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Unraveling the neurobiology of nicotine dependence using genetically engineered mice

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Abstract

This review article provides an overview of recent studies of nicotine dependence and withdrawal that used genetically engineered mice. Major progress has been made in recent years with mutant mice that include knockout and gain-of-function of specific neuronal nicotinic acetylcholine receptor (nAChR) subunit genes. Nicotine exerts its actions by binding to these neuronal nAChRs, which consist of five subunits. The different nAChR subunits that combine to compose a receptor determine the distinct pharmacological and kinetic properties of the specific nAChR. Recent findings in genetically engineered mice have indicated that while α4- and β2-containing nAChRs are involved in the acquisition and initial stages of nicotine dependence, α7 homomeric nAChRs appear to be involved in the later stages of nicotine dependence. In the medial habenula, α5-, α3- and β4-containing nAChRs were shown to be crucially important in the regulation of the aversive aspects of nicotine. Studies of the involvement of α6 nAChR subunits in nicotine dependence have only recently emerged. The use of genetically engineered mice continues to vastly improve our understanding of the neurobiology nicotine dependence and withdrawal.

Nicotinic acetylcholine receptor subunits and aspects of nicotine dependence

Nicotine binds to nicotinic acetylcholine receptors (nAChRs), which are pentameric structures that consist of a combinatorial assembly of five subunits. Neuronal nAChRs can either be heteromeric, consisting of a combination of α (α2-α6) and β subunits (β2-β4), or homomeric, consisting of only α subunits (α7-α10) [1]. The combination of nAChR subunits determines the distinct pharmacological and kinetic properties of specific nAChR subtypes [1]. To date, few pharmacological ligands have been identified that selectively target specific combinatorial assemblies of nAChR structures. Therefore, knockout and knock-in mice have been critical in the study of the roles of specific nAChR subunits in *in vivo* function. nAChRs are widely distributed throughout the central nervous system at presynaptic, postsynaptic, axonal, and somatodendritic locations. The activation of

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Conflict of Interest
AM has received contract research support from Bristol-Myers Squibb Co. and honoraria/consulting fees from Abbott GmbH and Company, AstraZeneca, and Pfizer during the past 2 years. AM has a patent on the use of metabotropic glutamate compounds for the treatment of nicotine dependence.

presynaptically located excitatory nAChRs results in the release of a wide range of neurotransmitters that critically modulate the function of several brain circuits and neurotransmitter systems, including dopamine, glutamate, γ-aminobutyric acid (GABA), acetylcholine, serotonin, and norepinephrine (for review, see [2]). Mice with null mutations of the α3, α4, α5, α6, α7, β2, β3, and β4 nAChR subunits have been created. This article reviews and summarizes the major discoveries concerning nicotine reinforcement and withdrawal made during recent years using genetically engineered mice.

Measures of the reinforcing effects of nicotine include nicotine self-administration and nicotine-induced conditioned place preference (CPP). The self-administration procedure is an operant paradigm in which a mouse is trained to self-administer nicotine, often either intravenously (e.g., [3]) or into a specific brain area that supports nicotine self-administration, such as the ventral tegmental area (VTA) (e.g., [4]). Conditioned place preference measures the reinforcing value of nicotine by pairing one environment with the contingent administration of nicotine and another environment with the administration of vehicle. When the mouse is allowed to freely explore both compartments in a drug-free state, it spends more time in the environment previously paired with nicotine if nicotine was previously rewarding to the mouse [5].

Although the reinforcing effects of drugs of abuse are considered important for the initiation and maintenance of drug dependence, drug dependence is characterized by the emergence of withdrawal symptoms once drug administration ceases [6]. Thus, in the study of the role of nAChR subunits in nicotine dependence, determining how nicotine withdrawal may be altered in mutant mice that lack specific nAChR subtypes is important. Signs of nicotine withdrawal in mice include “anhedonia,” somatic signs, conditioned place aversion (CPA), contextual fear conditioning, and hyperalgesia. Anhedonia, one of the affective signs of nicotine withdrawal, can be measured in mice using the intracranial self-stimulation (ICSS) procedure, in which anhedonia is reflected by elevations in brain reward thresholds [7]. Somatic signs of withdrawal include forelimb tremor, body or head shakes, scratching, and grooming (e.g., [8]). CPA is similar to the CPP procedure and involves pairing an environment with either nicotine withdrawal or vehicle withdrawal (e.g., [9]). The contextual fear conditioning procedure can be used to detect learning deficits that typically occur during nicotine withdrawal (e.g., [10]), whereas hyperalgesia reflects the pain associated with nicotine withdrawal (e.g., [11]).

Role of α4 and β2 nAChR subunits in nicotine dependence

α4β2-containing nAChRs have long been of interest in the study of nicotine dependence because these are among the most widely distributed nAChRs in the central nervous system, with high affinity for nicotine. One of the first gene-targeting studies of nicotine dependence reported that β2 knockout mice did not acquire intravenous nicotine self-administration behavior [12]. Later studies supported these results, suggesting the involvement of the β2 subunit in the reinforcing effects of nicotine by demonstrating that the β2 subunit is essential for the development of nicotine-induced CPP [13]. Additionally, self-administration of nicotine directly into the VTA did not occur in β2 knockout mice, whereas nicotine self-administration behavior was reinstated after lentiviral re-expression of the β2 nAChR subunit in this brain area [14]. Importantly, while the study by Maskos and colleagues provided important information about the role of β2 nAChR subunits in the VTA, humans are exposed to systemic nicotine rather than solely in the VTA. The above findings were therefore recently complimented by a study demonstrating recovery of intravenous self-administration of nicotine after lentiviral re-expression of the β2 subunit in the VTA of β2 knockout mice [3].

During withdrawal from chronic nicotine administration, β2 knockout mice, unlike wildtype mice, did not show withdrawal-induced CPA or anxiety-like behavior in the elevated plus maze [15]. Additionally, β2 knockout mice that were chronically treated with nicotine did not exhibit learning deficits in the contextual fear conditioning procedure when the nAChR antagonist dihydro-β-erythroidine was administered systemically or directly into the hippocampus [10,16]. These findings indicate that nAChRs that contain the β2 subunit are critical for the development of nicotine dependence that is expressed as withdrawal signs upon cessation of nicotine administration.

α4 knockout mice did not acquire intravenous nicotine self-administration ([17], but see [18]) or intra-VTA nicotine self-administration [19]. Interestingly, mice with a single point mutation in the α4 gene (α4-248F) administered nicotine at lower doses than their wildtype counterparts. These latter findings are consistent with results generated using a gain-of-function mouse that had hypersensitive α4 subunits. These mice exhibited CPP at very low nicotine doses [20]. Furthermore, deletion of the α4 subunit on dopaminergic neurons resulted in a loss of nicotine-induced CPP [21]. Altogether, these findings suggest a bidirectional modulatory role for the α4 subunit in nicotine reinforcement.

The data generated from α4 and β2 knockout mice, mice with a mutation in the α4 gene, and mice with α4 subunit hypersensitivity strongly suggest the crucial involvement of α4β2-containing nAChRs in the reinforcing effects of nicotine. The aversive aspects of nicotine withdrawal remain to be studied in α4 knockout mice, but an important role for the β2 subunit was shown in the mediation of the aversive effects of nicotine withdrawal using β2 knockout mice.

Role of α7 homomeric nAChRs in nicotine dependence

Homomeric α7 nAChRs are widely distributed throughout the brain, similar to α4β2 nAChRs. With a significantly lower affinity for nicotine, however, the effects of α7 nAChRs on nicotine reinforcement appear to be more subtle than those of α4β2 nAChRs. Importantly, α7 nAChRs rapidly recover from nicotine-induced desensitization [22]. This rapid recovery from desensitization suggests that α7 nAChRs, unlike α4β2 nAChRs, may remain sensitive to fluctuations in nicotine levels during continuous nicotine exposure and that these nAChRs may consequently be important in the maintenance of nicotine dependence. Nevertheless, nicotine-induced CPP [13] and the acquisition of nicotine self-administration in a single self-administration session [17] were unaffected in α7 knockout mice. Intra-VTA administration of nicotine over seven self-administration sessions, however, decreased in α7 knockout mice compared with wildtype mice [4], suggesting that α7 nAChRs in the VTA may be critical for the reinforcing effects of nicotine. Furthermore, a recent study found that α7 knockout mice initially consumed similar amounts of an oral nicotine solution as wildtype mice in a two-bottle choice procedure, but nicotine consumption slowly decreased after the initial three weeks in these knockout mice. Incontrast, β2 knockout mice initially consumed less nicotine and gradually increased their nicotine consumption over the course of two months of access to nicotine [23]. The involvement of the α7 subunit in the reinforcing effects of nicotine was supported by studies that showed that nicotine self-administration in rats was significantly reduced by the relatively selective α7 receptor antagonist methyllycaconitine (MLA) [24].

Studies from our laboratory suggested a role for the α7 receptor in nicotine withdrawal by showing that “anhedonia” expressed at the onset of spontaneous nicotine withdrawal in wildtype mice was absent in α7 knockout mice, whereas both α7 knockout and wildtype mice showed similar levels of “anhedonia” during the later stages of nicotine withdrawal [25]. A delay in the onset of withdrawal signs in α7 knockout mice was also shown when

the somatic signs associated with nicotine withdrawal were assessed. Somatic signs were decreased in α7 knockout mice at the onset of nicotine withdrawal [26] and similar in both α7 knockout and wildtype mice at 24 h [25] and 48 h of withdrawal [27]. Hyperalgesia induced by nicotine withdrawal was diminished in α7 knockout compared with wildtype mice [15,27], whereas contextual fear conditioning was unaffected in α7 knockout compared with wildtype mice during nicotine withdrawal [10]. In humans, aversive experiences during the early stages of tobacco withdrawal are an important contributor to the re-initiation of tobacco smoking after a period of abstinence. The attenuation of "anhedonia," somatic signs, and hyperalgesia during the early stages of nicotine withdrawal in α7 knockout mice, therefore, may decrease the re-initiation of nicotine seeking [28,29]. However, the α7 receptor antagonist MLA did not induce CPA to nicotine [15] and did not precipitate "anhedonia" or somatic signs of nicotine withdrawal in nicotine-dependent rats [24]. Altogether, these studies suggest the potential involvement of α7 nAChRs in the later stages of nicotine dependence, rather than the acquisition of nicotine-seeking behavior. Additionally, the involvement of α7 nAChRs was suggested in the very initial, rather than later, stages of several aspects of nicotine withdrawal. Although mice with a gain-of-function of α7 nAChRs have been created, these mice died within one day after birth [30].

Role of α5α3β4 nAChRs in nicotine dependence

Associations have been found between the *CHRNA5-CHRNA3-CHRNB4* nicotinic receptor subunit gene cluster [31,32] and D398N α5 variant [33–35] and nicotine dependence and lung cancer in humans. Several studies of α3, α5, and β4 knockout mice have reported altered behavioral responses to the aversive effects of nicotine and nicotine withdrawal. These studies emphasized the importance of the α3, α5, and β4 nAChR subunits in nicotine dependence and redirected the focus of nicotine withdrawal studies to the habenula-interpeduncular pathway where these nAChR subunits are highly expressed. The α3 and β4 subunits are often co-expressed, while the α5 subunit has been found to assemble into both α3β4- and α4β2-containing nAChR assemblies. The affinity for nicotine is significantly lower at α3β4-containing nAChRs than at α4β2-containing nAChRs, and α3β4-containing nAChRs recover more rapidly from nicotine-induced desensitization than α4β2 [22], suggesting that these receptors may remain sensitive to fluctuations in nicotine levels. Interestingly, the inclusion of the α5 subunit into α4β2-containing nAChRs decreased the duration of desensitization for these receptors [36].

α5 nAChR subunits were shown to mediate the aversive effects of nicotine. Specifically, α5 knockout mice vigorously self-administered high doses of nicotine at very high rates, whereas wildtype mice adjusted their self-administration rates when given access to high nicotine concentrations. Re-expression of α5 nAChR subunits in the medial habenula in knockout mice restored nicotine intake levels to those in wildtype mice [37]. Lower doses of nicotine induced similar CPP in α5 knockout and wildtype mice, but knockout mice continued to exhibit CPP at higher doses of nicotine for which wildtype mice did not show CPP [38]. The studies of α5 knockout mice indicate that deletion of the α5 subunit increases the reinforcing effects of high doses of nicotine, perhaps by attenuating the adverse effects associated with high nicotine concentrations in healthy subjects. During mecamylamine-precipitated nicotine withdrawal, somatic signs were decreased in α5 knockout compared with wildtype mice [15], further suggesting the involvement of the α5 subunit in nicotine dependence. Overall, these studies suggest the involvement of the α5 nAChR subunit in the mediation of the aversive effects of nicotine.

Transgenic Tabac reporter mice, which were created using a bacterial artificial chromosome to co-express the *CHRNA5-CHRNA3-CHRNB4* nicotinic receptor subunit gene cluster, exhibited increased activity of β4 subunits [39]. These Tabac transporter mice consumed

less nicotine than their wildtype littermates in a no-choice bottle procedure [39]. Additionally, these mice showed a strong aversion to nicotine in the CPA procedure, an effect that was reversed by lentiviral expression of the D398N α5 variant in the medial habenula [39]. Importantly, however, co-assembly of the α5 subunit with α3 and β4 subunits occurred only in a small percentage(approximately 15%)of α3β4-containing receptors in the medial habenula [40], suggesting that β4 subunit function does not solely depend on the α5 subunit.

In β4 knockout mice, mecamylamine-precipitated and spontaneous nicotine withdrawal was associated with decreased somatic signs compared with wildtype mice [25,41]. The onset of the anhedonic signs of nicotine withdrawal were also delayed in β4 knockout mice [25], and hyperalgesia was decreased during nicotine withdrawal [41]. These studies suggest the strong involvement of the β4 subunit in nicotine dependence and importance of the balance between α5 and β4 subunit activity in the regulation of nicotine dependence by β4 subunits.

Mice that lacked the α3 nAChR subunit died within weeks after birth, likely because of bladder dysfunction and growth impairments [42]. Such postnatal mortality has prevented the study of the role of the α3 subunit in nicotine dependence using knockout mice.

Role of α6 nAChRs in nicotine dependence

A possible role for the α6 subunit in nicotine dependence was suggested a decade ago [43], but the necessity of α6-containing receptors in nicotine self-administration behavior was only demonstrated recently. Interestingly, α6 knockout mice do not self-administer nicotine intravenously [17], and these mice readily self-administer high but not low doses of nicotine into the VTA to a similar extent as wildtype mice [19], suggesting a modulatory role for the α6 subunit in the VTA in nicotine reinforcement. Pharmacological blockade of the α6 subunit using the antagonist α-conotoxin H9A;L15A attenuated nicotine-induced CPP [9], further supporting a possible role for the α6 subunit in nicotine dependence.

Involvement of non-nicotinic receptors in nicotine dependence

In addition to nAChRs, an extensive body of literature has described the involvement of non-nicotinic neuronal receptors in nicotine dependence. Pharmacological ligands may be more readily available for some of these receptors, but genetically engineered mice provide insights into genetic variations that result in differential sensitivity to nicotine dependence. For example, mice null for metabotropic glutamate receptor 5 (mGlu5 receptor) differed from their wildtype counterparts during nicotine withdrawal, displaying an attenuation of withdrawal-induced “anhedonia” and somatic signs of withdrawal [44]. These findings are interesting when considering that the chromosomal region where the gene for mGlu5 receptor is located was linked to habitual smoking behavior in humans [45]. Importantly, pharmacological blockade of mGlu5 receptor attenuated nicotine self-administration in rats [46–48], indicating that pharmacological blockade of these receptors may have therapeutic potential for assisting people with quitting tobacco smoking.

Additional mice with null mutations of other central nervous system receptors have also been studied. The somatic signs of mecamylamine-precipitated nicotine withdrawal were attenuated in γ-aminobutyric acid-B1 receptor knockout mice [49]. Nicotine-induced CPP was attenuated in δ opioid receptor knockout mice [50] and Ca^{2+}/calmodulin-dependent kinase IV knockout mice [11]. Thus, additional central nervous system receptors and neurotransmitter systems, in addition to nAChRs and acetylcholine, may be involved in various aspects of nicotine dependence and potentially interact with acetylcholine neurotransmitter function.

Conclusion

Genetically modified mice have greatly impacted our knowledge of nicotine dependence. Table 1 provides a summary of the data summarized in the present article. The use of these mutant mice has provided significant insights into how genetic variations in humans may underlie individual differences in the acute effects of nicotine, the severity of withdrawal upon smoking cessation, and potentially responses to smoking cessation medications. Additionally, these mouse lines have provided valuable knowledge about the *in vivo* involvement of nicotinic and non-nicotinic receptors in nicotine reinforcement, dependence, and withdrawal.

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Highlights

- α4 and β2 nAChR subunits are crucially involved in nicotine reinforcement.
- The involvement of α7 subunits emerges in the later stages of nicotine dependence.
- α5, α3, and β4 subunits mediate the aversive effects of nicotine.
- A modulatory role for α6 subunits has been suggested in nicotine dependence.

Table 1

Genetic modifications in mice and their effects on nicotine reinforcement and nicotine withdrawal.

Subunit	Genetic modification	Nicotine reinforcement	Nicotine withdrawal
α2	Knockout		No somatic signs of nicotine withdrawal [51]
α3			
α4	Knockout	No acquisition of intravenous nicotine self- administration [17], but see [18]	
		No acquisition of intra-VTA nicotine self-administration [19]	
		Similar nicotine-induced CPP as wildtype mice [18]	
	Gain-of-function	Conditioned place preference for low doses of nicotine [20]	
	Point mutation in the α4 subunit (α4-S248F)	Self-administration of lower nicotine doses compared with wildtype mice [18]	
	α4 subunit deletion on dopaminergic neurons	Loss of nicotine-induced CPP [21]	
	Restriction fragment length polymorphism in the α4 subunit gene	Restriction fragment length polymorphism in the α4 gene affects only ethanol and not nicotine consumption in a four-bottle choice procedure [52]	
α5	Knockout	Self-administration of very high doses of nicotine [37]	Attenuation of somatic signs of nicotine withdrawal [15,51]
		Conditioned place preference for higher doses of nicotine compared with wildtype mice [38]	Conditioned place aversion similar to wildtype mice [15]
α6	Knockout	No acquisition of intravenous self-administration [17]	
		Only high and not low doses of nicotine administered into the VTA, similar to wildtype mice [19]	
α7	Knockout	Acquisition of intravenous nicotine self-administration unaffected [17]	Delayed onset of nicotine withdrawal-induced “anhedonia” [25]
		Acquisition of nicotine-induced CPP unaffected [13]	Similar contextual fear conditioning deficits as wildtype mice [10]
		Attenuation of intra-VTA administration of nicotine [4]	Attenuation of somatic signs at the start of nicotine withdrawal [26], but see [15]
		Steady attenuation of nicotine consumption over the course of several weeks in a two-bottle choice procedure [23]	Similar somatic signs compared with wildtype mice at 24 h [25] 48 h of nicotine withdrawal [27]
			Similar CPA and anxiety-like behavior in the elevated plus maze as wildtype mice [15]
			Attenuation of withdrawal-induced hyperalgesia [15,27]
β2	Knockout	No acquisition of nicotine self-administration [3,12]	No acquisition of CPA [15]
		No acquisition of intra-VTA nicotine self-administration [14]	No withdrawal-induced anxiety-like behavior [15]

Subunit	Genetic modification	Nicotine reinforcement	Nicotine withdrawal
		No acquisition of CPP [13]	Similar hyperalgesia [15] and somatic signs of withdrawal compared with wildtype mice [42]
			No deficits in contextual fear conditioning during nicotine withdrawal [10]
β3	Knockout	—	—
β4	Knockout		Attenuation of hyperalgesia[42] and somatic signs of nicotine withdrawal [25,42]
			Delayed onset of nicotine withdrawal-induced “anhedonia” [25]
	Gain-of-function	Conditioned place aversion to nicotine [39]	
		Less nicotine consumption in a no-choice procedure [39]	

—, not studied