Published in final edited form as:

Biochem Pharmacol. 2011 October 15; 82(8): 808–819. doi:10.1016/j.bcp.2011.06.014.

Recent advances in gene manipulation and nicotinic acetylcholine receptor biology

Anne Tammimäki*, William J. Horton*, and Jerry A. Stitzel

Institute for Behavioral Genetics, University of Colorado at Boulder

Abstract

Pharmacological and immunological methods have been valuable for both identifying some native nicotinic acetylcholine receptor (nAChR) subtypes that exist in vivo and determining the neurobiological and behavioral role of certain nAChR subtypes. However, these approaches suffer from shortage of subtype specific ligands and reliable immunological reagents. Consequently, genetic approaches have been developed to complement earlier approaches to identify native nAChR subtypes and to assess the contribution of nAChRs to brain function and behavior. In this review we describe how assembly partners, knock-in mice and targeted lentiviral re-expression of genes have been utilized to improve our understanding of nAChR neurobiology. In addition, we summarize emerging genetic tools in nAChR research.

Keywords

assembly partners; knock-in; lentivirus; localization; nicotinic acetylcholine receptors; targeted re-expression

1. Introduction

Neuronal nicotinic acetylcholine receptors (nAChRs) are members of the cysteine loop superfamily of ligand gated ion channels that includes ionotropic 5-hydroxytryptamine (5-HT), γ -aminobutyric acid (GABA) and glycine receptors. As their name implies, nAChRs are receptors for the endogenous neurotransmitter acetylcholine in the nicotinic branch of the cholinergic system. The existence of nAChRs was first hypothesized as the “receptive substance” for nicotine described by Langley in 1905 [1]. Much of the pioneering work was done at the neuromuscular junction and peripheral ganglia throughout the first half of the 20th century [2–4] using α -bungarotoxin (α -BTX) as an antagonist. During these early years, pharmacological studies also suggested that there may be nAChRs in brain responsible for some physiological and behavioral effects of nicotine. For example, in 1965 Domino and Yamamoto demonstrated that low doses of nicotine induced cortical activation as measured by EEG and behavioral arousal while large doses of the nicotine metabolite, cotinine, did not produce these effects [5]. For an early review of these behavioral studies see Silvette 1962 [6]. Building on these findings, the existence of nAChRs in the brain was first

© 2011 Elsevier Ltd. All rights reserved.

Corresponding author: Anne Tammimäki, Institute for Behavioral Genetics, University of Colorado at Boulder, UCB 447, Boulder CO, 80309, United States of America, Telephone: +1 303 735 6571, Fax: +1 303 303 492 8063, anne.tammimaki@colorado.edu.

* equal contribution

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

demonstrated by ligand binding studies around 1980s. Using radio-ligand binding techniques, several groups established that there were at least two distinct nAChR populations in the rodent brain: one that binds the ligand [125 I]- α -bungarotoxin with high affinity [7–9] and one that binds the ligands [3 H]-L-nicotine or [3 H]-acetylcholine with high affinity [7;10–13]. The two binding sites also were found to be expressed in overlapping yet distinct patterns in the brain [7;14; 15]. At the time of their identification, the functional relevance of these binding sites in the brain was not clear [10; 13; 16]. However, from the mid-1980s through the early 1990s cDNAs for multiple nAChR subunits were cloned from rat and chicken brain [17]. These studies not only led to the identification of eleven different genes (twelve in chickens) that code for neuronal nAChR subunits but also demonstrated that various subunit combinations could form functional nAChRs that could be activated by acetylcholine and nicotine. The subunit genes identified were named $\alpha 2$ – $\alpha 10$ ($\alpha 8$ only found in chickens) and $\beta 2$ – $\beta 4$ based on the presence (α subunit) or absence (β subunit) of vicinal cysteines in the N-terminal extracellular domain and the order in which they were cloned. Neuronal nAChRs, like nAChRs at the neuromuscular junction, also were found to be composed of five subunits that form a pentameric ring around a central cation pore. These early studies also demonstrated that some nAChRs are heteromeric, requiring both an α subunit ($\alpha 2$ – $\alpha 4$, $\alpha 6$) and a β subunit ($\beta 2$ or $\beta 4$) in order to form a functional receptor in vitro. The most abundant heteromeric nAChR in brain is comprised of the subunits $\alpha 4$ and $\beta 2$ [18;19]. The $\alpha 4\beta 2^*$ (the asterisk indicates that other subunits such as $\alpha 5$ can contribute to $\alpha 4\beta 2$ nAChRs) receptor exhibits high affinity for nicotinic agonists and has been demonstrated to be the [3 H]-L-nicotine binding site described in the early ligand binding studies [18–21]. Other nAChR α subunits were identified that could form functional pentameric receptors in vitro without a β subunit. The most prevalent of these so-called homomeric nAChRs in the brain is composed of $\alpha 7$ subunits. Homomeric $\alpha 7$ nAChRs exhibit low affinity for nicotinic agonists and immunological [22] and genetic studies [23] demonstrated that $\alpha 7$ nAChRs are the previously described [125 I]- α -BTX binding sites in brain.

Although $\alpha 4\beta 2^*$ nAChRs are the most abundant nAChR expressed in the brain, several other heteromeric nAChR subtypes exist in the brain. For example, within dopamine terminals there are at least five different heteromeric nAChRs composed of anywhere between two and four different subunits [24–28]. The nAChRs on dopamine terminals in the striatum include $\alpha 4\beta 2$, $\alpha 4\beta 2\alpha 5$, $\alpha 6\beta 2$, $\alpha 6\beta 2\beta 3$, and $\alpha 4\alpha 6\beta 2\beta 3$. Data also indicate that the nAChRs in GABAergic terminals are $\alpha 4\beta 2$ and $\alpha 4\beta 2\alpha 5$ [29–31] whereas nAChRs that modulate acetylcholine release in the interpeduncular nucleus are $\alpha 3\beta 4$ and $\alpha 3\beta 3\beta 4$ heteromers [32;33]. A combination of immunoprecipitation experiments and in situ hybridization studies also suggest the existence of additional heteromeric nAChR subtypes [34;35]. Pharmacological and immunological methods have been valuable for both identifying some native nAChR subtypes that exist in vivo and determining the neurobiological and behavioral role of some nAChR subtypes. However, these approaches suffer from a lack of subtype specific ligands and, for the most part, reliable immunological reagents. Consequently, genetic approaches have been developed to complement the pharmacological and immunological approaches to identify native nAChR subtypes and to assess the contribution of nAChRs to brain function and behavior. Initial efforts to utilize genetic tools as a means towards understanding nAChR neurobiology included standard transgenesis [36], traditional knock-out methods [37] and an anti-sense knock-down approach [38]. Timeline of the emergence of genetic tools in nicotine research is presented in Figure 1. The focus of this review is to describe some recent advances in genetic technology and how such technology has been utilized to improve our understanding of nAChR neurobiology.

2. Assembly Partners and Localization

The pharmacological and biophysical properties of nAChRs are determined by the subunit composition of the receptor. For example, the most common nAChR in the brain is the $\alpha 4\beta 2$ receptor which is only moderately calcium permeable while the second most common is the $\alpha 7$ receptor which has a much higher calcium permeability [39]. In addition, expression patterns for the different nAChR subunits vary substantially throughout the central nervous system [40]. Because the subunit composition can have dramatic effects on the properties of the receptor, understanding the neurobiology of nAChRs requires knowledge of which subunits are expressed in which brain regions as well as which subunits are assembled together.

As a further degree of complexity the most abundant neuronal nicotinic receptor, the $\alpha 4\beta 2$ has been found in two distinct stoichiometric forms that have distinct pharmacological properties [39]. Typically two $\alpha 4$ and three $\beta 2$ subunits form the functional receptor, however when $\beta 2$ is limited functional receptors with three $\alpha 4$ and two $\beta 2$ are formed. Previous in vitro studies have attempted to influence the production of one or another of these stoichiometries by altering the ratio of $\alpha 4$ to $\beta 2$ during transfection or lowering the temperature cells are grown at [41]. Unfortunately, this only favors one conformation or another and does not result in a pure population of a single receptor type. In an attempt to specifically characterize $\alpha 4\beta 2$ receptors with different stoichiometries, Zhou et al. linked $\alpha 4$ and $\beta 2$ cDNAs such that expression leads to a fusion protein of the two subunits with a linker between them [42]. By addition of an unlinked individual $\alpha 4$ or individual $\beta 2$ construct they were able to specifically express only the $(\alpha 4)_3(\beta 2)_2$ or the $(\alpha 4)_2(\beta 2)_3$ in oocytes. Recordings from these oocytes showed that the two receptors have very distinct properties. The $(\alpha 4)_3(\beta 2)_2$ has a low affinity for acetylcholine and high calcium permeability, while the $(\alpha 4)_2(\beta 2)_3$ has high affinity but low calcium permeability. With the multitude of possible subunit combinations, this technique could be used to determine how the pharmacological and biophysical properties of the receptor change with the addition of various subunits [43]. However, the utility of this method is restricted to in vitro systems.

Genetic techniques such as in situ hybridization in combination with ligand binding techniques have allowed for gross localization of subunits to different brain regions (see Baddick & Marks in this issue). For example, Zhao-Shea et al. were able to localize $\alpha 4\alpha 6^*$ receptors to dopaminergic neurons in the posterior ventral tegmental area (VTA) through the use of RT-PCR, radioligand binding and a mouse line with the $\alpha 4$ L9'A gain of function mutation [44]. However because of the lack of tools to specifically identify a particular subunit and the complexity of these techniques, spatial resolution has generally been limited and evidence for assembly partners has been restricted largely to co-immunoprecipitation experiments with relatively crude brain regions [40].

To more directly address the issue of assembly and localization Nashmi et al. successfully produced $\alpha 4$ and $\beta 2$ constructs that were engineered to contain either a yellow fluorescent protein (YFP) or cyan fluorescent protein (CFP) label, respectively, located within the intracellular loop of each subunit [45]. When these chimeric proteins were expressed in vitro, they produced functionally normal receptors as measured by whole cell electrophysiology recordings and the calcium sensitive indicator fura-2 and exhibited nicotine-induced upregulation of $\alpha 4\beta 2$ nAChRs. An $\alpha 4$ YFP knock-in transgenic mouse line where the wild-type $\alpha 4$ has been replaced with one that is labeled with yellow fluorescent protein also has been generated. The advent of mouse lines with labeled, but otherwise functionally normal nAChRs, provides a unique opportunity to further refine our knowledge of receptor localization. For example, Nashmi et al. (2006) demonstrated that the upregulation of $\alpha 4^*$ nAChRs in the VTA in the $\alpha 4$ YFP mice is restricted to GABAergic

neurons. Moreover, Fonck et al. used the $\alpha 4$ YFP to assess $\alpha 4$ expression in the medial habenula and fasciculus retroflexus [46]. The medial habenula has previously been shown to express mRNAs for nearly all nAChR subunit genes and high doses of nicotine have been shown to be neurotoxic to the fasciculus retroflexus which connects the medial habenula to the interpeduncular nucleus. However, it is not clear what specific nAChR subtypes are expressed in the medial habenula/fasciculus retroflexus. Using the $\alpha 4$ YFP mice the authors showed specific expression of functional $\alpha 4$ containing receptors that are restricted in expression to the ventrolateral aspect of the medial habenula and on axons of the fasciculus retroflexus.

Creation of other fluorescently labeled subunits is ongoing and should allow for sub-region and even neuron specific localization of functional receptors. In addition, mouse lines expressing multiple different tagged subunits should provide information on assembly partners.

3. Knock-in strategies

Since the first report of targeted homologous recombination in mouse embryonic stem cells by Thomas and Capecchi in 1987, the homologous recombination technique has been extensively used to engineer knock-out mice [47;48]. More recently, this method has also been used to generate knock-in mice in which gene sequences are altered rather than deleted. Knock-in mice also have been generated via transgenesis in which the gene of interest is randomly integrated into the genome. Comparison of these two techniques is presented in Figure 2. Although this approach has some drawbacks including potential abnormal spatial and temporal expression of the transgene, insertional mutagenesis which may disrupt the function of an endogenous gene, and the inability to control copy number, knock-in via standard transgenesis is a more rapid and less expensive alternative to knock-in mutagenesis via homologous recombination. Moreover, some concerns with this approach can be minimized by testing multiple lines and using constructs such as bacterial artificial chromosomes (BACs) which more reliably recapitulate endogenous expression of the transgene. In nAChR research, the most popular knock-in approach has been to introduce a gain-of-function mutation into a nAChR subunit gene. Some of these mutations [e.g. Leu9'Ser and Leu9'Ala in ChRNA4 [49;50]] are experimental whereas others are mutations found in human diseases [e.g. V287L in ChRNB2, a human mutation found in autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE) patients [51;52]]. Most of the human polymorphisms exist in those parts of exons that are conserved between humans and mice. Gain-of-function knock-ins exaggerate agonist responses of nAChRs harboring the particular subtype. Together with knock-out mice they can be used to analyze the roles of receptors that contain or lack a particular subunit in a certain behavior or function. In some cases they can also be used to decipher the subtype specificity of novel ligands. For example, $\alpha 4$ L9'A knock-in mice are hypersensitive to $\alpha 4^*$ -mediated locomotor stimulation [53] whereas $\alpha 6$ L9'S knock-in mice are hypersensitive to $\alpha 6^*$ -mediated hypothermia [54]. Thus, a compound that causes hypothermia in $\alpha 4$ L9'A mice and increased locomotion in $\alpha 6$ L9'S mice at low doses that are without affect in control animals is probably a full or partial agonist of both $\alpha 4\beta 2^*$ and $\alpha 6\beta 2^*$ receptors [55].

The following sections describe mouse lines that have a knock-in mutation in a nAChR subunit gene (Table 1). nAChR subunits for which knock-in animals have been described include $\alpha 3$, $\alpha 4$, $\alpha 6$, $\alpha 7$ and $\beta 2$ although the majority of knock-in mutants reported are in the $\alpha 4$ subunit. Figure 3 shows the approximate locations of these mutations in a nicotinic receptor. The exact locations are listed in Table 2.

3.1 Function of modified nicotinic receptors in vitro and in vivo

Chrna3 is the only neuronal nAChR subunit gene that produces a lethal phenotype when knocked out in mice [56]. Caffery and coworkers generated a knock-in mouse line expressing a chimeric $\alpha 3$ nAChR subunit ($\alpha 3[5]$ knock-in mice) [57]. Five amino acids in the loop C region of the mouse $\alpha 3$ subunit were substituted with the corresponding residues from the $\alpha 1$ subunit of the muscle type nAChR from *Torpedo californica*. This manipulation renders $\alpha 3^*$ receptors sensitive to the muscle and $\alpha 7$ nAChR antagonist α -BTX [58]. Staining of superior cervical ganglia with rhodamine-conjugated α -BTX as well as pre- and postsynaptic markers showed that $\alpha 3[5]^*$ receptors colocalized with presynaptic varicosities on the soma and dendrites of sympathetic neurons [57]. $\alpha 3[5]$ knock-in mice showed nerve-evoked compound action potentials and excitatory postsynaptic potentials that were smaller than in wild-type mice but blocked by α -BTX. Similar experiments in $\alpha 3[5]$ knock-in/ $\alpha 7$ knock-out mice verified that $\alpha 7$ receptors did not contribute to these findings. Electrophysiological studies suggested that the amino acid substitutions in $\alpha 3[5]$ may have a negative impact on receptor subunit assembly or surface expression, which may explain the observed attenuated potentials.

The $\alpha 4L9'S$ mouse line was the first attempt to create a Chrna4 gain-of-function knock-in mouse [49]. Since the $\alpha 4L9'S^*$ mutation proved problematic in vivo, $\alpha 4L9'A$ mutation that has a less robust gain-of-function effect was introduced into Chrna4 [50], see below. The $\alpha 4L9'S$ and $\alpha 4L9'A$ mice have a Leu to Ser or Leu to Ala point mutation in the 9' position of M2 transmembrane region, respectively, that renders the $\alpha 4^*$ receptor hypersensitive to agonist activation. In $\alpha 4L9'S^*$ mice, channels seem to be continuously activated, possibly by endogenous choline, which results in decreased numbers of dopaminergic neurons in the substantia nigra (SN) and the ventral tegmental area (VTA) of homozygous embryos [49], in the SN of heterozygous adults [59;60] and in adult $\alpha 4L9'S$ heterozygotes where the Neo cassette has been locally deleted in SN [60]. $\alpha 4L9'A^*$ receptors are apparently not significantly activated by physiological levels of choline [61] and they do not show detectable changes in the number of dopaminergic neurons [62]. Other mice that possess gain-of-function mutations in the $\alpha 4$ subunit, including two mutations that cause ADNFLE in humans, $\alpha 4S248F$ (or $\alpha 4S252F$) mice with Ser to Phe substitution in amino acid 248 or 252 of M2 region [63;64] and $\alpha 4+L264$ knock-in with an in-frame insertion of a Leu in 17' position of M2 region [63], also do not exhibit presumably excitotoxic death of dopaminergic neurons. In addition, an $\alpha 4T529A$ knock-in mouse line with a Thr to Ala substitution in the large cytoplasmic loop of the $\alpha 4$ subunit [65], that were developed to allow a more precise assessment of the role of this mouse polymorphism in nicotine sensitivity and nAChR function has not been reported to cause cell death.

Although it appears that the $\alpha 4L9'S$ gain-of-function mutation is the only $\alpha 4$ mutation introduced into mice that is neurotoxic, a gain-of-function mutation in Chrna7, the gene that codes for the nAChR $\alpha 7$ subunit, also is neurotoxic. Heterozygous and homozygous $\alpha 7L250T$ knock-in mice with a Leu to Thr substitution in the amino acid 250 of M2 region [corresponds to chicken $\alpha 7L247T$ [66] and human $\alpha 7L248T$ [67] polymorphisms] show increased apoptosis-related terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) staining in several brain regions [68], evidently due to increased intracellular Ca^{2+} levels [67]. However, no other nAChR knock-in mice including the $\alpha 6L9'S$ knock-in mice with a Leu to Ser substitution in the 9' position of M2 region [54], and the $\beta 2V287L$ knock-in mice with Val to Leu substitution in amino acid 287 in the M2 region [51;52] have been reported to be neurotoxic.

Binding and immunocytochemistry experiments in fetal and adult brain have shown that $\alpha 4L9'S$ and $\alpha 4L9'A$ knock-in mice have less $\alpha 4^*$ binding sites in their brain than do wild-type mice [50;59;61;69]. However, it is not clear if this downregulation of expression is due

to a physiological response to the heightened sensitivity of the receptors or to vector elements that remain in the $\alpha 4L9'S$ and $\alpha 4L9'A$ constructs. [^{125}I]- α -BTX binding assays and western blots also have shown that $\alpha 7L250T$ knock-in mice express less $\alpha 7$ receptors than do wild-type mice [68], [70]. In contrast, the $\alpha 4S248F$ [64], $\alpha 4T529A$ [65] and $\beta 2V287L$ [51] knock-ins have little effect on [^{125}I]-epibatidine binding, although homozygous $\alpha 4S248F$ knock-ins show enhanced binding in a few brain areas [64], and $\alpha 4T529A$ knock-ins decreased binding in thalamus [65]. Likewise, location and intensity of [^{125}I]- α -conotoxin binding is intact in $\alpha 6L9'S$ knock-in mice and their wild-type littermates [54;71].

The firing pattern and thus dopamine release and turnover patterns seem to be altered in $\alpha 6L9'S$ knock-in animals. Fast-scan cyclic voltammetry in brain slices confirmed that after burst stimulation dopamine overflow is increased in $\alpha 6L9'S$ knock-in mice but not in $\alpha 4$ knock-out/ $\alpha 6L9'S$ knock-in mice. Unlike wild-type slices, $\alpha 6L9'S$ brain slices do not exhibit synaptic depression for one pulse stimulation. Removal of $\alpha 4$ subunits re-establishes synaptic depression in $\alpha 6L9'S$ slices. Furthermore, part of the $\alpha 6L9'S^*$ nAChR channels seem to be tonically active, which affects dopamine release and turnover patterns. However, the pacemaker firing is intact in $\alpha 6L9'S$ dopaminergic neurons.

Functional experiments showed that thalamic, cortical or striatal synaptosomes prepared of adult heterozygous $\alpha 4L9'S$ [69], homozygous $\alpha 4L9'A$ [61], and $\alpha 4S248F$ [64] knock-in mice had less $^{86}Rb^+$ efflux than those obtained from wild-type mice. This effect was less evident at low acetylcholine and nicotine concentrations, suggesting gain of function at low agonist levels. In $\alpha 4L9'S$ and $\alpha 4L9'A$ mice this effect may be due to decreased nAChR number [61;69]. $^{86}Rb^+$ efflux studies in $\alpha 4A529T$ knock-in midbrain synaptosomes have also revealed decreased receptor function in comparison to wild-type $\alpha 4A529T^*$ receptors, with larger percentage of response derived from the high-affinity $\alpha 4\beta 2$ component [65]. Furthermore, synaptosomes obtained from striatum and olfactory tubercle of $\alpha 6L9'S$ mice release more [3H]-dopamine, but not [3H]-GABA than those obtained from wild-type animals [54]. Elimination of $\alpha 4$ subunits dramatically decreases the sensitivity of $\alpha 6L9'S^*$ receptors, which is seen as wild-type level [3H]-dopamine release from striatal and olfactory tubercle synaptosomes [71].

At present, there are no published results of electrophysiology or $\beta 2^*$ receptor function in $\beta 2V287L$ knock-in mice. In *Xenopus* oocytes, it has been found that $\alpha 4\beta 2V287L$ mutant receptors are more sensitive to acetylcholine than wild-type $\alpha 4\beta 2$ receptors [72;73]. Like $\alpha 4S248F$ and $\alpha 4+L264$ mutations, $\beta 2V287L$ also shows reduced Ca^{2+} potentiation of acetylcholine response due to altered allosteric modulation [74].

3.2 Viability and baseline behavior of knock-in mice

Most nAChR subunit knock-in lines are healthy and have normal life expectancy, but some strains exhibit viability problems. In the case of $\alpha 3[5]$ mice, the background strain affects viability quite dramatically [57]. Homozygous - but not heterozygous - animals maintained on mixed S129S7/C57BL/6 background die within three months of birth, 50 % of deaths occurring before the third week of life. Pathologic examination revealed mild hydronephrosis of the kidney but the exact cause of premature death is not known. Maintaining on outbred ICR or CD-1 background increases the lifespan of homozygous $\alpha 3[5]$ mice to an average of five months.

The $\alpha 4L9'S$ and $\alpha 7L250T$ mutations are lethal: homozygous animals do not feed and die within 24 h of birth [49;68]. The situation is further exacerbated if the production of $\alpha 4L9'S$ protein is enhanced by deleting the Neo cassette. In Neo-deleted $\alpha 4L9'S$ line even heterozygous pups die within the first postnatal day. In contrast, $\alpha 4L9'A$ knock-in mice are

viable and fertile [50] while the $\beta 2V287L$ knock-in mice are generally viable and fertile and develop normally [51;52] but adult homozygous animals have higher death rate than heterozygotes or their wild-type littermates [52].

The most common behavioral abnormalities in nAChR subunit knock-in mice are changes in some aspect of locomotor activity. In a novel environment, $\alpha 4L9'S$ heterozygotes [49], $\alpha 6L9'S$ homozygotes [54] and $\beta 2V287L$ knock-in mice [52] show hyperactivity. $\alpha 4L9'S$ heterozygotes also show impaired motor learning [49] and $\alpha 6L9'S$ [71] as well as $\beta 2V287L$ mice [52] habituate poorly to a novel environment. $\alpha 4$ knock-out/ $\alpha 6L9'S$ knock-in mice show wild-type behavior, suggesting that $\alpha 4\alpha 6^*$ receptors mediate the hyperactivity observed in $\alpha 6L9'S$ knock-in mice.

Due to the loss and dysfunction of dopaminergic cells, heterozygous $\alpha 4L9'S$ knock-in mice exhibit altered locomotor response to amphetamine. A 5-mg/kg dose of amphetamine was initially found to be intact in 3-months-old mice but reduced in 11-months-old mice [49]. However, another study reported that 3–8-months-old $\alpha 4L9'S$ mice actually show an increased response to amphetamine during the exploratory phase (the first 5 min of experiment) and a reduction in the response 30–45 min after injection [59]. Adult heterozygous $\alpha 4L9'S$ mice treated with a nigral injection of helper-dependent adenovirus (HDA)-Cre show reduced locomotion both under normal conditions and after amphetamine treatment [60].

$\beta 2V287L$ knock-in mice created by Xu et al. show an interesting behavioral phenotype [52]. In home cage recordings, they exhibit altered activity-rest patterns as well as fragmented activity and sleep periods with increased daytime activity [52]. Interestingly, similar disruption of diurnal patterns and have been found in $\alpha 4L9'A$ knock-in mice [61]. Homozygous $\beta 2V287L$ mice do not develop avid running-wheel turning behavior, and their diurnal activity counts are lower than in wild-type mice. However, the homozygous $\beta 2V287L$ knock-in mice outperform wild-type animals in accelerating rotarod test, suggesting that their motor skills are even better than those of the controls. Anxiety tests (open field, elevated plus maze and light-dark exploration box) indicate that they are less anxious than their wild-type littermates [52].

3.3 Response of the knock-in mice to nicotine and nicotinic drugs

Receptors with a gain-of-function mutation are hypersensitive to the acute effects of nicotine [49;50;61]. When tested in *Xenopus* oocytes, the $\alpha 4L9'S\beta 2$ receptors were 30-fold [49], $\alpha 4L9'A\beta 2$ receptors roughly 15-fold [61] and $\alpha 7L250T^*$ receptors 400-fold [68] more sensitive to acetylcholine and nicotine than corresponding wild-type receptors. In Ca^{2+} efflux experiments, ventral tegmental and thalamic primary neuronal cultures of $\alpha 4L9'A$ knock-in mice show a 12–40-fold leftward shift in EC_{50} and an approximately 4-fold increase in maximal signal [50;61].

Electrophysiological experiments have revealed an increase in spiking behavior and firing rate of dopaminergic neurons in response to nicotine in $\alpha 4L9'S$, $\alpha 4L9'A$ and $\alpha 6L9'S$ mutant midbrain neurons at doses that either activate only slightly, do not activate, or reduce activity in wild-type neurons [44;46;50;59]. Zhao-Shea et al. also demonstrated that in $\alpha 4L9'A$ knock in mice dopaminergic neurons of posterior but not anterior VTA are supersensitive to nicotine, suggesting differences in the nAChRs between distinct areas of VTA [44]. Pyramidal cells obtained from $\alpha 4S248F$ or $\alpha 4L264$ knock-in mice [63] as well as GABAergic interneurons, pyramidal neurons, granule cells and hippocampal slices from $\alpha 7L250T$ knock-in mice [68;70] show a marked increase in inhibitory postsynaptic current amplitude in response to nicotine, whereas wild-type cells do not show major responses in these measures.

Gain-of-function mutations also affect desensitization profiles of $\alpha 4^*$ or $\alpha 7$ nAChRs. $\alpha 4L9'A$ mutation increases nicotine-induced desensitization of $\alpha 4^*$ -containing channels when measured in synaptosomes from mouse thalamus and cortex [61]. $\alpha 4L9'A^*$ receptors are also hypersensitive to functional upregulation caused by chronic nicotine. When expressed in *Xenopus* oocytes, $\alpha 4S248F$, $\alpha 4+L264$ and $\beta 2V287L$ mutations have also been found to enhance nicotine-induced desensitization [64;74;75] and $\alpha 4+L264$ also increases the probability of receptor transition to the active state [75]. Desensitization properties of $\alpha 7L250T$ receptors may depend on the tissue sample as well as genotype: in homozygous $\alpha 7L250T$ knock-in hippocampal neuronal cultures nicotine produces a fast, rapidly desensitizing current [68], whereas hippocampal slices from heterozygous $\alpha 7L250T$ knock-in mice show slower nicotine-induced desensitization than wild-type slices [70]. Studies in *Xenopus* oocytes support faster desensitization rate in $\alpha 7L250T$ channels [76].

$\alpha 4L9'A$ and interestingly also $\alpha 4A529T$ knock-in mice are more sensitive to the nicotine-induced hypothermia than the wild-type mice [50;53;65]. Homozygous $\alpha 4L9'A$ mutants show hypothermia to the initial nicotine injection at a dose level that has no effect on wild-type animals. They develop tolerance to the hypothermic effect of nicotine at a similar pace as wild-type mice [53], but sensitization to nicotine-induced hyperactivity more efficiently than wild-type animals [50;53]. Nicotine-induced motor response is intact in $\alpha 4A529T$ knock-in mice [65]. Contrary to $\alpha 4$ gain-of-function mutants, $\alpha 6L9'S$ knock-in mice are hypersensitive to nicotine-induced locomotor activation but not hypothermia [54;71] and they do not develop tolerance or sensitization to the nicotine-induced activation [71].

Heterozygous $\alpha 7L250T$ knock-in mice show unique stereotypic behavior in response to low doses of nicotine [70]. This behavior is biphasic, the first phase consisting of head bobbing and the second phase showing rapid paw tapping, and often relatively long-lasting.

Nociception also has been assessed in one nAChR knock-in line. The baseline nociception of $\alpha 4L9'S$ heterozygotes is intact but they are six times more sensitive to the analgesic effect of nicotine than are wild-type mice [77]. This effect was only observed in hot plate test, not in tail flick test, indicating that $\alpha 4^*$ receptors are important in mediating nicotine analgesia on supraspinal but not spinal level.

3.4 Knock-in mice as epilepsy models

Dysfunction of certain nicotinic receptor subtypes is thought to be involved in some types of human epilepsy. One of them is ADNFLE, characterized by atypical seizures that originate from frontal lobe occur during sleep and include bizarre vocalizing, hyperkinesias, tonic contractions and dystonic posturing [78]. Seizures are usually brief, lasting less than one minute and they rarely occur during wakefulness. Occasionally they may evolve to generalized tonic clonic seizures. During seizures, ADNFLE patients exhibit changes in electroencephalograms (EEG) but more than half of the patients do not show interictal EEG changes.

Two mutations in human *CHRNA4* and one mutation of human *CHRNA2* that cause ADNFLE epilepsy have been introduced into mice: $\alpha 4S248F$ (in mouse gene; $S252F$ in rat *Chrna4* gene) [63;64], $\alpha 4+L264$ [63] and $\beta 2V287L$ [51], [52]. The $\alpha 4S248F$ and $\alpha 4+L264$ ADNFLE mutations reported by Klaassen et al. [63] as well as $\beta 2V287L$ ADNFLE mutation reported by Manfredi et al. [51] exhibit abnormal EEGs characterized by a considerable increase in δ and/or θ wave activities as well as recurrent short-lasting spontaneous seizures, which are accompanied by high-amplitude cortical EEG activity. The seizures of the $\alpha 4S248F$ mice by Klaassen et al. [63] consist of a wide variety of behavioral symptoms, ranging from short-lasting behavioral arrests to long periods of rhythmic jerking movements of limbs. This palette of symptoms is much more severe than that of a typical ADNFLE

case; although in some cases violent, prolonged hyperkinetic activity or epileptiform EEGs are found also in humans [79].

The phenotypes of $\alpha 4S248F$ knock-in mice developed by Teper et al. [64] and $\beta 2V287L$ knock-in mice generated by Xu et al. [52] are more moderate. Spontaneous seizures are rare in these animals [52;64] and epileptiform changes in EEG have not been found in $\alpha 4S248F$ mice [64]. When given nicotine, $\alpha 4S248F$ mice develop distinct two-phase stereotypic behavior called dystonic arousal complex [64]. The first phase involves aroused, jerky, exploration-like movements of head and body, whereas the second phase is characterized by forelimb dystonia, without concomitant changes in EEG. Interestingly, the heterozygous $\alpha 4S248F$ knock-in mice are less sensitive to severe nicotine-induced seizures than wild-type animals. However, $\beta 2V287L$ knock-in mice show increased sensitivity to nicotine-induced seizures [52].

Considerable differences in the two $\alpha 4S248F$ knock-in lines make it difficult to isolate the effect of the polymorphism from other, confounding factors. It is likely that the differences are due to different modifier genes stemming from the use of different background strains [C57BL/6J or 129S4/SvJae in Klaassen et al. [63], CD1 in Teper et al. [64]] and also different embryonic stem cells [129S4/SvJae in Klaassen et al. [63] vs. 129Sv W9.5 in Teper et al. [64]] used in the generation of these lines.

The $\alpha 4L9'S$ and $\alpha 4L9'A$ knock-in mutations are experimental mutations and not related to human ADNFLE. However, as M2 region gain-of-function mutations they result in alterations in seizure phenotype. $\alpha 4L9'S$ and $\alpha 4L9'A$ knock-in mice do not exhibit spontaneous seizures but they are 8–15-fold more sensitive to nicotine-induced seizures than are wild-type mice [61;69]. The seizures are also qualitatively different from wild-type seizures: they start earlier, are prevented by nicotine pretreatment, lack EEG spike-wave discharges, are partial and consist of fast repetitive movements [61]. Another seizure-like phenomenon in $\alpha 4L9'A$ mice is nicotine-sensitivity of neurons in hypoglossal nucleus [80]. When injected into the hypoglossal nucleus, low dose of nicotine causes seizure-like activity in knock-in animals but not in their wild-type littermates. Another feature in $\alpha 4L9'A$ mice that resembles ADNFLE models is that they exhibit more brief awakenings during the resting periods of the activity-rest cycle. In addition, microinjection of low concentrations of nicotine onto the inspiratory neurons of preBötzinger Complex increases respiratory frequency in $\alpha 4L9'A$ knock-in mice but not in wild-type mice.

$\alpha 7L250T$ knock-in mice are another, non-ADNFLE related mouse line that shows increases sensitivity to nicotine-induced seizures [70;81]. The latency to seizure is markedly shorter and the behavioral scores are higher in the knock-in animals than in their wild-type littermates [70]. The nicotine-induced seizures are accompanied by high-amplitude rhythmic discharges in EEG recordings [81]. Interestingly, nicotine-induced seizures affect gene expression differently in wild-type and $\alpha 7L250T$ knock-in mice [82]. In wild-type mice, nicotine-induced seizures altered the expression of 559 genes, whereas in $\alpha 7L250T$ mice 995 differentially expressed transcripts were found after nicotine treatment.

3.5 Knock-in mice and reinforcing effects of drugs

So far, the only nAChR knock-in mice that have been used to study the reinforcing effect of drugs are various $\alpha 4$ knock-in mouse lines. As can be expected of a gain-of-function mutation, $\alpha 4L9'A$ mice develop a place preference for nicotine at a 50-fold lower dose than wild-type mice [50]. Cahir et al. also demonstrated that the gain of function $\alpha 4 S248F$ knock-in mouse self-administer more nicotine than controls at a low dose of nicotine [83]. Since the $\alpha 4$ knock-in mice highlight the role of $\alpha 4^*$ receptors, it can be concluded that their selective activation is sufficient for the reinforcing effects of nicotine. As for the $\alpha 4T529A$

knock-in mice that show diminished responses for nicotinic agonists in biochemical assays, they consume less nicotine than their wild-type controls in a two-bottle free choice setup [65]. They also do not develop nicotine-induced conditioned place preference, unlike control animals.

Studies with knock-in mice also indicate that $\alpha 4^*$ receptors also are involved in the reinforcing properties of other addictive compounds. Varenicline, an nAChR agonist [84;85] that is used as a smoking cessation aid [86;87], has been recently found to reduce voluntary ethanol consumption in mice and rats [88;89]. The varenicline-induced reduction in alcohol consumption involves $\alpha 4^*$ nAChRs as $\alpha 4 L 9' A$ knock-in mice dramatically reduce their alcohol consumption at such doses of varenicline or nicotine that have virtually no effect in wild-type mice [90;91]. Local infusions showed that $\alpha 4^*$ receptors in posterior, but not anterior, VTA are necessary for this effect [44;90].

3.6 Other findings

When treated with dopamine D2 receptor agonist, quinpirole, $\alpha 4 L 9' A$ knock-in mice develop a severe motor dysfunction characterized by rigidity, catalepsy, akinesia and tremor [62]. Quinpirole did not affect tissue dopamine content, dopamine release or turnover differently in knock-in and wild-type mice. However, strong quinpirole-induced activation of striatal cholinergic interneurons was observed in knock-in animals only. This suggests that a dopamine D2 receptor– $\alpha 4^*$ nAChR functional interaction regulates the activity of cholinergic interneurons. Due to the imbalance in the function of cholinergic-dopaminergic innervation, this has been proposed as another mouse model for Parkinson's disease.

4. Targeted genetic rescue strategies in knock-out animals

With genetic rescue strategies a desired gene can be re-expressed in a brain area-specific manner in mice that are null mutants for that gene. Therefore, this technique allows exploring the role of nicotinic receptors containing a certain subunit in a certain brain area.

The most often used vectors in genetic rescue are lentiviral vectors. Their advantages include efficient gene transfer in vivo into a diverse range of tissues and organs [92;93], long-term stable transgene expression, stable integration into both dividing and nondividing cells, including adult neurons, as well as capacity to carry large inserts [94]. Lentiviral vectors are derived from the human immunodeficiency virus genome and they have been modified to prevent replication of viral DNA and to increase safety [95;96].

4.1 $\alpha 5$ knock-out mice re-expressing $\alpha 5$ subunit in medial habenula

The expression of the $\alpha 5$ subunit is restricted in the brain with the highest levels of $\alpha 5$ subunit RNA located in the habenulo-interpeduncular pathway, cortex, and hippocampus, and lower expression levels in VTA and SN [97]. Fowler et al. reported that $\alpha 5$ knock-out mice self-administer significantly more nicotine at high dose levels than do their wild-type littermates [98]. However, at low doses nicotine intake is not different from controls suggesting that the motivation that controls nicotine is not altered in $\alpha 5$ knock-out animals. Instead they may be relatively insensitive to the adverse effects of nicotine or have a higher limit for drug satiation, aspects that limit nicotine intake in wild-type animals. To assess the role of habenulo-interpeduncular $\alpha 5$ nAChRs in the high dose nicotine intake observed in $\alpha 5$ knock-out mice, Fowler et al. re-expressed the $\alpha 5$ subunit in medial habenula (MHb) neurons. Re-expression of the $\alpha 5$ subunit in the habenulo-interpeduncular pathway reduces nicotine intake to control levels demonstrating the role of habenulo-interpeduncular $\alpha 5$ nAChRs in regulating drug intake at high doses of nicotine.

Functional studies in synaptosomes have shown that agonist-induced $^{86}\text{Rb}^+$ efflux is attenuated in $\alpha 5$ knock-out mouse interpeduncular nucleus (IPN) [98]. Lentiviral re-expression of $\alpha 5$ subunit in MHb corrects this deficit. In addition, high doses of nicotine activate IPN in wild-type animals, detected as increased Fos immunostaining. This effect was almost completely eliminated in $\alpha 5$ knock-outs and again rescued in $\alpha 5$ re-expressing mice. Collectively, these findings suggest that the habenulo-interpeduncular pathway is involved in regulating nicotine intake.

4.2 $\beta 2$ knock-out mice re-expressing $\beta 2$ subunit in ventral tegmental area or substantia nigra pars compacta

The genetically rescued $\beta 2$ knock-out mice generated by Maskos et al. re-express $\beta 2$ subunit in VTA [99]. This brain area was chosen because the nAChRs in this region are involved in mediating nicotine's effects on cognition and reinforcement [100]. Later, $\beta 2$ subunit has also been reintroduced into the substantia nigra pars compacta (SNpc) of $\beta 2$ knock-out mice [101]. SN is part of the nigrostriatal dopaminergic pathway that is essentially involved in motor planning and motor learning [102]. Thus, together these mouse models allow comparing the roles of $\beta 2^*$ nAChRs in VTA and SN. Re-expression of $\beta 2$ subunit in VTA is sufficient to restore the spontaneous firing pattern of dopaminergic neurons that is disturbed in $\beta 2$ knock-out mice [103] as well as the effect of nicotine on firing of VTA dopaminergic neurons [99]. However, only firing frequency was restored, while bursting activity remained deficient [103]. In addition, the effect of nicotine was shorter lasting in $\beta 2$ re-expressing mice than in wild-type animals suggesting that sustained activity depends on $\beta 2^*$ receptors on other, possibly glutamatergic, structures [99].

In vivo microdialysis showed that local re-expression of $\beta 2$ subunit in VTA reinstate nicotine-induced dopamine release in nucleus accumbens shell [99; 104]. $\beta 2$ knock-out mice do not show an increase in extracellular dopamine in response to nicotine injection. The re-expression also restores varenicline-induced increase of extracellular dopamine in nucleus accumbens shell [104]. However, basal extracellular dopamine levels are not affected by either $\beta 2$ knock-out or re-expression. In striatum, $\beta 2$ knock-out mice have lower basal extracellular dopamine level than wild-type mice [101]. Re-expression of $\beta 2$ in SNpc restores dopamine concentration as well as nicotine response to wild-type level.

$\beta 2$ knock-out mice are hyperactive in the open field, showing increased distance travelled and time spent in fast, ambulatory movements and reduced time spent in slow, exploratory movements [101]. Re-expression of $\beta 2$ in SNpc reduced distance travelled and fast movements to wild-type levels [101]. However, slow movements were not restored. Re-expression of $\beta 2$ in VTA does not affect distance travelled or fast movements but it restores slow exploratory movements [99;101].

In an intra-VTA nicotine self-administration paradigm, wild-type mice learn rapidly to seek and self-administer nicotine [99]. This behavior is abolished in $\beta 2$ knock-out mice, but lentiviral rescue of VTA $\beta 2$ subunits re-establishes self-administration [99;105], although $\beta 2$ re-expressing mice acquire self-administration more slowly than wild-type animals. Re-expression of $\beta 2$ in SN does not rescue nicotine self-administration behavior [105]. Pons et al. also reported that the lack of nicotine self-administration in $\alpha 4$ and $\alpha 6$ knock-out mice is corrected by re-expression of $\alpha 4$ and $\alpha 6$ in VTA [105].

In social interaction task, $\beta 2$ knock-out mice spend increased time in social contact and overemphasized approach attempts towards other, previously unfamiliar mice [106]. Exploration of novel conspecifics but not novel objects activates c-Fos expression specifically in prelimbic area (PrL) of prefrontal cortex and lesions in PrL produce similar

social pattern in mice as deletion of $\beta 2$ subunit gene. Re-expression of $\beta 2$ in PrL rescues the social behavior up to wild-type levels.

5. Future directions

Current strategies for gene knock-ins are focused on understanding the function, specific subtype and localization of nAChRs. Data generated from these animals to date have proven to be extremely valuable in understanding nicotinic receptor biology. In contrast, little effort has been put forth to understand how the non-coding regulatory sequences in nAChR subunit genes determine their spatial and temporal expression. Understanding the role of non-coding sequences in nAChR subunit genes is critical since the vast majority of polymorphisms identified to date that are significantly associated with disease risk in humans, including polymorphisms in nAChR subunit genes, are in the non-coding regions of genes [107]. However, unlike coding sequences which are relatively highly conserved between humans, rats and mice, non-coding sequences in the nAChR subunit genes are generally poorly conserved between these species. Thus, using a traditional knock-in approach to replace one or a few conserved nucleotides in mice with variant nucleotides from the human ortholog that is associated with disease risk is not feasible.

One method that should be useful for understanding the underlying biology of disease associated non-coding polymorphisms in human nAChR subunit genes is the recombinase-mediated genomic replacement (RMGR) method recently described by Wallace et al. [108]. This technique allows for replacement of large sections of syntenic chromosomes including entire genes and their regulatory sequences. Furthermore, once the initial germ line transmitting embryonic stem cells are generated, electroporation with modifications of the original BAC allow for iterative studies of many regulatory elements within the region and/or different genetic variants within the gene that are associated with disease risk. The initial proof of principal experiment replaced the mouse α -globin gene with either a normal human α -globin gene and all of the cis-acting regulatory elements or the human α -globin gene with a deletion in a regulatory region known to cause α -thalassemia. As a possible example of use for the nicotinic acetylcholine field, nearly all single nucleotide polymorphisms (SNPs) that have been associated with smoking phenotypes in human studies have been found in non-coding regions of the genes. RMGR could be used to generate mouse models harboring major and minor alleles of these various SNPs, which could then be phenotyped for expression differences as well as behavioral characteristics.

Another difficulty that researchers have faced is detecting neural activity with both high temporal and spatial resolution. Calcium sensitive dyes have been in use for a number of years and provide some of these benefits, but prior to study the dyes must be loaded which makes analysis of deep brain regions difficult [109]. More recently a genetically encoded calcium sensor has been developed that would remove this difficulty. Transgenic mice harboring the gene for the calcium sensor GCaMP within specific tissues would allow for much more targeted detection of neural activity [110;111]. Maskos et al in 2008 showed development of fiber-optic imaging of the intact mouse brain using these transgenic GCaMP lines [109]. This allowed for visualization of brain activity with high spatial and temporal resolution in specific regions in an awake, freely moving animal during administration of nicotine.

While RMGR provides more finely tuned expression patterns and GCaMP allows for better visualization of activity, another technique that is under development in the neuroscience field at large actually provides a way to activate specific neurons on demand. To some extent, electrophysiological and micro-infusion techniques have given us a way to activate specific brain regions [112]. However, because both of these techniques use a stimulus that

any nearby cell can respond to, they both lead to complications in interpretation. The emerging field of optogenetics may resolve these issues. Optogenetics uses a family of light-sensitive receptors discovered in bacteria in 1971 called channelrhodopsins [113]. When light hits these channels, they open and allow for ion flow in or out the cell, as a normal receptor would. However, because the agonist is light, and because mammalian cells do not contain these receptors, only those cells that have been engineered to express the channelrhodopsins would be activated. For example, use of previously developed tissue- and cell type-specific Cre-recombinase lines of transgenic mice provides an avenue to express the channelrhodopsins in particular brain regions, or on particular cell types (eg: neurons but not glia). Light stimulation from fiber-optic cables would then open the channels allowing ion flow to produce or inhibit action potentials only within those cells that are expressing the transgene. This manipulation could be performed in freely moving animals to determine effects of specific neural activation on behavior without the concern of side effects from non-specific drug activity, perhaps providing much stronger evidence for the role of particular brain circuits (see Deisseroth, 2011 for a summary of the technique [114]).

6. Conclusions

Genetic techniques have provided invaluable insight into the biology of nicotinic acetylcholine receptors. In this review, we have focused on recent advances that have elucidated subunit localization and assembly partners and what the possible systemic and behavioral effects of these expression patterns might be. The more recent transgenic mouse lines developed have focused on modification of receptors (gain or loss of function, or targeted knock-down/knock-ins) rather than global knock-out of subunits. We have summarized the findings from these newer mutants, from region specific effects to behavioral alterations. Finally, we have highlighted a few novel genetic techniques that we think provide great possibilities for future advancements in understanding of the neurobiology of nAChRs.

Abbreviations

5-HT	5-hydroxytryptamine
α-BTX	α -bungarotoxin
ADNFLE	autosomal dominant nocturnal frontal lobe epilepsy
BAC	bacterial artificial chromosome
CFP	cyan fluorescent protein
CPP	conditioned place preference
Chrna3-7	α 3-7 nicotinic acetylcholine receptor subunit genes
Chrn2	β 2 nicotinic acetylcholine receptor subunit gene
EEG	electroencephalogram
ES	cell embryonic stem cell
GABA	γ -aminobutyric acid
HAD	helper-dependent adenovirus
IPN	interpeduncular nucleus
MHb	medial habenula
nAChR	nicotinic acetylcholine receptor

PrL	prelimbic area of prefrontal cortex
RMGR	recombinase-mediated genomic replacement
RT-PCR	real-time polymerase chain reaction
SN	substantia nigra
SNP	single nucleotide polymorphism
SNpc	substantia nigra pars compacta
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling
VTA	ventral tegmental area
YFP	yellow fluorescent protein

Acknowledgments

Financial support: Academy of Finland grant 135525 (AT), CA089392, DA017637, DA026918

References

1. Langley JN. On the reaction of cells and of nerve-endings to certain poisons, chiefly as regards the reaction of striated muscle to nicotine and to curari. *J Physiol.* 1905; 33:374–413. [PubMed: 16992819]
2. Chang CC, Lee CY. Isolation of neurotoxins from the venom of *Bungarus multicinctus* and their modes of neuromuscular blocking action. *Arch Int Pharmacodyn Ther.* 1963; 144:241–257. [PubMed: 14043649]
3. Changeux JP, Kasai M, Lee CY. Use of a snake venom toxin to characterize the cholinergic receptor protein. *Proc Natl Acad Sci U S A.* 1970; 67:1241–1247. [PubMed: 5274453]
4. Schmidt J. Biochemistry of nicotinic acetylcholine receptors in the vertebrate brain. *Int Rev Neurobiol.* 1988; 30:1–38. [PubMed: 3061965]
5. Yamamoto KI, Domino EF. Nicotine-induced EEG and behavioral arousal. *Int J Neuropharmacol.* 1965; 4:359–373. [PubMed: 5894257]
6. Silvette H, Hoff EC, Larson PS, Haag HB. The actions of nicotine on central nervous system functions. *Pharmacol Rev.* 1962; 14:137–173. [PubMed: 13912982]
7. Marks MJ, Collins AC. Characterization of nicotine binding in mouse brain and comparison with the binding of alpha-bungarotoxin and quinuclidinyl benzilate. *Mol Pharmacol.* 1982; 22:554–564. [PubMed: 7155123]
8. Morley BJ, Kemp GE, Salvaterra P. alpha-Bungarotoxin binding sites in the CNS. *Life Sci.* 1979; 24:859–872. [PubMed: 376997]
9. Oswald RE, Freeman JA. Alpha-bungarotoxin binding and central nervous system nicotinic acetylcholine receptors. *Neuroscience.* 1981; 6:1–14. [PubMed: 7012663]
10. Abood LG, Reynolds DT, Bidlack JM. Stereospecific 3H-nicotine binding to intact and solubilized rat brain membranes and evidence for its noncholinergic nature. *Life Sci.* 1980; 27:1307–1314. [PubMed: 7442444]
11. Romano C, Goldstein A. Stereospecific nicotine receptors on rat brain membranes. *Science.* 1980; 210:647–650. [PubMed: 7433991]
12. Schwartz RD, McGee R Jr, Kellar KJ. Nicotinic cholinergic receptors labeled by [3H]acetylcholine in rat brain. *Mol Pharmacol.* 1982; 22:56–62. [PubMed: 7121451]
13. Sershen H, Reith ME, Lajtha A, Gennaro J Jr. Noncholinergic, saturable binding of (+/-)-[3H]nicotine to mouse brain. *J Recept Res.* 1981; 2:1–15. [PubMed: 7288742]
14. Clarke PB, Schwartz RD, Paul SM, Pert CB, Pert A. Nicotinic binding in rat brain: autoradiographic comparison of [3H]acetylcholine, [3H]nicotine, and [125I]-alpha-bungarotoxin. *J Neurosci.* 1985; 5:1307–1315. [PubMed: 3998824]

15. Marks MJ, Stitzel JA, Romm E, Wehner JM, Collins AC. Nicotinic binding sites in rat and mouse brain: comparison of acetylcholine, nicotine, and alpha-bungarotoxin. *Mol Pharmacol.* 1986; 30:427–436. [PubMed: 3534542]
16. Abood LG, Reynolds DT, Booth H, Bidlack JM. Sites and mechanisms for nicotine's action in the brain. *Neurosci Biobehav Rev.* 1981; 5:479–486. [PubMed: 6119664]
17. Boyd RT. The molecular biology of neuronal nicotinic acetylcholine receptors. *Crit Rev Toxicol.* 1997; 27:299–318. [PubMed: 9189657]
18. Flores CM, Rogers SW, Pabreza LA, Wolfe BB, Kellar KJ. A subtype of nicotinic cholinergic receptor in rat brain is composed of alpha 4 and beta 2 subunits and is up-regulated by chronic nicotine treatment. *Mol Pharmacol.* 1992; 41:31–37. [PubMed: 1732720]
19. Anand R, Conroy WG, Schoepfer R, Whiting P, Lindstrom J. Neuronal nicotinic acetylcholine receptors expressed in *Xenopus* oocytes have a pentameric quaternary structure. *J Biol Chem.* 1991; 266:11192–11198. [PubMed: 2040627]
20. Marubio LM, del Mar Arroyo-Jimenez M, Cordero-Erausquin M, Lena C, Le Novère N, de Kerchove d'Exaerde A, et al. Reduced antinociception in mice lacking neuronal nicotinic receptor subunits. *Nature.* 1999; 398:805–810. [PubMed: 10235262]
21. Picciotto MR, Zoli M, Lena C, Bessis A, Lallemand Y, Le Novère N, et al. Abnormal avoidance learning in mice lacking functional high-affinity nicotine receptor in the brain. *Nature.* 1995; 374:65–67. [PubMed: 7870173]
22. Chen D, Patrick JW. The alpha-bungarotoxin-binding nicotinic acetylcholine receptor from rat brain contains only the alpha7 subunit. *J Biol Chem.* 1997; 272:24024–24029. [PubMed: 9295355]
23. Orr-Urtreger A, Goldner FM, Saeki M, Lorenzo I, Goldberg L, De Biasi M, et al. Mice deficient in the alpha7 neuronal nicotinic acetylcholine receptor lack alpha-bungarotoxin binding sites and hippocampal fast nicotinic currents. *J Neurosci.* 1997; 17:9165–9171. [PubMed: 9364063]
24. Champiaux N, Han ZY, Bessis A, Rossi FM, Zoli M, Marubio L, et al. Distribution and pharmacology of alpha 6-containing nicotinic acetylcholine receptors analyzed with mutant mice. *J Neurosci.* 2002; 22:1208–1217. [PubMed: 11850448]
25. Cui C, Booker TK, Allen RS, Grady SR, Whiteaker P, Marks MJ, Heinemann SF, et al. The beta3 nicotinic receptor subunit: a component of alpha-conotoxin MII-binding nicotinic acetylcholine receptors that modulate dopamine release and related behaviors. *J Neurosci.* 2003; 23:11045–11053. [PubMed: 14657161]
26. Klink R, de Kerchove d'Exaerde A, Zoli M, Changeux JP. Molecular and physiological diversity of nicotinic acetylcholine receptors in the midbrain dopaminergic nuclei. *J Neurosci.* 2001; 21:1452–1463. [PubMed: 11222635]
27. Marubio LM, Gardier AM, Durier S, David D, Klink R, Arroyo-Jimenez MM, et al. Effects of nicotine in the dopaminergic system of mice lacking the alpha4 subunit of neuronal nicotinic acetylcholine receptors. *Eur J Neurosci.* 2003; 17:1329–1337. [PubMed: 12713636]
28. Salminen O, Murphy KL, McIntosh JM, Drago J, Marks MJ, Collins AC, et al. Subunit composition and pharmacology of two classes of striatal presynaptic nicotinic acetylcholine receptors mediating dopamine release in mice. *Mol Pharmacol.* 2004; 65:1526–1535. [PubMed: 15155845]
29. Lu Y, Grady S, Marks MJ, Picciotto M, Changeux JP, Collins AC. Pharmacological characterization of nicotinic receptor-stimulated GABA release from mouse brain synaptosomes. *J Pharmacol Exp Ther.* 1998; 287:648–657. [PubMed: 9808692]
30. McClure-Begley TD, King NM, Collins AC, Stitzel JA, Wehner JM, Butt CM. Acetylcholine-stimulated [3H]GABA release from mouse brain synaptosomes is modulated by alpha4beta2 and alpha4alpha5beta2 nicotinic receptor subtypes. *Mol Pharmacol.* 2009; 75:918–926. [PubMed: 19139153]
31. Zhu PJ, Chiappinelli VA. Nicotine modulates evoked GABAergic transmission in the brain. *J Neurophysiol.* 1999; 82:3041–3045. [PubMed: 10601439]
32. Grady SR, Meinerz NM, Cao J, Reynolds AM, Picciotto MR, Changeux JP, et al. Nicotinic agonists stimulate acetylcholine release from mouse interpeduncular nucleus: a function mediated by a different nAChR than dopamine release from striatum. *J Neurochem.* 2001; 76:258–268. [PubMed: 11145999]

33. Grady SR, Moretti M, Zoli M, Marks MJ, Zanardi A, Pucci L, et al. Rodent habenulo-interpeduncular pathway expresses a large variety of uncommon nAChR subtypes, but only the alpha3beta4* and alpha3beta3beta4* subtypes mediate acetylcholine release. *J Neurosci.* 2009; 29:2272–2282. [PubMed: 19228980]
34. Gotti C, Moretti M, Bohr I, Ziabreva I, Vailati S, Longhi R, et al. Selective nicotinic acetylcholine receptor subunit deficits identified in Alzheimer's disease, Parkinson's disease and dementia with Lewy bodies by immunoprecipitation. *Neurobiol Dis.* 2006; 23:481–489. [PubMed: 16759874]
35. Liu Q, Huang Y, Xue F, Simard A, DeChon J, Li G, et al. A novel nicotinic acetylcholine receptor subtype in basal forebrain cholinergic neurons with high sensitivity to amyloid peptides. *J Neurosci.* 2009; 29:918–929. [PubMed: 19176801]
36. Daubas P, Salmon AM, Zoli M, Geoffroy B, Devillers-Thiery A, Bessis A, et al. Chicken neuronal acetylcholine receptor alpha 2-subunit gene exhibits neuron-specific expression in the brain and spinal cord of transgenic mice. *Proc Natl Acad Sci U S A.* 1993; 90:2237–2241. [PubMed: 8460128]
37. Mineur YS, Picciotto MR. Genetics of nicotinic acetylcholine receptors: Relevance to nicotine addiction. *Biochem Pharmacol.* 2008; 75:323–333. [PubMed: 17632086]
38. Le NN, Zoli M, Lena C, Ferrari R, Picciotto MR, Merlo-Pich E, et al. Involvement of alpha6 nicotinic receptor subunit in nicotine-elicited locomotion, demonstrated by in vivo antisense oligonucleotide infusion. *Neuroreport.* 1999; 10:2497–2501. [PubMed: 10574359]
39. Albuquerque EX, Pereira EF, Alkondon M, Rogers SW. Mammalian nicotinic acetylcholine receptors: from structure to function. *Physiol Rev.* 2009; 89:73–120. [PubMed: 19126755]
40. Gotti C, Clementi F, Fornari A, Gaimarri A, Guiducci S, Manfredi I, et al. Structural and functional diversity of native brain neuronal nicotinic receptors. *Biochem Pharmacol.* 2009; 78:703–711. [PubMed: 19481063]
41. Nelson ME, Kuryatov A, Choi CH, Zhou Y, Lindstrom J. Alternate stoichiometries of alpha4beta2 nicotinic acetylcholine receptors. *Mol Pharmacol.* 2003; 63:332–341. [PubMed: 12527804]
42. Zhou Y, Nelson ME, Kuryatov A, Choi C, Cooper J, Lindstrom J. Human alpha4beta2 acetylcholine receptors formed from linked subunits. *J Neurosci.* 2003; 23:9004–9015. [PubMed: 14534234]
43. Kuryatov A, Onksen J, Lindstrom J. Roles of accessory subunits in alpha4beta2(*) nicotinic receptors. *Mol Pharmacol.* 2008; 74:132–143. [PubMed: 18381563]
44. Zhao-Shea R, Liu L, Soll LG, Improgo MR, Meyers EE, McIntosh JM, et al. Nicotine-mediated activation of dopaminergic neurons in distinct regions of the ventral tegmental area. *Neuropsychopharmacology.* 2011; 36:1021–1032. [PubMed: 21289604]
45. Nashmi R, Dickinson ME, McKinney S, Jareb M, Labarca C, Fraser SE, et al. Assembly of alpha4beta2 nicotinic acetylcholine receptors assessed with functional fluorescently labeled subunits: effects of localization, trafficking, and nicotine-induced upregulation in clonal mammalian cells and in cultured midbrain neurons. *J Neurosci.* 2003; 23:11554–11567. [PubMed: 14684858]
46. Fonck C, Nashmi R, Salas R, Zhou C, Huang Q, De Biasi M, et al. Demonstration of functional alpha4-containing nicotinic receptors in the medial habenula. *Neuropharmacology.* 2009; 56:247–253. [PubMed: 18789953]
47. Thomas KR, Capecchi MR. Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells. *Cell.* 1987; 51:503–512. [PubMed: 2822260]
48. Stephens DN, Mead AN, Ripley TL. Studying the neurobiology of stimulant and alcohol abuse and dependence in genetically manipulated mice. *Behav Pharmacol.* 2002; 13:327–345. [PubMed: 12394407]
49. Labarca C, Schwarz J, Deshpande P, Schwarz S, Nowak MW, Fonck C, et al. Point mutant mice with hypersensitive alpha 4 nicotinic receptors show dopaminergic deficits and increased anxiety. *Proc Natl Acad Sci U S A.* 2001; 98:2786–2791. [PubMed: 11226318]
50. Tapper AR, McKinney SL, Nashmi R, Schwarz J, Deshpande P, Labarca C, et al. Nicotine activation of alpha4* receptors: sufficient for reward, tolerance, and sensitization. *Science.* 2004; 306:1029–1032. [PubMed: 15528443]

51. Manfredi I, Zani AD, Rampoldi L, Pegorini S, Bernascone I, Moretti M, et al. Expression of mutant beta2 nicotinic receptors during development is crucial for epileptogenesis. *Hum Mol Genet.* 2009; 18:1075–1088. [PubMed: 19153075]
52. Xu J, Cohen BN, Zhu Y, Dziewczapolski G, Panda S, Lester HA, et al. Altered activity-rest patterns in mice with a human autosomal-dominant nocturnal frontal lobe epilepsy mutation in the beta2 nicotinic receptor. *Mol Psychiatry.* 2010 advance online publication 6 July 2010.
53. Tapper AR, McKinney SL, Marks MJ, Lester HA. Nicotine responses in hypersensitive and knockout alpha 4 mice account for tolerance to both hypothermia and locomotor suppression in wild-type mice. *Physiol Genomics.* 2007; 31:422–428. [PubMed: 17712039]
54. Drenan RM, Grady SR, Whiteaker P, McClure-Begley T, McKinney S, Miwa JM, et al. In vivo activation of midbrain dopamine neurons via sensitized, high-affinity alpha 6 nicotinic acetylcholine receptors. *Neuron.* 2008; 60:123–136. [PubMed: 18940593]
55. Grady SR, Drenan RM, Breining SR, Yohannes D, Wageman CR, Fedorov NB, et al. Structural differences determine the relative selectivity of nicotinic compounds for native alpha 4 beta 2*, alpha 6 beta 2*, alpha 3 beta 4* and alpha 7-nicotine acetylcholine receptors. *Neuropharmacology.* 2010; 58:1054–1066. [PubMed: 20114055]
56. Xu W, Gelber S, Orr-Urtreger A, Armstrong D, Lewis RA, Ou CN, et al. Megacystis, mydriasis, and ion channel defect in mice lacking the alpha3 neuronal nicotinic acetylcholine receptor. *Proc Natl Acad Sci U S A.* 1999; 96:5746–5751. [PubMed: 10318955]
57. Caffery PM, Krishnaswamy A, Sanders T, Liu J, Hartlaub H, Klysik J, et al. Engineering neuronal nicotinic acetylcholine receptors with functional sensitivity to alpha-bungarotoxin: a novel alpha3-knock-in mouse. *Eur J Neurosci.* 2009; 30:2064–2076. [PubMed: 20128845]
58. Levandoski MM, Lin Y, Moise L, McLaughlin JT, Cooper E, Hawrot E. Chimeric analysis of a neuronal nicotinic acetylcholine receptor reveals amino acids conferring sensitivity to alpha-bungarotoxin. *J Biol Chem.* 1999; 274:26113–26119. [PubMed: 10473561]
59. Orb S, Wieacker J, Labarca C, Fonck C, Lester HA, Schwarz J. Knockin mice with Leu9/Ser alpha4-nicotinic receptors: substantia nigra dopaminergic neurons are hypersensitive to agonist and lost postnatally. *Physiol Genomics.* 2004; 18:299–307. [PubMed: 15199190]
60. Schwarz J, Schwarz SC, Dorigo O, Stutzer A, Wegner F, Labarca C, et al. Enhanced expression of hypersensitive alpha4* nAChR in adult mice increases the loss of midbrain dopaminergic neurons. *FASEB J.* 2006; 20:935–946. [PubMed: 16675851]
61. Fonck C, Cohen BN, Nashmi R, Whiteaker P, Wagenaar DA, Rodrigues-Pinguet N, et al. Novel seizure phenotype and sleep disruptions in knock-in mice with hypersensitive alpha 4* nicotinic receptors. *J Neurosci.* 2005; 25:11396–11411. [PubMed: 16339034]
62. Zhao-Shea R, Cohen BN, Just H, McClure-Begley T, Whiteaker P, Grady SR, et al. Dopamine D2-receptor activation elicits akinesia, rigidity, catalepsy, and tremor in mice expressing hypersensitive {alpha}4 nicotinic receptors via a cholinergic-dependent mechanism. *FASEB J.* 2010; 24:49–57. [PubMed: 19720621]
63. Klaassen A, Glykys J, Maguire J, Labarca C, Mody I, Boulter J. Seizures and enhanced cortical GABAergic inhibition in two mouse models of human autosomal dominant nocturnal frontal lobe epilepsy. *Proc Natl Acad Sci U S A.* 2006; 103:19152–19157. [PubMed: 17146052]
64. Teper Y, Whyte D, Cahir E, Lester HA, Grady SR, Marks MJ, et al. Nicotine-induced dystonic arousal complex in a mouse line harboring a human autosomal-dominant nocturnal frontal lobe epilepsy mutation. *J Neurosci.* 2007; 27:10128–10142. [PubMed: 17881519]
65. Wilking JA, Hesterberg KG, Crouch EL, Homanics GE, Stitzel JA. ChRNA4 A529 knock-in mice exhibit altered nicotine sensitivity. *Pharmacogenet Genomics.* 2010; 20:121–130. [PubMed: 20061993]
66. Bertrand D, Devillers-Thiery A, Revah F, Galzi JL, Hussy N, Mulle C, et al. Unconventional pharmacology of a neuronal nicotinic receptor mutated in the channel domain. *Proc Natl Acad Sci U S A.* 1992; 89:1261–1265. [PubMed: 1741378]
67. Fucile S, Palma E, Mileo AM, Miledi R, Eusebi F. Human neuronal threonine-for-leucine-248 alpha 7 mutant nicotinic acetylcholine receptors are highly Ca²⁺ permeable. *Proc Natl Acad Sci U S A.* 2000; 97:3643–3648. [PubMed: 10716716]

68. Orr-Urtreger A, Broide RS, Kasten MR, Dang H, Dani JA, Beaudet AL, et al. Mice homozygous for the L250T mutation in the alpha7 nicotinic acetylcholine receptor show increased neuronal apoptosis and die within 1 day of birth. *J Neurochem.* 2000; 74:2154–2166. [PubMed: 10800961]
69. Fonck C, Nashmi R, Deshpande P, Damaj MI, Marks MJ, Riedel A, et al. Increased sensitivity to agonist-induced seizures, straub tail, and hippocampal theta rhythm in knock-in mice carrying hypersensitive alpha 4 nicotinic receptors. *J Neurosci.* 2003; 23:2582–2590. [PubMed: 12684443]
70. Broide RS, Salas R, Ji D, Paylor R, Patrick JW, Dani JA, et al. Increased sensitivity to nicotine-induced seizures in mice expressing the L250T alpha 7 nicotinic acetylcholine receptor mutation. *Mol Pharmacol.* 2002; 61:695–705. [PubMed: 11854451]
71. Drenan RM, Grady SR, Steele AD, McKinney S, Patzlaff NE, McIntosh JM, et al. Cholinergic modulation of locomotion and striatal dopamine release is mediated by alpha6alpha4* nicotinic acetylcholine receptors. *J Neurosci.* 2010; 30:9877–9889. [PubMed: 20660270]
72. Bertrand D, Picard F, Le HS, Weiland S, Favre I, Phillips H, et al. How mutations in the nAChRs can cause ADNFLE epilepsy. *Epilepsia.* 2002; (43 Suppl 5):112–122. [PubMed: 12121305]
73. Rodrigues-Pinguet N, Jia L, Li M, Figl A, Klaassen A, Truong A, et al. Five ADNFLE mutations reduce the Ca²⁺ dependence of the mammalian alpha4beta2 acetylcholine response. *J Physiol.* 2003; 550:11–26. [PubMed: 12754307]
74. Rodrigues-Pinguet NO, Pinguet TJ, Figl A, Lester HA, Cohen BN. Mutations linked to autosomal dominant nocturnal frontal lobe epilepsy affect allosteric Ca²⁺ activation of the alpha 4 beta 2 nicotinic acetylcholine receptor. *Mol Pharmacol.* 2005; 68:487–501. [PubMed: 15901849]
75. Bertrand S, Weiland S, Berkovic SF, Steinlein OK, Bertrand D. Properties of neuronal nicotinic acetylcholine receptor mutants from humans suffering from autosomal dominant nocturnal frontal lobe epilepsy. *Br J Pharmacol.* 1998; 125:751–760. [PubMed: 9831911]
76. Revah F, Bertrand D, Galzi JL, Devillers-Thiery A, Mulle C, Hussy N, et al. Mutations in the channel domain alter desensitization of a neuronal nicotinic receptor. *Nature.* 1991; 353:846–849. [PubMed: 1719423]
77. Damaj MI, Fonck C, Marks MJ, Deshpande P, Labarca C, Lester HA, et al. Genetic approaches identify differential roles for alpha4beta2* nicotinic receptors in acute models of antinociception in mice. *J Pharmacol Exp Ther.* 2007; 321:1161–1169. [PubMed: 17371806]
78. Marini C, Guerrini R. The role of the nicotinic acetylcholine receptors in sleep-related epilepsy. *Biochem Pharmacol.* 2007; 74:1308–1314. [PubMed: 17662253]
79. Combi R, Dalpra L, Tenchini ML, Ferini-Strambi L. Autosomal dominant nocturnal frontal lobe epilepsy--a critical overview. *J Neurol.* 2004; 251:923–934. [PubMed: 15316796]
80. Shao XM, Tan W, Xiu J, Puskar N, Fonck C, Lester HA, et al. Alpha4* nicotinic receptors in preBotzinger complex mediate cholinergic/nicotinic modulation of respiratory rhythm. *J Neurosci.* 2008; 28:519–528. [PubMed: 18184794]
81. Gil Z, Sack RA, Kedmi M, Harmelin A, Orr-Urtreger A. Increased sensitivity to nicotine-induced seizures in mice heterozygous for the L250T mutation in the alpha7 nicotinic acetylcholine receptor. *Neuroreport.* 2002; 13:191–196. [PubMed: 11893908]
82. Kedmi M, Orr-Urtreger A. Expression changes in mouse brains following nicotine-induced seizures: the modulation of transcription factor networks. *Physiol Genomics.* 2007; 30:242–252. [PubMed: 17456735]
83. Cahir E, Pillidge K, Drago J, Lawrence AJ. The necessity of alpha4(*) nicotinic receptors in nicotine-driven behaviors: Dissociation between reinforcing and motor effects of nicotine. *Neuropsychopharmacology.* 2011; 36:1505–1517. [PubMed: 21430644]
84. Mihalak KB, Carroll FI, Luetje CW. Varenicline is a partial agonist at alpha4beta2 and a full agonist at alpha7 neuronal nicotinic receptors. *Mol Pharmacol.* 2006; 70:801–805. [PubMed: 16766716]
85. Papke RL, Wecker L, Stitzel JA. Activation and inhibition of mouse muscle and neuronal nicotinic acetylcholine receptors expressed in *Xenopus* oocytes. *J Pharmacol Exp Ther.* 2010; 333:501–518. [PubMed: 20100906]
86. Coe JW, Brooks PR, Vetelino MG, Wirtz MC, Arnold EP, Huang J, et al. Varenicline: an alpha4beta2 nicotinic receptor partial agonist for smoking cessation. *J Med Chem.* 2005; 48:3474–3477. [PubMed: 15887955]

87. Jorenby DE, Hays JT, Rigotti NA, Azoulay S, Watsky EJ, Williams KE, et al. Efficacy of varenicline, an alpha4beta2 nicotinic acetylcholine receptor partial agonist, vs placebo or sustained-release bupropion for smoking cessation: a randomized controlled trial. *JAMA*. 2006; 296:56–63. [PubMed: 16820547]
88. Steensland P, Simms JA, Holgate J, Richards JK, Bartlett SE. Varenicline, an alpha4beta2 nicotinic acetylcholine receptor partial agonist, selectively decreases ethanol consumption and seeking. *Proc Natl Acad Sci U S A*. 2007; 104:12518–12523. [PubMed: 17626178]
89. Kamens HM, Andersen J, Picciotto MR. Modulation of ethanol consumption by genetic and pharmacological manipulation of nicotinic acetylcholine receptors in mice. *Psychopharmacology*. 2010; 208:613–626. [PubMed: 20072781]
90. Hendrickson LM, Zhao-Shea R, Pang X, Gardner PD, Tapper AR. Activation of alpha4* nAChRs is necessary and sufficient for varenicline-induced reduction of alcohol consumption. *J Neurosci*. 2010; 30:10169–10176. [PubMed: 20668200]
91. Hendrickson LM, Gardner P, Tapper AR. Nicotinic acetylcholine receptors containing the alpha4 subunit are critical for the nicotine-induced reduction of acute voluntary ethanol consumption. *Channels*. 2011:5.
92. Jakobsson J, Ericson C, Jansson M, Bjork E, Lundberg C. Targeted transgene expression in rat brain using lentiviral vectors. *J Neurosci Res*. 2003; 73:876–885. [PubMed: 12949915]
93. Kafri T, van Praag H, Ouyang L, Gage FH, Verma IM. A packaging cell line for lentivirus vectors. *J Virol*. 1999; 73:576–584. [PubMed: 9847362]
94. Blomer U, Naldini L, Kafri T, Trono D, Verma IM, Gage FH. Highly efficient and sustained gene transfer in adult neurons with a lentivirus vector. *J Virol*. 1997; 71:6641–6649. [PubMed: 9261386]
95. Naldini L, Blomer U, Gage FH, Trono D, Verma IM. Efficient transfer, integration, and sustained long-term expression of the transgene in adult rat brains injected with a lentiviral vector. *Proc Natl Acad Sci U S A*. 1996; 93:11382–11388. [PubMed: 8876144]
96. Miyoshi H, Blomer U, Takahashi M, Gage FH, Verma IM. Development of a self-inactivating lentivirus vector. *J Virol*. 1998; 72:8150–8157. [PubMed: 9733856]
97. Marks MJ, Pauly JR, Gross SD, Deneris ES, Hermans-Borgmeyer I, Heinemann SF, et al. Nicotine binding and nicotinic receptor subunit RNA after chronic nicotine treatment. *J Neurosci*. 1992; 12:2765–2784. [PubMed: 1613557]
98. Fowler CD, Lu Q, Johnson PM, Marks MJ, Kenny PJ. Habenular alpha5 nicotinic receptor subunit signalling controls nicotine intake. *Nature*. 2011; 471:597–601. [PubMed: 21278726]
99. Maskos U, Molles BE, Pons S, Besson M, Guiard BP, Guilloux JP, et al. Nicotine reinforcement and cognition restored by targeted expression of nicotinic receptors. *Nature*. 2005; 436:103–107. [PubMed: 16001069]
100. Granon S, Faure P, Changeux JP. Executive and social behaviors under nicotinic receptor regulation. *Proc Natl Acad Sci U S A*. 2003; 100:9596–9601. [PubMed: 12876201]
101. Avale ME, Faure P, Pons S, Robledo P, Deltheil T, David DJ, et al. Interplay of beta2* nicotinic receptors and dopamine pathways in the control of spontaneous locomotion. *Proc Natl Acad Sci U S A*. 2008; 105:15991–15996. [PubMed: 18832468]
102. Pisani A, Bonsi P, Picconi B, Tolu M, Giacomini P, Scarnati E. Role of tonically-active neurons in the control of striatal function: cellular mechanisms and behavioral correlates. *Prog Neuropsychopharmacol Biol Psychiatry*. 2001; 25:211–230. [PubMed: 11263753]
103. Mameli-Engvall M, Evrard A, Pons S, Maskos U, Svensson TH, Changeux JP, et al. Hierarchical control of dopamine neuron-firing patterns by nicotinic receptors. *Neuron*. 2006; 50:911–921. [PubMed: 16772172]
104. Reperant C, Pons S, Dufour E, Rollema H, Gardier AM, Maskos U. Effect of the alpha4beta2* nicotinic acetylcholine receptor partial agonist varenicline on dopamine release in beta2 knock-out mice with selective re-expression of the beta2 subunit in the ventral tegmental area. *Neuropharmacology*. 2010; 58:346–350. [PubMed: 19887076]
105. Pons S, Fattore L, Cossu G, Tolu S, Porcu E, McIntosh JM, et al. Crucial role of alpha4 and alpha6 nicotinic acetylcholine receptor subunits from ventral tegmental area in systemic nicotine self-administration. *J Neurosci*. 2008; 28:12318–12327. [PubMed: 19020025]

106. Avale ME, Chabout J, Pons S, Serreau P, De Chaumont F, Olivo-Marin JC, et al. Prefrontal nicotinic receptors control novel social interaction between mice. *FASEB J*. 2011
107. Manolio TA. Genomewide association studies and assessment of the risk of disease. *N Engl J Med*. 2010; 363:166–176. [PubMed: 20647212]
108. Wallace HA, Marques-Kranc F, Richardson M, Luna-Crespo F, Sharpe JA, Hughes J, et al. Manipulating the mouse genome to engineer precise functional syntenic replacements with human sequence. *Cell*. 2007; 128:197–209. [PubMed: 17218265]
109. Maskos, U.; Cressant, A.; Brito, M.; Granon, S.; Vincent, P. 2008 Neuroscience Meeting Planner. Washington, DC: Society for Neuroscience; 2008. Functional fibered fluorescence imaging in freely moving mice. Program 598.8, Poster VV30
110. Diez-Garcia J, Matsushita S, Mutoh H, Nakai J, Ohkura M, Yokoyama J, et al. Activation of cerebellar parallel fibers monitored in transgenic mice expressing a fluorescent Ca²⁺ indicator protein. *Eur J Neurosci*. 2005; 22:627–635. [PubMed: 16101744]
111. Tian L, Hires SA, Mao T, Huber D, Chiappe ME, Chalasani SH, et al. Imaging neural activity in worms, flies and mice with improved GCaMP calcium indicators. *Nat Methods*. 2009; 6:875–881. [PubMed: 19898485]
112. Zhang F, Wang LP, Boyden ES, Deisseroth K. Channelrhodopsin-2 and optical control of excitable cells. *Nat Methods*. 2006; 3:785–792. [PubMed: 16990810]
113. Oesterheld D, Stoerkenius W. Rhodopsin-like protein from the purple membrane of *Halobacterium halobium*. *Nat New Biol*. 1971; 233:149–152. [PubMed: 4940442]
114. Deisseroth K. Optogenetics. *Nat Methods*. 2011; 8:26–29. [PubMed: 21191368]

Figure 1.
Timeline of the emergence of genetic tools in nicotine research. Red indicates a novel transgenic mouse. Blue indicates a novel molecular technique used.

A. Transgenic techniques

Advantages:

Fast, production of transgenic animals starts within 3-6 months. Low cost.

Disadvantages:

Using traditional transgenic approach leads to random incorporation of the insert. Therefore, the insert can interrupt other genes or it can be integrated into area where gene expression is suppressed or under the control of locus control region. The insert also tends to get silenced over time. In addition, traditional transgenic technique does not allow controlling for the copy number. These problems are less likely when BAC vectors are used.

B. Knock-out and knock-in techniques

Advantages:

Both knock-out and knock-in techniques are replacement methods in which the native gene is modified either by deletion (knock-out) or by modification of coding sequence (knock-in). Thus, there is always only one copy of the gene of interest.

Disadvantages:

Slow, production of mutant mice starts within 1-3 years. High-cost, labor intensive and technically challenging methods.

Figure 2.
Comparison of transgenic and knock-out/knock-in techniques.

Figure 3.

Approximate locations of mutations in knock-in mouse strains. ACh = acetylcholine binding site, C = C-terminus, M1-M4 = transmembrane regions, N = N-terminus, SS = Cysteine residues in the Cys loop. Red areas show the approximate locations of mutations. Numbers refer to knock-in mouse strains: 1 = $\alpha 3[5]$, 2 = $\alpha 4L9'S$, 3 = $\alpha 4L9'A$, 4 = $\alpha 4S248F$, 5 = $\alpha 4T529A$, 6 = $\alpha 6L9'S$, 7 = $\alpha 7L250T$, 8 = $\beta 2V287L$

Table 1

Main findings of the properties of nAChR knock-in mouse lines.

Mouse line	Homozygotes viable?	Changes in behavior	Biochemical, neurochemical and electrophysiological findings
$\alpha 3[5]$	Yes*	No apparent changes	Receptors blocked by α -BTX. $\alpha 3[5]$ receptors colocalize with presynaptic varicosities on the soma and dendrites of sympathetic cells.
$\alpha 4L9'S$ <i>Neo</i> intact	No	Increased anxiety, increased activity, deficiency in motor learning. Diminished locomotor response to amphetamine. Increased sensitivity to nicotine in hot-plate test. Increased sensitivity to nicotine-induced seizures.	Less dopaminergic neurons, less $\alpha 4^*$ binding sites. Increased sensitivity to nicotine-induced $^{86}\text{Rb}^+$ efflux.
$\alpha 4L9'A$	Yes	CPP to nicotine at 50-fold lower doses than wt mice. Increased sensitivity to nicotine-induced seizures.	Less $\alpha 4^*$ binding sites. Enhanced sensitivity to nicotine-induced Ca^{2+} efflux and increase in firing rate of dopaminergic cells. Decreased maximal response of $^{86}\text{Rb}^+$ efflux. Enhanced desensitization.
$\alpha 4S248F$	Yes	Spontaneous seizures in Klaassen et al. (2006) line, not in Teper et al. (2007) line. Heterozygous mice relatively protected from nicotine-induced seizures. Dystonic arousal syndrome in response to nicotine.	Decreased function of synaptosomal nAChRs. Increase in nicotine-induced inhibitory postsynaptic currents in pyramidal cells.
$\alpha 4T529A$	Yes	No apparent changes in baseline behavior. Increased sensitivity to hypothermic effect of nicotine. Decreased consumption of nicotine solutions. Do not develop nicotine-induced CPP.	Decreased agonist-induced $^{86}\text{Rb}^+$ in midbrain synaptosomes. Decreased [^{125}I]-epibatidine binding in thalamus.
$\alpha 6L9'S$	Yes	Increased activity especially in dark, impaired habituation to novel environment. Increased sensitivity to nicotine-induced hyperactivity. Do not develop tolerance or sensitization to nicotine-induced motor activation.	Increased [^3H]-dopamine but not [^3H]-GABA release from striatum and olfactory tubercle. No synaptic depression for one pulse stimulation in fast-scan cyclic voltammetry. Altered firing pattern of dopamine neurons and dopamine release and turnover pattern. Part of $\alpha 6L9'S^*$ channels tonically active.
$\alpha 7L250T$	No	No changes in baseline behavior. Increased sensitivity to nicotine-induced seizures.	Decreased [^{125}I]- α -BTX binding. Altered agonist-induced $\alpha 7$ currents, no desensitization or slow desensitization. Increased apoptotic cell death in several brain regions.
$\beta 2V287L$	Yes	Altered activity-rest pattern. Decreased wheel-running behavior, enhanced rotarod performance, increased exploratory activity, impaired habituation to novel environment. Decreased anxiety. Increased sensitivity to nicotine-induced seizures.	N/A

α -BTX = α -bungarotoxin, CPP = conditioned place preference, GABA = γ -aminobutyric acid, N/A = not available, nAChR = nicotinic acetylcholine receptor, wt = wild-type.

* life span on average 5 months when maintained on outbred background, < 3 weeks if maintained on inbred background.

Table 2

Locations of mutations in knock-in mouse strains

Knock-in strain	Location of mutation	Structure
$\alpha 3[5]$	Amino acid changes Tyr184Try, Glu187Try, Ile188Val, Lys189Tyr and Asn191Thr in exon 5 of Chrna3	C-loop of agonist binding site
$\alpha 4L9'S$	Amino acid change Leu142Ser in exon 5 of Chrna4	M2 transmembrane region, channel domain
$\alpha 4L9'A$	Amino acid change Leu142Ala in exon 5 of Chrna4	M2 transmembrane region, channel domain
$\alpha 4S248F$	Amino acid change Ser248Phe in exon 5 of Chrna4	M2 transmembrane region, channel domain
$\alpha 4T529A$	Amino acid change Thr529Ala in exon 5 of Chrna4	Large intracellular loop between M3 and M4
$\alpha 6L9'S$	Amino acid change Leu280Ser in exon 5 of Chrna6	M2 transmembrane region, channel domain
$\alpha 7L250T$	Amino acid change Leu250Thr in exon 8 of Chrna7	M2 transmembrane region, channel domain
$\beta 2V287L$	Amino acid change Val287Leu in exon 5 of Chrnb2	M2 transmembrane region, channel domain