cyp2a5 expression[g] and corresponding changes in metabolic rates and increased nicotine self-administration. Basically, slow metabolism may produce an accumulation of nicotine and toxicity (e.g., activation of muscle receptors or autonomic neurons), and thereby contribute to limited intake. Rapid clearance, however, decreases the effective pharmacological dose.

[a]Swan, G. E., N. L. Benowitz, C. N. Lessov, P. Jacob 3rd, R. F. Tyndale, and K. Wilhelmsen. 2005. Nicotine metabolism: The impact of CYP2A6 on estimates of additive genetic influence. *Pharmacogenetics and Genomics* 15 (2): 115–25.

[b]Svensson, C. K. 1987. Clinical pharmacokinetics of nicotine. *Clinical Pharmacokinetics* 12 (1): 30–40.

[c]Messina, E. S., R. F. Tyndale, and E. M. Sellers. 1997. A major role for CYP2A6 in nicotine C-oxidation by human liver microsomes. *Journal of Pharmacology and Experimental Therapeutics* 282 (3): 1609–14.

[d]Terry Jr., A. V., C. M. Hernandez, E. J. Hohnadel, K. P. Bouchard, and J. J. Buccafusco. 2005. Cotinine, a neuroactive metabolite of nicotine: Potential for treating disorders of impaired cognition. *CNS Drug Reviews* 11 (3): 229–52.

[e]Perez-Stable, E. J., B. Herrera, P. Jacob 3rd, and N. L. Benowitz. 1998. Nicotine metabolism and intake in black and white smokers. *JAMA: The Journal of the American Medical Association* 280 (2): 152–56.

[f]Siu, E. C., and R. F. Tyndale. 2007. Non-nicotine therapies for smoking cessation. *Annual Review of Pharmacology and Toxicology* 47:541–64.

[g]Nakajima, Y., A. M. DelliPizzi, C. Mallouh, and N. R. Ferreri. 1996. TNF-mediated cytotoxicity and resistance in human prostate cancer cell lines. *Prostate* 29 (5): 296–302.

conditions that would otherwise be intolerable because of inflammatory complications. In effect, alternative routes of nicotine administration can affect pro-inflammatory systems differently; this may compromise both reproducibility and possibly the translatability of results to other systems. Of course, this remains to be clearly demonstrated experimentally, but the possibility that the route and rate of administration can influence experiments and even modulate strain-specific responses to this drug remains an important open question.

The Mouse Model of Nicotine Dependence

Basics of Experimental Design

Researchers using animal models have often observed that "mice are not little rats." Similarly, a mouse is not just a mouse; different strains can vary dramatically in characteristics[2–4,7] that may directly or indirectly affect phenotypic measures

(figure 4.5). Although the laboratory rat has been used extensively for examining the behavioral effects of nicotine, the mouse as a model system is relatively new. The basic premise that the mouse model can be successfully exploited to reveal human-related traits is supported by more than three decades of successful translational research conducted by immunologists and cancer biologists. Although the application of the mouse to complex behavioral traits is relatively new, the popularity of books such as *What's Wrong with My Mouse?*[151] and similarly oriented introductory "how-to" Web sites is indicative of the growing interest in this model system.

Numerous mouse-rat differences make it hazardous to extrapolate across these species. For instance, the mouse is, in general, much less sensitive to nicotine than is the rat.[8] Nevertheless, the mouse has several advantages. First, the mouse model has a long and detailed record for being used successfully to measure the effects of nicotine on behavior, physiology, biochemistry, and a variety of diseases. Second, the mouse is particularly amenable to well-defined genetic and pharmacological experimental manipulation, and this model has been used successfully to reveal key nAChRs important in mediating the effects of nicotine. Third, heterogeneity in the response of different mouse strains to nicotine provides a valuable opportunity to identify strain-linked genetic differences that affect the magnitude and persistence of nicotine's effects. Fourth, genes can be readily manipulated through methods of homologous recombination. Finally, mice are much cheaper to acquire and maintain in large numbers. Although the many mouse varieties provide a remarkable array of experimental opportunities, the selection of the strain appropriate to the experimental paradigm is crucial, and this topic is discussed below.

Selecting a Mouse Strain

Ultimately, the goal for using mice in the study of nAChR biology is to understand how nicotine use leads to dependence. Chronic nicotine use and the phenotypes of dependence are closely associated, in both humans and other animals, with concurrent physiological changes in nAChR function and expression. The measurement of acute and chronic effects of nicotine administration in at least 19 mouse strains has yielded a remarkable database. This database quantitatively describes multiple genetically influenced physiological and behavioral differences in the effects of nicotine exposure.[7]

Studies using genetic manipulation of mouse nAChR subunits in combination with pharmacological and functional measures are beginning to add experimental details that contribute to the understanding of strain-related results and to the design of future genetic analyses of nicotine dependence. Excellent resource information is available regarding these selections in an extensive and growing database including hundreds of strain- and gender-specific behavioral and physiological traits of mice that can be accessed on the Web from the Mouse Genome Informatics database.[152,153]

Upon selecting the best strain, it is important to ensure it has the desired phenotype and genotype. For example, certain inbred colonies can undergo subtle genetic drift relative to a colony at another institution, making it important that the strain of mouse selected actually exhibits the reported traits. One example of the influence of strain-related genetic drift is that very young DBA/2 mice exhibit sensitivity to auditory seizure if purchased from Jackson Laboratory but not if obtained from Charles River Laboratories.[154] The problem of unknown genotype can be particularly serious in studies that routinely mix multiple strain backgrounds during the production of knockout or knockin mice. This can lead to unexpected phenotypes. Problems caused by strain mixture may arise when a newly

made transgenic mouse is crossed with a parent from a different strain. For instance, transgenic mice may be crossed to CBA mice to increase hybrid vigor and enhance the possibility of obtaining offspring. However, CBA mice experience hereditary retinal degeneration that, despite their dark black eyes, renders them severely visually impaired, if not blind. When these animals are used in experiments that require the use of visual cues for the behavioral endpoint, they can produce spurious results of limited value (e.g., see discussion by Crawley and colleagues[7]).

Similar problems of unexpected phenotypes can occur in homologous recombination experiments that are most easily accomplished in stem cells from two mouse strain backgrounds (129 and FVB/N) and then commonly backcrossed into the C57BL/6 mouse. Notable, and often substantial, differences in the basic neuroanatomy between the 129 and B6 strains are of sufficient relevance to warrant one mouse brain atlas to show these differences side by side (e.g., corpus callosum agenesis in strain 129[155]). As a consequence, it is not surprising that 129/Sv mice are impaired on many learning tasks.[7] Crossing them with other mouse strains produces a complex background in which the respective parental gene interactions and related but ill-defined environmental interactions[156] can impart a significant range of interactive effects not necessarily controlled for by litter mates.

When using backcrossed mice, an important issue is how many backcrosses ensure genetic background homogeneity. One common approach is to use mice following 6 to 10 backcrosses into the desired parental strain. However, this method is highly subjective. Detailed analyses suggest that even after 10 backcrosses, which can take three years to complete, there is little guarantee that strain purity will exceed the optimal target of approximately 99%. The purity of the parental background can vary considerably among offspring; by some estimates, as much as ~5% of the genetic variation may remain even after as many as 50 random backcrosses.[156] Ideally, marker-assisted, accelerated, backcrossing strategies, also referred to as speed congenics, should be used to optimize backcrossing efficiency, minimize the time required to accomplish the optimal genetic background, and ensure optimal genetic uniformity among the animals being compared. In this method, strain-specific PCR-based procedures that are commercially available (e.g., Charles River Laboratories, Harlan Sprague, Dawley, Inc., or Jackson Laboratory) permit assessment of strain-specific DNA marker density of at least 15 centimeters on all chromosomes for no fewer than 15 commonly used strains. This quantifies background contamination and permits selection of strains with more than 95% of the desired parental strain background, often with fewer than five backcrosses.

Nicotine and Behavioral Changes

Addictive substances share common features, including an ability to produce behavioral reinforcement, promote self-administration, and alter reward-related behavior. The mouse model recapitulates each of these basic features of dependence and has facilitated the identification of genetic, neural, and behavioral substrata promoting these changes. This section will review mouse models of reinforcement, self-administration, reward, and tolerance, with emphasis on the genetic and neural systems that are implicated by these models.

Reinforcement

The reinforcing properties of nicotine are often assessed by studies that measure the ability of nicotine to maintain

self-administration. In mice, both IV and oral nicotine self-administration models have demonstrated nicotine reinforcement. Both will be discussed below. As a note, the reporting of nicotine doses varies across studies. Some studies report nicotine doses as base weight, and some studies using nicotine tartrate salt report nicotine doses as salt weight. To facilitate comparisons across studies, doses are standardized to reflect base weight. In addition, one of the difficulties in comparing genetic influences across studies is that methodologies often vary. Factors that can vary across studies include strains used, doses used and effective doses, routes of administration, and treatment of the mice. This chapter attempts to provide information on doses tested, strains used, and methodological variables for the self-administration studies and the studies examining reward and tolerance. Factors that influence reinforcement include nAChR properties, genetics, developmental changes, and nonnicotinic neural mechanisms. The following sections provide an overview of these factors.

Intravenous Nicotine Self-Administration

Inferences Regarding Critical nAChRs

Self-administration has helped both to reveal the behavioral properties of nicotine reinforcement and to elucidate the neural substrates of reinforcement from the level of neural area, to receptor subtypes, to underlying cell-signaling cascades. Multiple early studies suggest that high-affinity nAChRs are involved in the reinforcing effects of nicotine. First, IV self-administration of nicotine and other high-affinity nAChR agonists was examined in NMRI mice.[157] Each self-administration session began with administration of a priming infusion of the test compound for that session. A range of nicotine doses was tested (0.01, 0.03, 0.04, and 0.06 milligrams per kilogram [mg/kg]/infusion); the 0.01 and 0.03 mg/kg/infusion doses of nicotine were both associated with increased nose pokes (the drug-contingent response), but the rate of nose pokes for higher doses (0.04 and 0.06 mg/kg/infusion) was no different from the rate in yoked controls. Self-administration was also seen for the high-affinity nAChR agonists cytisine (at doses of 0.025, 0.05, and 0.075 mg/kg/infusion) and lobeline (at doses of 0.25, 0.5, and 0.75 mg/kg/infusion); however, self-administration of the high-affinity nAChR agonists ABT-418 and epibatidine was not seen at the doses tested.

Studies in nAChR subunit knockout and knockin mice have produced direct evidence in support of the high-affinity nAChRs, especially β2-containing nAChRs, in promoting the reinforcing effects of nicotine. Mice deficient in β2 nAChRs failed to self-administer nicotine and did not develop behaviors consistent with reinforcement.[96,158,159] In these studies, β2 knockout mice were trained first to execute nose pokes for cocaine (0.8 mg/kg/infusion) by responding to an active versus inactive port in an operant chamber. After achieving an asymptotic level of response, wild-type mice were switched to saline or 0.03 mg/kg/infusion of nicotine and β2; knockout mice were switched to 0.03 mg/kg/infusion of nicotine. The rates of nose pokes significantly decreased for wild-type mice receiving saline and β2 knockout mice receiving nicotine, but not for wild-type mice receiving nicotine.

Direct drug infusion has been a powerful approach for identifying nAChRs subtypes involved in the reinforcing effects and also for identifying the associated neural substrata. Direct infusion of pharmacological agents into the ventral tegmental area (VTA), an area shown to be involved in drug-seeking behavior, has established VTA nAChR involvement in self-administration.[160] In rats, direct infusion of the α4β2 nAChR-favoring

antagonist dihydro-β-erythroidine (DHβE) into the VTA disrupted IV nicotine self-administration, suggesting that the reinforcement effect of nicotine emanated from high-affinity nAChRs in the VTA, such as the α4β2 nAChR.[161]

Studies in mice have similarly demonstrated the involvement of VTA high-affinity nAChRs in the reinforcing effects of nicotine. Besson and colleagues[162] examined if wild-type and β2 knockout mice would self-administer either the vehicle, 100 ng of nicotine, or 200 ng of nicotine directly into the VTA. This study used a hybrid self-administration paradigm that required the mice to navigate a Y-maze. One arm of the Y-maze was associated with direct infusion of nicotine into the VTA. Wild-type mice showed a high level of self-administration for both doses of nicotine, and self-administration decreased when nicotine was replaced with the vehicle. In contrast, β2 knockout mice had equal levels of the vehicle and nicotine self-administration. However, when nicotine was switched to morphine, the level of drug self-administration in β2 knockout mice increased. This suggests that β2-containing nAChRs in the VTA are not involved in generalized reinforcing effects of drugs of abuse but are specifically involved in the reinforcing effects of nicotine. This was further substantiated[163] by showing that viral-mediated reexpression of the β2 subunit in the VTA of β2 mice would restore IV nicotine self-administration. This study also used the hybrid Y-maze self-administration paradigm with one arm associated with direct infusion of 36 ng of nicotine into the VTA. Infusion of nicotine into the VTA of wild-type mice, but not β2 knockout mice, was reinforcing. However, the reexpression of the β2 subunit in the β2 knockout mice was associated with increased intra-VTA nicotine self-administration.

Because β2 is widely expressed in the nervous system and is a promiscuous subunit, subsequent studies explored the roles that different alpha subunits play in processes that may mediate self-administration. Such studies have revealed that α6 is particularly important to neurons of the VTA and basal ganglia,[107,164–167] where it forms receptors composed of α4α6β2β3 subunits.[68] In fact, receptors harboring α6 may be disproportionately upregulated,[102] or possibly downregulated,[107,108] in response to chronic nicotine. In either case, this reinforces the hypothesis that this subunit plays a special role in the effects of nicotine related to self-administration and reinforcement.

In summary, studies demonstrate an important role of β2-containing nAChRs in the reinforcing effects of nicotine. Factors that influence β2-containing nAChR function, such as the inclusion of other subunits (e.g., α6) or other less defined genetic considerations, should alter nicotine self-administration. However, genetic influences on β2-containing nAChR function may not be the only factors that contribute variation in nicotine self-administration.

Behavioral Genetics of Self-Administration

Behavioral genetics studies that have compared oral nicotine self-administration across inbred strains of mice have helped to advance understanding of the genetics of nicotine dependence. For instance, in a study that compared oral self-administration of nicotine, ethanol, amphetamine, and aspartame between C57BL/6 inbred mice and DBA/2 inbred mice, clear differences were observed.[168] Because the focus of this monograph is on nicotine, only the treatment procedure for nicotine will be presented.

After eight days of habituation to the two-bottle-choice cages, one bottle was replaced with a 0.38-microgram per milliliter (μg/mL) nicotine bottle. After two days, the nicotine concentration was increased to 0.61 μg/mL for two days, followed by

0.96 µg/mL for two days. The 0.96-µg/mL concentration was followed by four days at 1.54 µg/mL, six days at 2.42 µg/mL, and then eight days at each of the following concentrations: 3.84, 6.14, 9.60, 15.36, 24.19, and 38.39 µg/mL of nicotine. C57BL/6 mice displayed a greater preference for nicotine than did the DBA/2 mice. This was also true for ethanol and amphetamine, but not for aspartame, which DBA/2 mice preferred. Because C57BL/6 and DBA/2 mice are inbred strains, variance in behavior between the strains reflects the influence of genetics on the behavior. Thus, the results from this study demonstrate the existence of genetic influences on nicotine, ethanol, amphetamine, and aspartame oral self-administration. The study also suggests that the genetic influences on aspartame self-administration differ from those affecting nicotine, ethanol, and amphetamine self-administration.

A similar procedure was used in an expanded inbred mouse-strain survey of oral nicotine self-administration.[6] C57BL/6, C3H, DBA/2, BUB, A, and ST/b mice were presented with a two-bottle choice: nicotine versus vehicle. The vehicle was either water or 2% saccharin. The concentration of nicotine changed from 10 to 20 to 35 to 50 to 65 to 80 to 100 to 125 to 160 to 200 µg/mL every four days. Increased nicotine concentration was inversely related to nicotine consumption, and this relationship was influenced by genetics. The concentration of nicotine that produced a 50% decrease in consumption relative to the 10 µg/mL concentration was compared across strains. This concentration was highest in C57BL/6 mice, followed by DBA mice, then BUB, A, C3H, and ST/b mice, respectively. Saccharin influenced nicotine intake only in C57BL/6 mice at low nicotine concentrations and in ST/b mice across all nicotine concentrations.

Comparable results were found in another strain survey of oral nicotine self-administration.[169] A two-bottle-choice paradigm was used to compare oral self-administration of water and escalating doses of nicotine (1.75, 3.51, 8.77, 17.54, 26.31, and 35.08 µg/mL; five days of administration per level) across C57BL/6, C3H/J, DBA/2, ST/b inbred mice, NMRI outbred mice, and an A/J×NMRI cross. As reported before, strain differences existed in amount of nicotine consumed. C57BL/6 consumed the most nicotine, followed by C3H/J mice, the A/J×NMRI cross, DBA/2 mice, NMRI mice, and ST/b mice. Clearly, genetics contributes to differences in oral nicotine self-administration; however, in all three studies nicotine consumption was lower than vehicle consumption. Therefore, in these studies, it is unclear if genetics is influencing preference for nicotine or sensitivity to aversive effects of nicotine. One strategy for addressing this question would be to compare oral nicotine self-administration and IV nicotine self-administration across strains of inbred mice to determine if genetics of oral and IV nicotine self-administration are similar. This type of study remains to be done; most mouse behavioral genetic studies of nicotine self-administration have used oral nicotine self-administration.

One study directly tested if preference for oral self-administration of nicotine over water could be established.[170] Outbred CD-1 mice were maintained on a water-restriction schedule with water access limited to two hours. Mice were then presented with a two-bottle choice of water versus 10 mg/L nicotine for a two-hour period. In a follow-up experiment, the concentration of nicotine was reduced to the following levels every two days: 7, 5, 3.5, and 2.5 mg/liter (L). Mice preferred nicotine-containing solutions to water, and as the concentration of nicotine decreased, the fluid intake for nicotine, but not for water, increased. Individual differences in nicotine preference were then assessed. Mice were given another choice of a vehicle or 10 mg/L of nicotine; however, the vehicle

used a 10% sucrose solution to mask the bitter taste of the drug. After six days of training, the vehicle was switched to water and preference for nicotine was measured. A significant preference for nicotine was seen on day seven. This preference decreased over subsequent days. However, when mice were segregated by preference for nicotine on day seven, two subpopulations emerged: one showed preference for nicotine over all days of testing (days 7–10), and the other showed no preference for nicotine. This study suggests that in outbred mice, naturally occurring genetic variance could contribute to preference for oral nicotine; however, follow-up studies are needed to determine if polymorphisms exist between the two subpopulations.

A study that strongly suggests that genetic variance influences oral nicotine self-administration examined if expression of *Cyp2a5*—the homologue of the human gene *CYP2A6,* which codes for an enzyme involved in the metabolism of nicotine—is correlated with levels of oral nicotine self-administration.[171] Because C57BL/6 mice show high levels of nicotine consumption and ST/b mice show low levels, F2 mice from a C57BL/6×ST/b cross were segregated into high and low oral nicotine consumers to test if levels of Cyp2a5 protein similarly segregated. In male F2 mice, high nicotine consumption was associated with higher levels of Cyp2a5 protein and, not surprisingly, faster metabolism of nicotine, suggesting that the genes involved in nicotine metabolism can influence oral nicotine self-administration. The same effect was not seen in female mice; thus, the expression of this phenotype may be linked to gender. The findings from this study, combined with the other behavioral genetics studies reviewed here, demonstrate how polymorphisms can alter nicotine self-administration and potentially influence nicotine dependence. Although genetic factors influence nicotine self-administration, environmental and developmental factors most likely interact with genetics to alter nicotine self-administration.

Modeling Developmental Factors in Nicotine Reinforcement

A relationship exists between childhood exposure to tobacco industry promotional activities and risk for initiation of tobacco use (for a review, see DiFranza and colleagues[172]). Thus, identifying variables that contribute to adolescent nicotine consumption may prove critical for the successful treatment and prevention of nicotine dependence. A limited number of studies in mice have examined factors that influence oral nicotine consumption in adolescent mice. One study compared oral nicotine consumption in outbred CD-1 mice across early (24–35 days), middle (37–48 days), and late (50–61 days) adolescence.[170] Water-restricted mice were given two-hour access to two bottles—water versus a 10 mg/L solution of nicotine—for six days, after which the nicotine concentration was reduced to 7 mg/L for the next three days, and then to 5 mg/L for the last three days. The youngest group demonstrated a preference for nicotine; nicotine and water consumption was equal in the mid-adolescence group, and the late adolescence group showed a trend for avoidance of the nicotine solution. These results suggest that early adolescence may be a critical period for increased risk of nicotine consumption.

Two factors that may contribute to adolescent nicotine intake are gender and novelty-seeking behavior. Gender differences in oral nicotine consumption by adolescent C57BL/6 mice (35 days of age) were examined using a two-bottle-choice paradigm.[173] Mice had access to the vehicle (2% saccharin) and one of six doses of nicotine (10, 25, 50, 75, 100, or 200 µg/mL) for seven days. When adjusted for body weight, female adolescent mice consumed more nicotine than did males. Adolescent smoking in humans can be associated with increased novelty-seeking

behavior or attempts to mitigate teenage angst and anxiety. A study in C57BL/6 mice directly examined if individual differences in novelty-seeking behavior or anxiety correlated with oral nicotine self-administration.[174] At postnatal day 30, novelty-seeking behavior and anxiety were assessed using the hole-board activity box. After testing, nicotine consumption was measured for 10 days using a two-bottle-choice paradigm: water versus 10 μg/L nicotine. No correlations existed between novelty seeking and anxiety. However, adolescent mice classified as high novelty seeking consumed more nicotine than did adolescent mice classified as low novelty seeking; no relation was found between anxiety levels and nicotine consumption. These results agree with results from human research that show that novelty seeking, or a personality trait of disinhibition, is a risk factor for smoking (chapters 3 and 5).

Involvement of Extra-Nicotinic Mechanisms

An interaction among nAChRs and other neurotransmitter systems, most notably changes in dopamine signaling, appears to be critical to the reinforcing effects of nicotine in the VTA. One study examined involvement of dopamine D1 receptors in the reinforcing effects of nicotine infused into the VTA.[162] Four groups of C57BL/6 mice were trained in the Y-maze intra-VTA nicotine self-administration paradigm. Mice received either the vehicle infused into the VTA, 10 ng of nicotine infused into the VTA, 100 ng of nicotine infused into the VTA, or 100 ng of nicotine infused 2.3 millimeters dorsal of the VTA. The 10-ng and 100-ng doses of nicotine infused into the VTA were more reinforcing compared to vehicle and nicotine infusions dorsal to the VTA. The D1 dopamine receptor antagonist SCH 23390 and the high-affinity α4β2 nAChR antagonist DHβE blocked the reinforcing effects of intra-VTA nicotine self-administration. These results suggest that nicotine activation of high-affinity nAChRs (e.g., α4β2 nAChRs) in the VTA may modulate D1 dopamine receptor activity and reinforcement.

Studies suggest that both metabotropic and *N*-methyl-D-aspartic acid (NMDA) glutamate receptors are also involved in the reinforcing effects of nicotine. Sorger and colleagues investigated the role of the metabotropic glutamate receptor 5 (mGluR5) in nicotine self-administration in DBA/2 mice.[175] DBA/2 mice were trained to execute nose pokes for either saline or one of four doses of nicotine (0.016, 0.048, 0.16, 0.48 μg/infusion, IV self-administration). Only the 0.048-μg dose of nicotine was associated with increased nose pokes compared to the yoked control. This increase was blocked by administration of 2-methyl-6-(phenylethynyl)-pyridine, an mGluR5 antagonist. The involvement of the glutamate system in IV nicotine self-administration was further investigated by examining the effects of the NMDA receptor channel blocker memantine on self-administration.[176] A yoked experimental design was used, and Swiss mice in the active chamber executed nose pokes for either 0, 0.03, 0.06, or 0.11 μg of nicotine. The 0.06-μg dose of nicotine was associated with the greatest increase in nose pokes. This effect was blocked by memantine. Subsequently, however, the pharmacological specificity of this agent has been extended to include α7 nAChRs,[177] which could cloud the interpretation of this effect as being mediated solely through NMDA receptors. However, as reviewed earlier, the reinforcing effects of nicotine appear to be largely dependent on high-affinity nAChRs and relatively independent of α7 nAChRs.[11,158,162,178]

Clearly, the nicotinic and the glutamate systems interact to support self-administration of nicotine; however, the mechanism underlying this interaction remains unclear and possibly multifaceted. Certainly, glutamate receptor (GluR)

function is modified by nicotine acting through nAChRs, as reported by multiple electrophysiological examinations.[62,64,179–181] However, additional cell-mediated mechanisms, including alteration of GluR transport by the neurons[182] and the modification of susceptibility to proteolysis,[183] must be included in considerations of how nicotine affects this major excitatory system.

Finally, in addition to dopamine and glutamate involvement in the reinforcing effects of nicotine, GABA may also be involved. The effects of the $GABA_B$ receptor agonist baclofen on IV nicotine self-administration was assessed in mice; unfortunately, the study did not specify the strain of mice.[184] Mice in the chamber with the active nicotine (0.03 mg/kg/infusion)-associated port executed significantly more nose pokes than did yoked controls. Baclofen decreased responding at the nicotine-associated port, suggesting that activation of $GABA_B$ receptors decreases the reinforcing effects of nicotine. A thorough explanation for the reinforcing effects of nicotine acting through other neurotransmitter systems, including dopaminergic, glutamate, and GABA, requires more detailed investigation. It remains clear, however, that modulation of these systems through both nAChRs and related polymorphisms is likely to form the basis of the complex genetic components that combine to define the reinforcing effects of nicotine for individuals.

The cellular and molecular changes triggered by activation of nAChRs and other neuropharmacological systems that underlie the reinforcing effect of nicotine (e.g., dopamine, glutamate, and GABA systems) may involve altered calcium-mediated cell signaling. Swiss albino mice were trained to execute a nose poke for 0, 0.01, 0.02, 0.03, or 0.04 mg/kg/infusion of nicotine.[185] The dose-response curve was an inverted function; the 0.02- and 0.03-mg/kg/infusion doses of nicotine produced significant increases in nose pokes, with the 0.03-mg/kg/infusion dose producing the largest change. A 2.4-mg/kg dose of the nAChR antagonist mecamylamine blocked the reinforcing effects of 0.03 mg/kg/infusion of nicotine but had no effect on responding in the saline control group. In addition, the L-channel calcium antagonist isradipine dose dependently inhibited the reinforcing properties of 0.03 mg/kg/infusion of nicotine without altering baseline levels of nose pokes. Additional work is needed to further elucidate the molecular substrata of the reinforcing effects of nicotine because genetic influences on these substrata could contribute to variability in the reinforcing effects of nicotine.

In summary, self-administration studies in mice that examined the reinforcing effects of nicotine have demonstrated that mice will self-administer nicotine and have identified the neural and genetic substrata involved. For example, these studies have demonstrated that high-affinity nAChRs in the VTA are involved in nicotine self-administration and that calcium-mediated cell signaling is also involved. In addition, these studies have shown that genetic variation contributes to variation in nicotine self-administration, and they have identified the *Cyp2a5* gene, which is involved in nicotine metabolism, as a gene potentially linked to nicotine self-administration. Finally, these studies have identified potential risk factors that may, in general, contribute to adolescent nicotine use: age, gender, and risk-taking behavior.

Nicotine and Reward

Conditioned place preference (CPP) is used as a model to investigate the rewarding effects of nicotine.[186] Nicotine administration is repeatedly paired with one chamber, and saline administration is repeatedly paired with a second. The mouse or rat is then given access to both chambers,

and greater time spent in the chamber previously paired with nicotine is taken as a measure of preference for nicotine. In addition to measuring the rewarding properties of nicotine, CPP also measures the ability to form associations between the effects of nicotine and a contextual environment. Thus, for any manipulation that disrupts CPP, it must be determined if the manipulation is altering learning or reward processes. Experiments using CPP to investigate the rewarding effects of nicotine in mice have identified procedural variables that affect the development of nicotine CPP and have identified underlying neural and genetic substrata involved in CPP.

Research using CPP provides a complementary perspective to research on self-administration. Although self-administration should, in theory, reflect the hedonic or rewarding impact of nicotine on the mouse, it may also reflect other factors as well. One such factor is nicotine metabolism, which might affect tolerance to the repeated doses of nicotine used in self-administration studies, but be somewhat less relevant to the effects of acute doses delivered in CPP studies. Other differences between the self-administration and CPP paradigms could "select out" different genetic associates; for example, different sorts of learning are involved (Pavlovian versus instrumental), and only the self-administration paradigm permits the organism control over drug administration. Thus, it is quite likely that the two approaches will show different data patterns across strains and involvement of different neurotransmitter systems. However, the fact that all addictive drugs support both drug self-administration and CPP acquisition suggests that both are sensitive to drug reinforcement.

External variables that can influence CPP include the dose of nicotine and prehandling. The effects of four different doses of nicotine (0.25, 0.5, 1.0, or 2.0 mg/kg) on CPP were tested in Swiss-Webster mice.[187] Mice showed a preference for the chamber previously paired with 0.5 mg/kg of nicotine but avoided the chamber previously paired with 2.0 mg/kg of nicotine. No significant preference or avoidance was seen for the 0.25-mg/kg or 1.0-mg/kg doses. This study demonstrates that the effects of nicotine can shift from rewarding to aversive, depending on the dose of nicotine used in mice. Another study examined both the effects of prehandling on CPP in ICR mice and the effects of different doses of nicotine on CPP.[188] CPPs for multiple doses of nicotine (0.1, 0.25, 0.35, 0.5, 0.7, or 1.0 mg/kg) were measured; only the 0.5-mg/kg dose was associated with CPP, but only for prehandled mice. Both studies suggest that a 0.5-mg/kg dose of nicotine is rewarding, as measured by CPP. In addition, prehandling can affect the development of CPP, but it is unclear if this is a strain-specific effect and specifically related to CPP or anxiety levels of the mice.

Strain Differences

As discussed, external variables such as prehandling can affect CPP, but internal factors such as genetics also influence nicotine CPP. Studies comparing inbred strains of mice and studies using selective breeding have shown that differences in nicotine CPP are associated with genotype.

In a study using selective breeding, three lines of mice derived from heterogeneous stock mice were tested in CPP: a line in which 0.75 mg/kg of nicotine depressed locomotor activity, a line in which the same dose increased locomotor activity, and a randomly bred line.[189] A 0.75-mg/kg dose of nicotine produced CPP in the line generated by random breeding and the line bred for the stimulatory effects of nicotine on locomotor activity. In contrast, the same dose produced conditioned place aversion in the line bred for sensitivity to the locomotor depressant effects of nicotine. These results suggest that genes involved in the psychostimulant effects

of nicotine may also be involved in the rewarding effects of nicotine. Another study compared CPP across inbred strains of mice to test if natural genetic variance contributed to differences in the rewarding effects of nicotine. Multiple doses of nicotine were tested for CPP in C57BL/6 mice (0.05, 0.1, 0.3, 0.5, or 0.7 mg/kg) and in DBA/2J mice (0.3, 0.7, or 1 mg/kg[188]). The C57BL/6 mice showed significant CPP for the 0.3-mg/kg dose of nicotine, but the DBA/2J mice did not show even a trend toward CPP. Both of these studies demonstrate that genotype contributes to phenotype for nicotine CPP.

A direct comparison between the genetic influences on CPP versus nicotine self-administration can be made by comparing results from studies that contrasted these measures in DBA/2 and C57BL/6 mice. Studies previously discussed in this chapter found that C57BL/6 mice consumed more nicotine than did DBA/2 mice.[6,168,169] Similar differences were found for CPP. These results suggest that C57BL/6 mice are more sensitive to the effects of nicotine that may support dependence. Furthermore, these results could suggest that common genes are involved in CPP and oral nicotine self-administration; however, caution must be exercised because an extensive comparison across multiple inbred strains, using multiple nicotine doses and behavioral assays, is necessary to strengthen this argument.

Involvement of Receptor and Neurotransmitter Systems

In addition to identifying genetic influences on nicotine CPP, mouse studies have also examined receptor subtype involvement in CPP. The effects of the broad-spectrum nAChR antagonist mecamylamine, the high-affinity nAChR antagonist DHβE, and the α7 nAChR antagonist methyllycaconitine citrate (MLA) on nicotine CPP were assessed in ICR mice.[188] Both mecamylamine and DHβE significantly decreased nicotine CPP for a 0.5-mg/kg dose of nicotine, whereas a nonsignificant trend toward attenuated CPP was seen with MLA. This study suggests that high-affinity nAChRs, such as the α4β2 nAChR, are involved in the rewarding effects of nicotine.

Another study examined the nAChR subtypes involved in nicotine CPP by using both pharmacological and genetic inhibition of nAChR subunits.[190] Multiple nicotine doses were tested for CPP in (1) C57BL/6 mice, (2) β2 nAChR subunit knockout mice and corresponding wild-type mice, and (3) α7 nAChR subunit knockout mice and corresponding wild-type mice. C57BL/6 mice showed significant CPP for 0.3 and 0.5 mg/kg of nicotine but not for 0.1, 0.7, or 1.0 mg/kg nicotine. In β2 knockout mice, neither 0.5, 1.0, nor 2.0 mg/kg of nicotine produced CPP, but in wild-type mice, both 0.5 and 1.0 mg/kg produced CPP. The α7 knockout mice and wild-type mice both showed nicotine CPP. Further demonstrating a critical role of β2-containing but not α7-containing nAChRs in CPP, the α4β2 antagonist DHβE blocked nicotine (0.5 mg/kg) CPP in C57BL/6 mice, but the α7 nAChR antagonist MLA had no effect on nicotine CPP. Thus, β2-containing nAChRs appear to be involved in nicotine CPP.

In addition to the nicotinic acetylcholinergic system, other neurotransmitter systems may also be involved in CPP. In mice, studies have suggested that the adenosine, endogenous cannabinoid, and neuropeptide systems may all be involved in the effects of nicotine on reward. Adenosine 2_A (A_{2A}) knockout mice and wild-type mice were tested for the development of CPP to a 0.18- and a 0.35-mg/kg dose of nicotine.[191] Wild-type mice developed CPP for 0.18 mg/kg of nicotine but not for 0.35 mg/kg of nicotine. The A_{2A} knockout mice did not develop CPP for either dose. Also, both wild-type mice and A_{2A} knockout mice showed conditioned taste aversion to saccharin that was paired with a 1.75-mg/kg intraperitoneal (IP)

dose of nicotine. The A_{2A} receptor appears to be involved in the rewarding effects of nicotine but not its aversive effects. This also suggests that A_{2A} is involved in mediating the appetitive effects of nicotine and not in nicotine-based associative processes.

Another study from the same laboratory examined the role of the endogenous cannabinoid CB1 receptor in CPP for nicotine.[191] CPP was tested for 0.04, 0.09, or 0.18 mg/kg of nicotine in CB1 knockout mice and wild-type mice. The 0.18-mg/kg dose of nicotine produced CPP in wild-type mice, but no dose of nicotine produced CPP in the CB1 knockout mice. The antinociceptive effects of nicotine, however, were not disrupted in CB1 knockout mice. Thus, CB1 receptors may modulate the rewarding effects of nicotine, and drugs altering the cannabinoid system, such as the CB1 antagonist rimonabant, may have therapeutic potential for assisting in smoking cessation (for a review see Siu and Tyndale[171]).

Multiple studies suggest that the rewarding effects of nicotine can be modulated by neuropeptides, perhaps through effects at the mu opioid receptor. CPP for 0.09, 0.18, or 0.35 mg/kg of nicotine was compared between preproenkephalin knockout mice and wild-type mice.[192] Wild-type mice developed CPP for the 0.18-mg/kg dose, whereas the preproenkephalin knockout mice did not show CPP for any dose tested. The 0.18-mg/kg dose of nicotine increased dopamine levels in the nucleus accumbens of wild-type, but not knockout, mice. Thus, the endogenous enkephalin system may be involved in the rewarding effects of nicotine through altering dopamine signaling. Preproenkephalin stimulates mu opioid receptors, and consequently, mu opioid receptors may be involved in the effects of nicotine on CPP. Pharmacological studies and studies in mu opioid receptor knockout mice have directly tested if the mu opioid receptor is involved in the rewarding effects of nicotine. In NMR1 mice, nicotine CPP was successfully demonstrated for 1 mg/kg and 2 mg/kg of nicotine but not for 0.5 or 0.75 mg/kg of nicotine.[193] The mu opioid receptor antagonist naloxone blocked CPP for 1 mg/kg of nicotine, providing evidence for the involvement of mu opioid receptors in nicotine CPP. Genetic inhibition of mu opioid receptor function also disrupts nicotine CPP. No CPP for nicotine was seen in mu opioid knockout mice for all doses of nicotine tested (0.09, 0.18, and 0.35 mg/kg), which contrasts with findings with the wild-type mice that showed CPP for the 0.18-mg/kg dose but not for the 0.09- or 0.35-mg/kg doses of nicotine.[194] The mu opioid receptor does not appear to be involved in all of the effects of nicotine because deletion of the mu opioid gene did not alter the locomotor depressive effects of nicotine. The processes activated by mu opioid receptors, however, that are involved in nicotine CPP are not well understood but may involve changes in gene expression.

The transcription factor CREB is involved in learning and memory,[195] and in dependence;[196] mu opioid receptors may mediate the rewarding effects of nicotine through activation of CREB.[190] In wild-type mice, 0.35 mg/kg of nicotine produced CPP, but 0.70 mg/kg of nicotine produced conditioned place aversion. The 0.35-mg/kg dose of nicotine was associated with increased levels of phosphorylated CREB in the nucleus accumbens and VTA. Both CPP and the increased levels of phosphorylated CREB were reduced by pretreatment with naloxone. In addition, mu opioid knockout mice did not show increased levels of phosphorylated CREB after treatment with the same dose of nicotine. These results suggest that mu opioid receptor activation of CREB may be critically involved in CPP. In support, the same study found the $CREB^{\alpha\delta}$ knockout mice did not show CPP for 0.35 mg/kg of nicotine but did show conditioned place aversion for 0.7 mg/kg of

nicotine. Thus, cell-signaling cascades that activate CREB may be critically involved in the rewarding effects of nicotine, and activation of the mu opioid receptor may be one pathway that leads to reward-related increased activation of CREB. Not all effects of nicotine, however, involve activation of CREB, as demonstrated by intact, conditioned place aversion for nicotine in $CREB^{\alpha\delta}$ knockout mice.

Another transcription factor that may be involved in CPP is Fosb.[197] Both wild-type and Fosb knockout mice were tested for the development of CPP. At 0.2 mg/kg, wild-type mice showed CPP, which shifted to aversion at doses of 0.8 and 2.0 mg/kg. In contrast, Fosb knockout mice did not develop CPP for any dose tested (0.025–2.0 mg/kg) but did show aversion for doses of 0.6 mg/kg and higher. These results show that while Fosb knockout mice can learn (i.e., they show conditioned place aversion), they do not seem sensitive to the rewarding effects of nicotine. Furthermore, the knockout mice also showed reduced oral intake of 50-µg/mL nicotine in a two-bottle-choice paradigm, suggesting that Fosb is involved in processes common to CPP and choice of nicotine consumption.

Two studies examined the role of the cell-signaling molecule nitric oxide in nicotine CPP in Swiss-Webster mice. Nitric oxide is critically involved in some forms of synaptic plasticity[198,199] and may contribute to the addictive effects of drugs of abuse such as nicotine.[200] In one study, mice successfully developed CPP for 0.5 mg/kg of nicotine,[201] unless given the nitric oxide synthase inhibitor 7-nitroindazole (25 mg/kg). However, 7-nitroindazole had no effect on lithium-chloride conditioned place aversion, suggesting that the effects of 7-nitroindazole on CPP were not due to a generalized learning deficit. Another study investigated if the nitric oxide precursor L-arginine would enhance nicotine CPP.[202] Multiple nicotine doses (0.25, 0.5, 0.75, 1.0, and 2.0 mg/kg) were tested in CPP, and both the 1.0- and 2.0-mg/kg dose produced nicotine CPP. Interesting, L-arginine alone also produced CPP at doses of 200 and 500 mg/kg, but not at doses of 50, 100, or 150 mg/kg. When ineffective doses of nicotine and L-arginine were paired together, CPP resulted. Both the nAChR antagonist mecamylamine and the nitric oxide synthase inhibitor L-nitro-amino-methyl-ester blocked the acquisition, but not the expression of, CPP for the 1.0-mg/kg dose of nicotine. Together, the results from these studies suggest that nitric oxide mediates important functions associated with acquisition of nicotine CPP.

In summary, mouse research shows that nicotine reward is highly dose dependent. Specifically, as dose is increased from inert levels, mice first show robust CPPs, but ultimately, place aversions develop as doses are progressively increased. In addition, even within a strain, doses effective for establishing CPP can vary across studies. This suggests that CPP is sensitive to methodological and environmental factors, such as the construction of the apparatus and handling. Studies comparing inbred mice suggest that C57BL/6 mice may be particularly sensitive to the rewarding effects of nicotine. Further genetic studies are needed to elucidate strain differences in the rewarding effects of nicotine and to determine if common genetic substrata mediate the rewarding, aversive, and activating effects of nicotine. For instance, the stimulatory effects of nicotine and the rewarding effects of nicotine may be genetically linked. In addition, mouse studies have aided in identifying the neural substrates of the rewarding effects of nicotine. High-affinity nAChRs, such as the $\alpha4\beta2$ nAChR, appear to be critically involved in the rewarding effects of nicotine. The effects of nicotine at nAChRs may result in the activation of cell-signaling molecules such as nitric oxide and CREB that have been shown to be involved in drug dependence.

Tolerance

The rewarding and reinforcing effects of nicotine are not the only effects of nicotine that contribute to nicotine dependence; physiological adaptations that occur with chronic nicotine administration may lead to nicotine dependence and tolerance. Tolerance is a shift in the dose-response curve to the right following exposure to the drug in question. That is, with increased drug exposure, and resulting tolerance, an increasing amount of drug is required to produce a given magnitude of effect.

Tolerance has been demonstrated after both acute and chronic nicotine administration for the effects of nicotine on multiple behaviors and physiological responses. Studies in mice have elucidated the neural and genetic substrata associated with the development of tolerance and have helped identify neural adaptations that occur with chronic nicotine administration (figure 4.5). Much of the research discussed below addresses the topic of behavioral tolerance—that is, tolerance to behavioral or physiological effects of a drug that is not accounted for by enhanced drug clearance. Animals also acquire dispositional tolerance, which means enhanced clearance or metabolism of a drug as a function of prior exposure. The latter phenomenon has been discussed in the context of *Cyp2a5* expression.[171] The following sections review both acute and chronic tolerance. Significantly more is known about chronic tolerance, permitting a review of behavioral, genetic, and neural factors (from the level of the receptor to downstream cell-signaling cascades) involved in tolerance.

Acute Tolerance

Single injections of nicotine can produce tolerance to some of the direct effects of nicotine. The development and duration of such acute tolerance for the effects of nicotine on antinociception, body temperature, and motor activity were investigated in ICR mice.[203] Mice were treated with 4 mg/kg of nicotine and then challenged with a 2-mg/kg subcutaneous (SC) dose. The time to maximum tolerance and the duration of acute tolerance varied across tasks; maximum acute tolerance for the antinociceptive effects of nicotine was seen at between 30 and 60 minutes, and recovery from acute tolerance occurred after 6 hours; maximum acute tolerance for nicotine-induced motor impairments occurred between 3 and 6 hours and dissipated by 24 hours, and maximum acute tolerance for nicotine-induced hypothermia occurred between 2 and 4 hours and lasted 6 hours. The effect of intrathecal administration (i.e., injection into cerebral spinal fluid at the spinal cord) on the development of acute tolerance for the antinociceptive effects of nicotine was also tested. Maximal acute tolerance was seen at 5–10 minutes, and effective doses for the initial induction of tolerance ranged between 0.5 to 1 µg. Acute tolerance was disrupted by the calcium channel blocker nimodipine administered either via SC or intrathecal injections, suggesting that changes in calcium levels contribute to the development of acute tolerance.

Genetics may contribute to variability in acute tolerance. Miner and Collins[204] found that pretreating DBA mice with doses of a nicotine subthreshold for inducing seizures (1 or 2 mg/kg) either 15 or 30 minutes before testing for nicotine-induced seizures produced acute tolerance; by 60 minutes, acute tolerance was lost. In C3H mice, only pretreatment with the 2-mg/kg dose of nicotine resulted in acute tolerance and only when pretreatment was 7.5 minutes before testing for nicotine-induced seizures. These results suggest that genetic differences between DBA and C3H mice account for the increased sensitivity for acute tolerance in the DBA mice. The authors propose that nicotine inactivation of nAChRs may

account for the observed acute tolerance and that strain differences in desensitization or inactivation of nAChRs may underlie the strain differences in acute tolerance.

Less is known about the genetic factors that influence the likelihood or magnitude of such acute tolerance compared with chronic tolerance. In addition, there is little evidence from the human literature that indicates a role for tolerance in dependence.

Chronic Tolerance

Tolerance that develops after chronic treatment of nicotine has also been demonstrated on numerous measures. In theory, chronic tolerance might be related to dependence because higher levels of tolerance may permit higher rates of self-administration, which, in turn, result in greater effects on dependence processes. Although not a great deal of evidence links degree of chronic tolerance with tendency to self-administer nicotine, there are data showing that prolonged exposure to nicotine inures mice to the aversive effects of nicotine that they experience secondary to self-administration. The effects of chronic nicotine exposure on subsequent nicotine IV self-administration and the development of tolerance to the aversive effects of nicotine were assessed in DBA/2 mice.[205] Nose pokes delivered either saline, or one of four doses of nicotine (0.016, 0.048, 0.16, 0.48 µg/infusion), to the mouse executing the nose poke and to a yoked control. The 0.048 µg/infusion dose of nicotine was associated with a higher rate of nose pokes by mice in the chamber with the active port compared to the yoked mice. After self-administration, one-half of the mice were implanted with SC minipumps that delivered 6.3 mg/kg/day of nicotine or saline for 14 days. After removal of the pumps, the highest dose of nicotine (0.48 µg/infusion) self-administered was aversive in mice chronically treated with saline but not in mice chronically treated with nicotine. These data suggest that chronic nicotine exposure renders the organism less sensitive to the aversive effects of nicotine that is self-administered.

Behavioral Analysis of Tolerance

In many tolerance studies, the experimenter controls nicotine administration; however, oral self-administration can also induce tolerance. Tolerance for the acute effects of nicotine (1 mg/kg) on the depression of locomotor activity and on the induction of hypothermia was measured in mice that had access for 30 days to either 2% saccharin, 50 µg/mL of nicotine in 2% saccharin, 100 µg/mL of nicotine in 2% saccharin, or 200 µg/mL of nicotine in 2% saccharin.[140] The 200-µg/mL, but not the 100-µg/mL, oral nicotine group showed tolerance for the effects of acute nicotine on both locomotor activity and body temperature. In another study that assessed the development of tolerance with oral nicotine self-administration, ICR mice had access for 42 days to either 2% saccharin, 50 µg/mL of nicotine in 2% saccharin, 100 µg/mL of nicotine in 2% saccharin, or 200 µg/mL of nicotine in 2% saccharin.[146] On day 43, tolerance for the effects of 2.5 mg/kg of nicotine on nociception and body temperature was measured. Tolerance was seen for all doses of oral nicotine with the 200-µg/mL dose of nicotine producing the most tolerance. This dose produced a plasma nicotine level of 15.85 ± 10.54 ng/mL. Both of these studies demonstrate that self-administration of nicotine can produce tolerance for the effects of nicotine on multiple behaviors.

The majority of the studies examining the development of tolerance in mice have focused on tolerance for the effects of nicotine on physiological and locomotor responses, but nicotine can also alter cognition. A series of experiments has examined how the effects of nicotine on learning change as nicotine administration is shifted from acute

to chronic administration. In C57BL/6 mice, acute nicotine enhanced contextual conditioning[206,207]—that is, learning to associate a specific context with a stimulus such as a foot shock (for a review see Gould[208]). If, however, C57BL/6 mice are treated for 14 days with a chronic dose of nicotine (6.3 mg/kg/day, SC) producing the same plasma nicotine level as seen with the acute dose of nicotine (0.09 mg/kg) that enhanced contextual conditioning, no enhancement of contextual conditioning is seen. Thus, even though plasma nicotine levels were similar, the behavioral effects of the acute and chronic nicotine were not the same; acute nicotine treatment enhanced contextual conditioning, whereas chronic nicotine treatment failed to enhance contextual conditioning, suggesting the development of tolerance.[209] It should be noted that plasma nicotine levels in mice treated acutely and chronically with nicotine (13 ng/mL) were within the range of plasma nicotine levels (10–50 ng/mL) demonstrated by smokers.[210,211]

The above results demonstrate the development of tolerance for the effects of nicotine on cognition. The neural adaptations responsible for this behavioral change are unknown, but studies examining tolerance for the effects of nicotine on locomotor activity and physiological responses have identified accompanying changes in receptor density and function. This research is consistent with other research showing that behavioral tolerance cannot be explained by degree of dispositional tolerance. It must involve separate CNS neural adaptations that permit one animal to compensate for the disruptive effects of a drug, while another animal with the same level of the drug in its body shows greater drug effects. This phenomenon has, of course, many precedents with other drugs of abuse, with studies showing that most adaptation to drug-induced behavioral disruption is caused by learning mechanisms, rather than dispositional tolerance.[212,213]

Behavioral Genetics of Tolerance and Putative Substrata

Experiments conducted three decades ago provided early evidence of strain differences in tolerance. In one study,[214] DBA/2 and C57BL/6 mice were compared on the development of tolerance to nicotine. Mice received three daily IP injections of 1 mg/kg of nicotine for two, four, or seven days. Genotype and gender both contributed to variance in developing tolerance to the effects of nicotine on Y-maze activity. C57BL/6 male mice developed tolerance most rapidly, and DBA/2 male mice had the latest onset of tolerance; female mice of both genotypes developed tolerance at the same rate, but C57BL/6 female mice showed greater tolerance. A subsequent study examined the effects of chronic IV administration of 2, 4, or 6 mg/kg of nicotine for 10 days on nicotine and α-bungarotoxin binding as well as the effects of acute nicotine on Y-maze activity and rears, acoustic startle, heart rate, respiration rate, and body temperature in both DBA/2 and C3H/2 inbred mice.[215] DBA/2 and C3H/2 mice differed in the development of tolerance, but not in ^{3}H-nicotine binding or α-bungarotoxin binding, the two means available at that time for quantitation of nAChR expression.

The effects of chronic nicotine treatment were also extended to compare effects of chronic nicotine on tolerance and nAChR binding in C57BL/6, DBA/2, C3H/2, and BALB/cBy mice.[5] Mice were treated with 3 mg/kg/hour of nicotine intravenously for 10 days. Assays included tolerance to the acute effects of nicotine on Y-maze activity, startle response, heart rate, respiratory rate, and body temperature. This research revealed substantial interspecific variability in response to chronic nicotine exposure. Only C3H mice developed tolerance for the effects of nicotine on acoustic startle. However, C57BL/6, DBA/2, and BALB/cBy mice, but not C3H mice, all showed tolerance to the effects of nicotine on Y-maze

activity and body temperature. In addition, BALB/cBy mice showed tolerance for the effects of nicotine on heart rate. No strains showed tolerance for the effects of nicotine on respiratory rate, a measure of nAChR function in the autonomic nervous system. All four strains showed similar increases in nicotine binding in the cortex, hippocampus, midbrain, striatum, hypothalamus, and hindbrain after chronic nicotine treatment. Another marker of nicotine receptor expression, α-bungarotoxin binding, varied across strains in those areas of the brain after chronic nicotine treatment. DBA/2 mice showed increased binding in the cortex, hippocampus, and hypothalamus; C57BL/6 mice showed increased binding in the hindbrain and hippocampus; BALB/cBy mice showed increased binding in the hindbrain and hypothalamus, and C3H mice showed increased binding only in the hypothalamus. In sum, this early research showed considerable variability in tolerance development across inbred mouse strains and across physiological systems in response to nicotine exposure.

Genetic analysis was also used to examine the dose-dependent effects of chronic nicotine treatment on tolerance and changes in binding in A, C57BL/6, DBA/2, C3H/2, and BUB/Bn mice.[5] Mice were chronically infused with 0, 0.5, 1.0, 2.0, 4.0, or 6.0 mg/kg/hour of nicotine intravenously for 10 days and then tested for tolerance to the acute effects of nicotine on Y-maze activity and rears, acoustic startle, heart rate, respiration rate, and body temperature. C57BL/6 mice were more sensitive to the effects of chronic nicotine than C3H/2 and BUB/Bn mice in that the latter showed tolerance for only the highest doses tested; A and DBA/2 mice were intermediate. Changes in nAChR binding were measured in the cortex, cerebellum, colliculi, hindbrain, hippocampus, hypothalamus, midbrain, and striatum. All strains showed increased nicotine binding after chronic nicotine treatment, but variability across strains was seen for sensitivity to doses and for brain regions affected. For instance, A mice showed less change in binding across brain regions, and changes in binding were seen at higher doses, whereas C57BL/6 mice showed changes in binding in all brain regions and the lowest dose of nicotine-increased binding in six of the eight regions tested. Changes in α-bungarotoxin binding associated with chronic nicotine treatment were also seen but to a lesser extent than with nicotine binding.

Interestingly, changes in receptor binding may not exclusively explain tolerance. The time course for the development of tolerance for the effects of nicotine on locomotor activity, as measured by Y-maze activity and rears, body temperature, and heart rate, were compared with the time course for the effects of chronic treatment on nAChR binding in DBA mice.[14] DBA mice were infused with 4 mg/kg/hour of nicotine, and tolerance was assessed after 1, 2, 4, 8, or 12 days of treatment. Maximal tolerance to the effects of an acute dose of 0.75 mg/kg of nicotine was seen after four days of treatment, and the development of tolerance corresponded to increased binding of nicotine in the cortex, midbrain, hindbrain, hippocampus, and hypothalamus. Chronic nicotine treatment was also associated with increased α-bungarotoxin binding in the cortex and hippocampus, but the increase in low-affinity nAChR binding occurred before the development of tolerance. Tolerance for the effects of nicotine on Y-maze locomotor activity and rears was lost after 8 days, tolerance to the acute effects of nicotine on body temperature was lost after 12–16 days, and tolerance to the acute effects of nicotine on heart rate was lost after 20 days. Nicotine binding, however, returned to control levels after 8 days, and α-bungarotoxin binding returned to control levels after only 4 days. These results suggest that changes in nAChR density may, in part, contribute to tolerance, but may not be the only mechanism involved because receptor binding returned to control

levels before all of the physiological measures of tolerance returned to control levels. This result is consistent with a great deal of other evidence that behavioral tolerance involves complex learning processes.[212,213]

Although the above studies collectively established a genetic basis for the response to nicotine and changes in receptor properties, they also preceded molecular studies revealing that beyond simply those systems detected by nicotine and α-bungarotoxin binding, there is a diverse genetic richness in the genes that constitute the nAChR family and that collectively contribute to the functional and regionally specific effects of nicotine on the organism.

Involvement of nAChR and Other Neurotransmitter Systems

It is important to establish which nAChRs are implicated in chronic tolerance phenomena. As reviewed earlier, α4β2 nAChRs are involved in CPP and nicotine self-administration; thus, a logical question is whether the same receptors are involved in tolerance. Although early studies, based almost entirely upon ligand-binding measurements, indicated that a variety of receptors appear to underlie the development of tolerance, subsequent directed genetic studies have helped elucidate the nAChR subtypes that play a central role in the development and/or expression of tolerance. β2 nAChR subunit knockout mice treated chronically with 0, 1, 2, or 4 mg/kg/hour of IV nicotine for 10 days did not develop tolerance for the effects of nicotine on Y-maze activity and body temperature, but instead, showed increased sensitivity to the acute effects of nicotine after chronic treatment, suggesting that β2-containing nAChRs are involved in the development of tolerance for these measures.[97] Furthermore, mice with a single point mutation (Leu9′→Ala9′) that was associated with increased sensitivity of α4-containing nAChRs exhibited heightened development of tolerance.[178] Mice were treated daily with a single 15-μg/kg nicotine IP injection for nine days, and body temperature was measured. The Leu9′ mutant mice developed tolerance to the effects of nicotine on body temperature by day nine, but the wild-type mice did not develop tolerance. In contrast to the β2 and α4 nAChR subunits, which appear to be involved in tolerance, the α7 nAChR subunit may not be involved in tolerance because α7-null mice exhibit normal development of tolerance to the effects of nicotine on schedule reinforcement.[216] Although the α7 subunit does not appear to be as important as once thought in this process, caution must be exercised; these studies used different measures of tolerance, and it is possible that the nAChRs involved in tolerance are measurement specific.

In sum, the results of the behavioral genetic analysis of tolerance provide several important insights into the effects of nicotine. First, these studies demonstrate that genetic variation contributes to the development of tolerance for the effects of nicotine. Second, these studies illustrate the potential for the use of the nAChR subunit null mouse in that they accurately complement most pharmacological and functional studies and demonstrate how mutations to genes coding for nAChR subunits can alter sensitivity to nicotine.

As in self-administration, other nonnicotinic systems may interact during tolerance development. For instance, there is evidence in DBA/2 mice of an interaction between the nicotinic and muscarinic acetylcholinergic systems. DBA/2 mice were treated chronically with IV administration of 8 mg/kg/hour of nicotine, 1 mg/kg/hour of oxotremorine (muscarinic agonist), or a vehicle for 10 days.[217] After chronic treatment, the acute effects of 2 mg/kg of nicotine or 0.2 mg/kg of oxotremorine on rotorod performance, Y-maze activity, heart rate, respiratory rate, and body temperature were measured, along with

nicotine and α-bungarotoxin binding. For all tests, tolerance was seen for both drugs. In addition, mice chronically treated with oxotremorine showed cross-tolerance with nicotine for nicotine-induced heart rate and body temperature change. Interestingly, mice chronically treated with oxotremorine showed decreased binding at muscarinic receptors, but no change in nicotine and α-bungarotoxin binding. In contrast, mice chronically treated with nicotine showed increased nicotine and α-bungarotoxin binding, but no change in muscarinic receptor binding. In addition to demonstrating cross-tolerance between muscarinic and nicotinic agonists, this study once again demonstrates that tolerance to the effects of nicotine can develop independent of changes in nAChR binding; mice treated chronically with oxotremorine showed tolerance for the effects of nicotine on heart rate and body temperature but did not show changes in binding at nAChRs.

The mu opioid receptor may also be involved in the development of tolerance to at least one effect of nicotine. C57BL/6 mice were treated chronically with nicotine (three daily SC injections of 1.75 mg/kg) for 12 days. Locomotor responses and nociception were measured on even-numbered days for 5 minutes (locomotor activity) and for 15 minutes (nociception) after nicotine injection.[218] After the last test, mu opioid binding was assessed. Tolerance was seen for nicotine-induced antinociception but not for the disruptive effects of nicotine on locomotor activity. Chronically treated mice had decreased mu opioid binding in the caudate-putamen and in the nucleus accumbens. Tolerance was also tested in mu opioid receptor knockout mice. These mice developed tolerance to the antinociceptive effects of nicotine faster than did wild-type mice. These results suggest that nicotine-mediated changes in mu opioid receptor function may contribute to the development of tolerance for the antinociceptive effects of nicotine.

Cell Signaling

Studies in mice have demonstrated that chronic nicotine treatment is often associated with an increase in nAChR density but a decrease in the function of those nAChRs.[11,64] However, such changes in the nAChRs do not always correlate with the onset and duration of tolerance. This suggests that effects downstream of nAChR activation may be involved in tolerance. Changes in calcium-related cell signaling may be involved in the development of tolerance. The relationship between calcium signaling and the development of tolerance for the effects of nicotine on locomotor activity and nociception was measured in ICR mice.[219] Mice were treated chronically for 10 days with 2 mg/kg of SC nicotine twice daily. Tolerance was seen for the locomotor-impairing effects of nicotine and for the antinociceptive effects of nicotine. Mice that developed tolerance also showed cross-tolerance for the effects of BAY K 8644, a calcium channel agonist, and thapsigargin, which increases intracellular calcium concentrations, on locomotor activity and nociception. These results suggest that calcium signaling (possibly an α3 nAChR subtype) may be involved in the development of tolerance for some effects of nicotine. In further support of the involvement of calcium signaling in the development of tolerance to the effects of nicotine, drugs that alter calcium signaling altered tolerance.[220] Mice treated chronically with 24 mg/kg/day (SC, minipump) of nicotine for 14 days were concurrently treated with a calcium channel antagonist, a calcium channel agonist, or a vehicle, and tolerance for the antinociceptive effects of nicotine was then measured. Twice daily injections of the L-type calcium channel antagonists nimodipine and verapamil blocked the development of tolerance; whereas twice-daily injections of BAY K 8644 enhanced the development of tolerance. In addition, the study found that tolerant mice had

higher levels of calcium calmodulin protein kinase II in the spinal cord, and infusion of the calcium calmodulin protein kinase II antagonist KN-62 into the spinal cord decreased tolerance for the antinociceptive effects of nicotine. These results strongly suggest that calcium-mediated cell signaling is involved in the development of tolerance for the effects of nicotine on nociception.

The involvement of calcium in nicotine tolerance has also been demonstrated for tolerance to the anxiogenic effects of nicotine.[221] Swiss mice treated with daily injections of 0.04 mg/kg of nicotine for seven days showed tolerance for the anxiogenic affects of nicotine. However, in mice that received nicotine injections paired with injections of L-type, voltage-dependent, calcium channel antagonists (nimodipine, flunarizine, diltiazem, or verapamil), tolerance was blocked. Thus, while chronic nicotine treatment is associated with receptor level changes and the development of tolerance, changes in intracellular calcium cell signaling may also be critically involved in such tolerance development.

In summary, studies of the neural and cellular substrates of tolerance in mice have identified receptor subtypes and cell-signaling molecules involved in tolerance. The α4-containing and β2-containing nAChRs appear to be critically involved in tolerance to the effects of nicotine, although the role of α7 nAChRs may be less direct. In addition to nAChRs, muscarinic acetylcholinergic receptors and mu opioid receptors may also be involved in tolerance to the effects of nicotine. The cellular mechanisms involved in tolerance appear to involve calcium-mediated cell signaling because calcium channel antagonists decreased tolerance, and agonists increased tolerance.

It is important to bear in mind, however, that the functional role of tolerance to human nicotine dependence remains unclear. It is unclear that dispositional tolerance to nicotine[222] or behavioral tolerance[223,224] are causally determinant of nicotine reinforcement and dependence. Future research should address the extent to which the different types of tolerance are related to core features of dependence, such as a pervasive pattern of drug use. Further understanding of the neural and genetic substrata of tolerance, and how these compare with other causal influences on dependence, may elucidate the role of tolerance in dependence development.

Additional Directions for Research on the Nicotine-Dependence Phenotype in Mice

Given the tremendous potential created by the availability of well-characterized mouse strains and both knockout and knockin preparations, there is a great need to use such tools to explore genetic influences on phenotypes that provide additional insight into the processes involved in nicotine dependence. Additional assays, both physiological and behavioral, should be used to expand understanding of the genetic contributors to the critical motivational processes of dependence.

Extended Central and Peripheral Effects of Nicotine Observed in Mice

Alteration in nAChR function may also provide insights into nicotine effects on central and peripheral components of complex behaviors. For example, the roles of α7 and α4β2 receptors are implicated in nicotine-induced enhancement of cognition, including working memory, learning, and attention.[225] This relation is particularly strong in rodents; the loci of this effect

appear to be the hippocampus and the amygdala.[206,226–229] In C57BL/6 mice, acute nicotine enhanced hippocampus-dependent, but not hippocampus-independent, fear conditioning.[207,229] This enhancement of hippocampus-dependent forms of fear conditioning by nicotine is mediated by α4β2 nAChRs. DHβE, the high-affinity nAChR antagonist, blocked the nicotine enhancement;[230] β2 knockout mice did not show the enhancement of hippocampus-dependent fear conditioning, but α7 knockout mice did.[230,231] The mechanisms that modulate fear-based learning may be relevant to nicotine dependence, given the substantial evidence that affect control is a powerful motive for smoking in humans.[232]

Glutamate receptor systems are directly involved in learning and synaptic plasticity.[233–235] Accordingly, one mechanism through which nicotine can enhance learning and memory, in addition to modulating inhibitory tone and circuitry in regions such as the hippocampus (above), is via interaction with the glutamate system.[236] For example, chronic nicotine increases the phosphorylation state of the NR2B subunit, which correlates with a long-lasting component of long-term potentiation.[237] Similarly, chronic nicotine self-administration in rats corresponds with region-specific increases in NR2A mRNA expression (e.g., the auditory cortex), whereas thalamic NR2B messenger RNA (mRNA) levels decline.[238] In addition, protein levels that these subunits share also increased particularly in mesocorticolimbic regions.[239] Nicotine can also act on dopamine cell bodies to regulate glutamatergic inputs to these distal neurons that do not experience direct nAChR activation.[80,142,183,240,241] Finally, nicotine modulation of activity-dependent limited proteolysis of the GluR1 C-terminus has been described.[183] Because the C-terminus of this AMPA-GluR subunit is critical to association with proteins of the synaptic spine, it is possible that nicotine increases GluR1 expression through altering trafficking of the receptor. The common feature of these studies is, however, that nicotine's effects via glutamate receptor expression (even acting via the same receptor) may be very different, or even opposite, within the same learning or memory paradigm, depending upon the anatomical location of nicotine's actions.

Nicotine influences on gene-transcription cascades could be very important and highly strain specific. Genes and their protein products do not work alone; they are part of complex metabolic cascades that impart a change in "state" to the cell, eventually resulting in a change in function or behavior in the organism. Researchers in the field are aware of examples that extend from the regulation of the classic pathways of intermediary metabolism to later discoveries of complex cascades that regulate cell functions, including the induction of gene transcription and proteolytic cascades that determine cell survival or death through apoptosis.[242] This also means that changing the function of just one element, possibly because of a dysfunction of a pathway external to the one being examined, can change how these cascades proceed and how they eventually influence ultimate end points or states. In mice, the administration of nicotine can lead to persistent *c-Fos* activation[243,244] as well as to changes in fibroblast growth factor-2 mRNA,[245,246] nerve growth factor and tyrosine kinase B in the hippocampus,[247] and activation of CREB.[88] In tissue culture, reports indicate that nicotine increases the corticotropin-releasing factor and, as noted above, inhibits lipopolysaccharide induction of certain inflammatory cytokines (e.g., interleukin (IL)-1 and IL-8;[150,248]) or signaling through the receptor. This latter study suggests that nicotine inhibits the nuclear factor-kappa B transcriptional system,[249] although in other cell types, antagonism between α7 activity and tumor necrosis factor-α (TNF-α)-initiated, ceramide-related metabolic cascades has been reported.[144]

Distinguishing strain-specific systems that differ in ways relevant to nicotine dependence from those that vary because of unrelated genetic differences (e.g., the original reasons many of these strains were selected, such as H2 functions) is not straightforward. Factors that also influence nicotine dependence, such as drug metabolism or absorption, increase the complexity of the problem of genetic dissection. This is particularly true in mouse strains that are particularly "sensitive" (or possibly very "insensitive") to nicotine for which toxicity or seizure sensitivity due to particularly robust catabolism or clearance, versus compound accumulation, may mask correlations in seeking signaling cascades relevant to receptor function. The advent of microchip analysis of whole-genome quantitative transcript screening (e.g., Affymetrix) seems a likely future direction to begin the experimental dissection of the magnitude and specificity of the strain response to defined drug administration.

Aging

One measure of normal age-related decline in the CNS is the diminishment and eventual dysfunction of the limbic cholinergic system that, in its most severe form, manifests in pathologies of dementia, including Alzheimer's disease (AD; figure 4.1). Although studies that examine the state of the cholinergic neurotransmitter receptors in aging and dementia often focus upon muscarinic receptor expression, the loss of neuronal nAChRs precedes muscarinic receptor loss and is often much more extensive in human brains afflicted with AD relative to age matched controls.[17,250–253]

Mouse strains, like humans, exhibit a striking range in life span, ranging from two to three years in non-cancer-prone strains,[254] and they exhibit an onset of age-related decline in nAChR expression that is strain specific.[139,147,255] One example of this is seen in the hippocampus of aged CBA and C57BL/6 mice. In both strains, the expression of the α4 nAChR is diminished with age, but this loss is much more severe in CBA than in C57BL/6 when compared with adults of the same strain.[147] Also observed in the hippocampal CA1 region is a significant loss of α7 nAChR expression by aged CBA/J but not by C57BL/6 mice. In contrast, the β4 nAChR is preferentially diminished in C57BL/6 mice. Coincident with the loss of the α4 nAChR in the CBA/J strain is a significant age-related increase in nAChR staining of astrocytes,[69,81,147] which has also been reported in cases with AD.[256–258] These results suggest that mouse strains of different genetic backgrounds undergo dissimilar age-related changes in the expression of nAChRs.

The strain-related differences noted above have implications for how age will affect an animal's response to various toxic assaults. For example, either nicotine or acutely administered TNF-α can be neuroprotective when applied individually, but when applied together, neuroprotection is abolished.[142] In contrast, α4-receptor subtypes provide neuroprotection to assault produced by the toxic amyloid beta-peptide 25-35 (Abeta 25-35).[144] Therefore, loss of receptors containing α4 would significantly increase susceptibility to age-associated assault by Abeta 25-35. At the same time, loss of α7 activation would enhance susceptibility to excitotoxic challenges (e.g., NMDA) such as those associated with ischemic damage or with the presence of TNF-α, including reduced apoptosis. Combining these findings suggests that in the aged brain, a CBA mouse is likely to be relatively more susceptible to Abeta toxicity, while the C57BL/6 is more susceptible to excitotoxicity. More to the point, these studies indicate that early genetic predispositions may have important impacts upon the lifelong dynamics of nAChR function, and hence, dependence processes. Research shows age-related changes in quitting success;[259,260] it is possible that

age-related sensitivities to toxins could affect trajectories of dependence across the life span. Finally, therapeutic interventions in patients, including the use of memantine, which has been proposed to inhibit glutamate receptors and which interacts with α7,[177] could have widely differing impacts on the recipient that are consistent with the individual's genetic background.

Novel Behavioral Phenotypes

Although research with mouse models has already yielded very valuable information about the nature of nicotine dependence and its genetic substrata, progress might be enhanced by use of new phenotypic measures that could be used along with manipulations of strain differences, knockout/knockin status, agonist and antagonist administration, and other strategies designed to implicate particular physiological and genetic mechanisms. Several behavioral paradigms may assess relatively more specific, dependence-linked motivational processes than are assessed by traditional CPP or self-administration paradigms. In a sense, these would represent phenotypes similar to the intermediate and mature subphenotypes discussed in chapter 3. One such novel phenotype would be to examine the ability of nicotine to enhance either the incentive value or reinforcing value of nonpharmacological stimuli. For instance, in regard to the latter effect, Caggiula and colleagues[261,262] have shown that nicotine enhances the execution of behavior maintained by salient nonnicotine reinforcers. Thus, nicotine appears to modulate the reinforcing value of other stimuli. This may be one reason that nicotine produces such an intransigent dependence, despite its being a relatively weak primary reinforcer.[261] Similarly, activity in mesotelecephalic dopamine structures could be monitored to assess how nicotine modulates the incentive value of nonpharmacological stimuli (as opposed to their reinforcing effects).[263]

Another potentially useful phenotype might be the increase in reward threshold for electrical brain stimulation produced by nicotine withdrawal. Research by Epping-Jordan and colleagues has shown that nicotine withdrawal elevates the magnitude of stimulation required to sustain reliable self-stimulation.[264] Subsequent research suggests that cues associated with withdrawal may similarly decrease activity in brain reward systems via associative mechanisms.[265] A well-defined association between cues and withdrawal may provide a sensitive index of the motivational impact of withdrawal, which appears to be an important determinant of ability to quit smoking.[232] Notably, some studies also demonstrated little impact of the β2-knockout mouse on behaviors related to somatic signs of withdrawal,[162,266] indicating that these behaviors are separable from those of reinforcement and subject to dissection through additional genetic approaches. Future development and use of phenotypic assessment should reflect a triangulation of theories of human drug motivation, data on implicated genetic variants and their functions, and evidence regarding the behavioral and biological processes that are implicated in the various behavioral paradigms.

Summary

Given the tremendous potential created by the availability of well-characterized mouse strains and knockout and knockin preparations, it is vital that such tools be used to explore genetic influences on phenotypes that provide additional insight into the processes involved in nicotine dependence. The reviewed evidence shows that nAChR expression and function is customized through interplay with genetic background to ensure optimal modulation of neurotransmitter receptor functions important to survival and the specialized needs of the organism. Therefore, there is

a need to recognize that behavioral tests must be customized to the mouse, and care must be taken when findings with mice are extrapolated to other rodents or humans. Such translational validation also requires that both similarities and differences in nAChR expression and function be considered in experimental design. Finally, although the potential value of mouse models has not yet been realized, the available data show that such models can display principal behavioral and biological features of nicotine dependence. In addition, such models have already implicated particular genetic variants and biological systems in the development and expression of nicotine dependence.

Conclusions

1. Substantial differences exist between mouse strains in their response to the acute or chronic administration of nicotine. These differences implicate specific neuronal nicotinic acetylcholine receptors within a broader genetic context, which suggests a central role for these genetic variants in nicotine dependence in humans.
2. The three most common routes of administration (intravenous, subcutaneous, and oral) for nicotine in rodents vary in the degree to which they model key features of human nicotine dependence, such as the behavioral features of self-administration and the acute and chronic physiological effects of nicotine. Each administration route offers advantages and disadvantages. Intravenous self-administration permits self-administration but may entail receptor-level response artifacts due to high dosages. Subcutaneous administration allows experimenter control of dosage and withdrawal over long time periods at a cost of precluding self-administration. Oral administration via drinking water permits chronic nicotine exposure and produces evidence of dependence, but is subject to specific possible side effects, making this issue an important variable in research design.
3. While mice generally are less sensitive to nicotine than are rats, mouse models now have a strong research base for nicotine effects. Mice are amenable to genetic and pharmacological experimental manipulation. They exhibit heterogeneity in strain-specific responses to nicotine, and methods of homologous recombination permit manipulation of specific genes. Data now link specific mouse strains to genetically influenced differences in the effects of nicotine exposure that can facilitate further study of nicotinic acetylcholine receptor biology in mice.
4. Mouse models link nicotine self-administration to high-affinity nicotinic acetylcholine receptors, genetic differences, developmental factors, and other potential mechanisms of dependence. These models have, in addition, linked nicotine reward in the form of conditioned place preference with genetic strain differences and specific receptor subtypes and have linked acute and chronic nicotine tolerance with other genetic and receptor differences. The models have also linked the $\alpha 7$ and $\alpha 4\beta 2$ receptors with nicotine enhancement of working memory, learning, and attention and have shown strain-specific aging effects on nicotinic acetylcholine receptor expression.
5. Although substantial differences exist in the biology of nicotinic acetylcholine receptor expression and function between mice, other rodents, and humans, nascent research in mouse models for nicotine dependence shows considerable promise in furthering understanding of the biology and genetics of nicotine dependence.

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