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Review

Recent advances in gene manipulation and nicotinic acetylcholine receptor biology

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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.

Contents

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, α3–7 nicotinic acetylcholine receptor subunit genes; Chrnb2, β2 nicotinic acetylcholine receptor subunit gene; EEG, electroencephalogram; ES cell, embryonic stem cell; GABA, γ-aminobutyric acid; HDA, 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 deoxytransferase-mediated dUTP-biotin nick end-labeling; VTA, ventral tegmental area; YFP, yellow fluorescent protein.

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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 Yamamoto and Domino [5] 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. For an early review of these behavioral studies see Silvette et al. [6]. Building on these findings, the existence of nAChRs in the brain was first 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 α2–α10 (α8 only found in chickens) and β2–β4 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 (α2–α4, α6) and a β subunit (β2 or β4) in order to form a functional receptor *in vitro*. The most abundant heteromeric nAChR in brain is comprised of the subunits α4 and β2 [18,19]. The α4β2* (the asterisk indicates that other subunits such as α5 can contribute to α4β2 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 α7 subunits. Homomeric α7 nAChRs exhibit low affinity for nicotinic agonists and immunological [22] and genetic studies [23] demonstrated that α7 nAChRs are the previously described [^{125}I]-α-BTX binding sites in brain.

Although α4β2* 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 α4β2, α4β2α5, α6β2, α6β2β3, and α4α6β2β3. Data also indicate that the nAChRs in GABAergic terminals are α4β2 and α4β2α5 [29–31] whereas nAChRs that modulate acetylcholine release in the interpeduncular nucleus are α3β4 and α3β3β4 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 Fig. 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 α4β2 receptor which is only moderately calcium permeable while the second most common is the α7 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 α4β2 has been found in two distinct stoichiometric forms that have distinct pharmacological properties [39]. Typically two α4 and three β2 subunits form the functional receptor, however, when β2 is limited functional receptors with three α4 and two β2 are formed. Previous *in vitro* studies have attempted to influence the production of one or another of these stoichiometries by altering the ratio of α4 to β2 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 α4β2 receptors with different stoichiometries, Zhou et al. [42] linked α4 and β2 cDNAs such that expression leads to a fusion protein of the two subunits with a linker between them. By addition of an unlinked individual α4 or individual β2 construct they were able to specifically express only the $(\alpha4)_3(\beta2)_2$ or the $(\alpha4)_2(\beta2)_3$ in oocytes. Recordings from these oocytes showed that the two receptors have very distinct properties. The $(\alpha4)_3(\beta2)_2$ has a low affinity for acetylcholine and high calcium permeability, while the $(\alpha4)_2(\beta2)_3$ 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

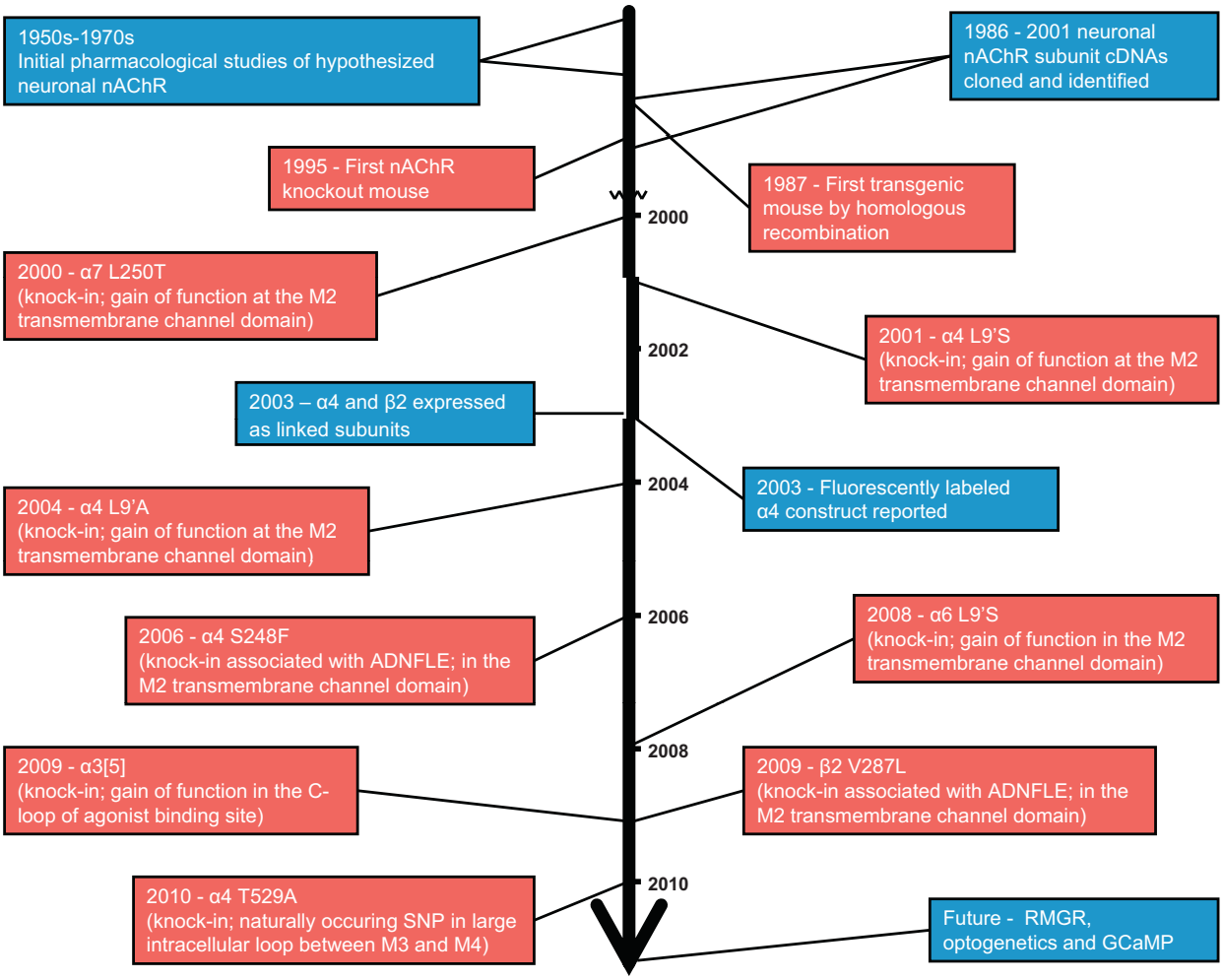

Fig. 1. Timeline of the emergence of genetic tools in nicotine research. Red indicates a novel transgenic mouse. Blue indicates a novel molecular technique used. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

of subunits to different brain regions (see Baddick and Marks, in this issue). For example, Zhao-Shea et al. [44] were able to localize α4α6* 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 α4 L9′A gain of function mutation. 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. [45] successfully produced α4 and β2 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. 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 α4β2 nAChRs. An α4YFP knock-in transgenic mouse line where the wild-type α4 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. [46] demonstrated that the upregulation of α4* nAChRs in the VTA in the α4YFP mice is restricted to GABAergic neurons. Moreover, Fonck et al. used the α4YFP to assess α4 expression in the medial habenula and fasciculus retroflexus. 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 α4YFP mice the authors showed specific expression of functional α4 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 [47], the homologous recombination technique has been extensively used to engineer knock-out mice [48]. More recently, this method has also been used to generate knock-in mice in which gene

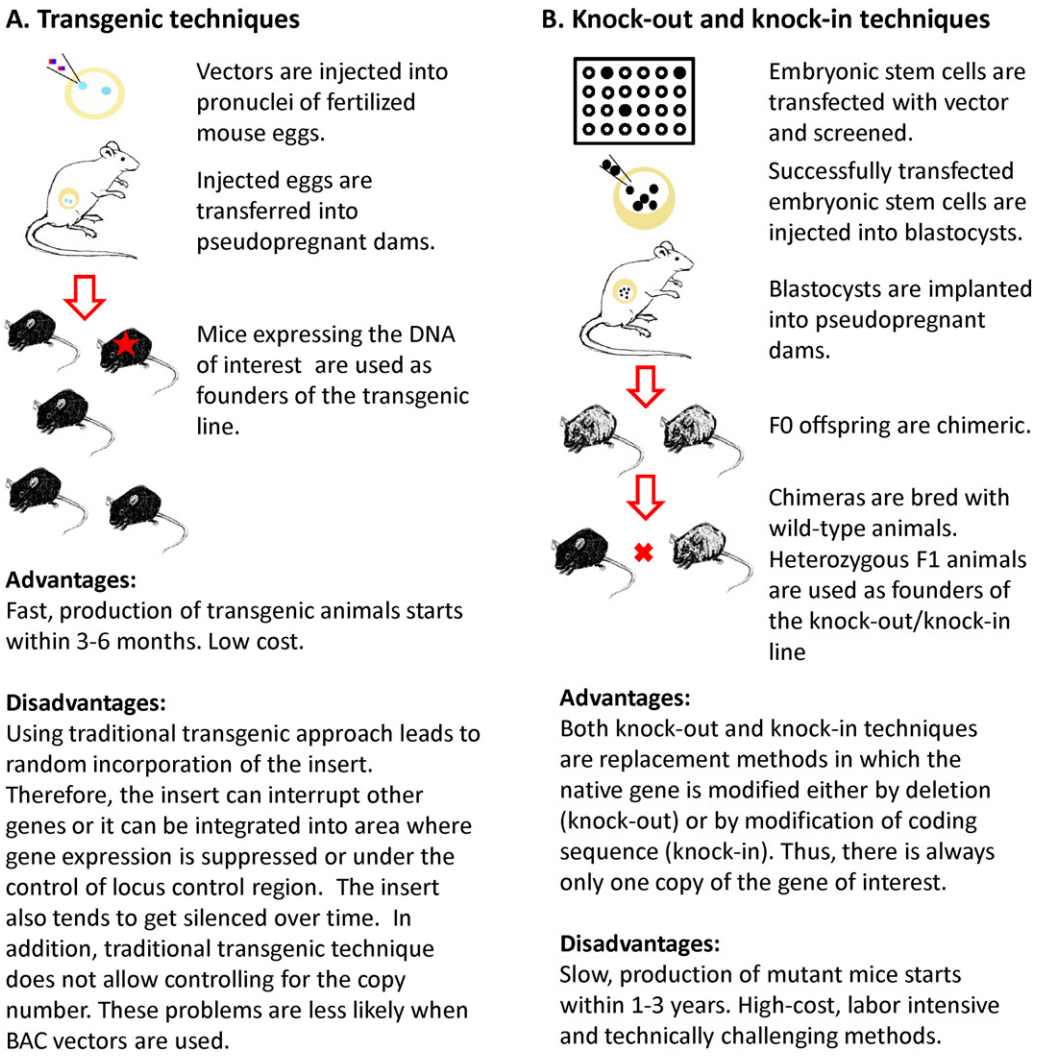

Fig. 2. Comparison of transgenic and knock-out/knock-in techniques.

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 Fig. 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, α4L9′A knock-in mice are hypersensitive to α4*-mediated locomotor stimulation [53] whereas α6L9′S knock-in mice are hypersensitive to α6*-mediated hypothermia [54]. Thus, a compound that causes hypothermia in α4L9′A mice and increased locomotion in α6L9′S mice at low doses that are without affect in control animals is probably a full or partial agonist of both α4β2* and α6β2* 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 α3, α4, α6, α7 and β2 although the majority of knock-in mutants reported are in the α4 subunit. Fig. 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 et al. [57] generated a knock-in mouse line expressing a chimeric α3 nAChR subunit (α3[5] knock-in mice). Five amino acids in the loop C region of the mouse α3 subunit were substituted with the corresponding residues from the α1 subunit of muscle type nAChR

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
α3[5]	Yes[a]	No apparent changes	Receptors blocked by α-BTX. α3[5] receptors colocalize with presynaptic varicosities on the soma and dendrites of sympathetic cells.
α4L9′S *Neo* 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 α4* binding sites. Increased sensitivity to nicotine-induced $^{86}Rb^{+}$ efflux.
α4L9′A	Yes	CPP to nicotine at 50-fold lower doses than wt mice. Increased sensitivity to nicotine-induced seizures.	Less α4* binding sites. Enhanced sensitivity to nicotine-induced Ca^{2+} efflux and increase in firing rate of dopaminergic cells. Decreased maximal response of $^{86}Rb^{+}$ efflux. Enhanced desensitization.
α4S248F	Yes	Spontaneous seizures in Klaassen et al. [63] line, not in Teper et al. [64] 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.
α4T529A	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 $^{86}Rb^{+}$ in midbrain synaptosomes. Decreased [^{125}I]-epibatidine binding in thalamus.
α6L9′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 [^{3}H]-dopamine but not [^{3}H]-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 α6L9′S* channels tonically active.
α7L250T	No	No changes in baseline behavior. Increased sensitivity to nicotine-induced seizures.	Decreased [^{125}I]-α-BTX binding. Altered agonist-induced α7 currents, no desensitization or slow desensitization. Increased apoptotic cell death in several brain regions.
β2V287L	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.
[a] Life span on average 5 months when maintained on outbred background, <3 weeks if maintained on inbred background.

from Torpedo californica. This manipulation renders α3* receptors sensitive to the muscle and α7 nAChR antagonist α-BTX [58]. Staining of superior cervical ganglia with rhodamine-conjugated α-BTX as well as pre- and postsynaptic markers showed that α3[5]* receptors colocalized with presynaptic varicosities on the soma and dendrites of sympathetic neurons [57]. α3[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 α3[5] knock-in/α7knock-out mice verified that α7 receptors did not contribute to these findings. Electrophysiological studies suggested that the amino acid substitutions in α3[5] may have a negative impact on receptor subunit assembly or surface expression, which may explain the observed attenuated potentials.

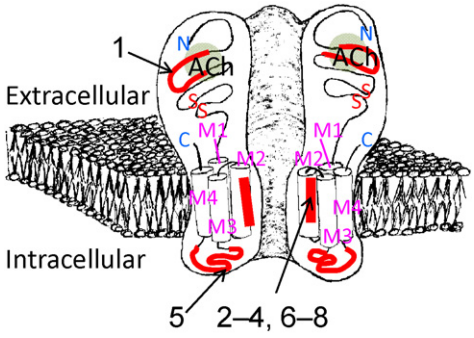

Fig. 3. Approximate locations of mutations in knock-in mouse strains. ACh = acetylcholine binding site, C = C-terminus, M1-M4 = transmembrane regions, N = N-terminus, SS = Cysteine residues in the Cys loop. Red areas show the approximate locations of mutations. Numbers refer to knock-in mouse strains: 1 = α3[5], 2 = α4L9′S, 3 = α4L9′A, 4 = α4S248F, 5 = α4T529A, 6 = α6L9′S, 7 = α7L250T, 8 = β2V287L. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

The α4L9′S mouse line was the first attempt to create a *Chrna4* gain-of-function knock-in mouse [49]. Since the α4L9′S* mutation proved problematic *in vivo*, α4L9′A mutation that has a less robust gain-of-function effect was introduced into *Chrna4* [50], see below. The α4L9′S and α4L9′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 α4* receptor hypersensitive to agonist activation. In α4L9′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 α4L9′S heterozygotes where the *Neo* cassette has been locally deleted in SN [60]. α4L9′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 α4 subunit, including two mutations that cause ADNFLE in humans, α4S248F (or α4S252F) mice with Ser to Phe substitution in amino acid 248 or 252 of M2 region [63,64] and α4+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 α4T529A knock-in mouse line with a Thr to Ala substitution in the large cytoplasmic loop of the α4 subunit [65], that were developed to allow a more precise assessment of the role

Table 2
Locations of mutations in knock-in mouse strains.

Knock-in strain	Location of mutation	Structure
α3[5]	Amino acid changes Tyr184Try, Glu187Try, Ile188Val, Lys189Tyr and Asn191Thr in exon 5 of Chrna3	C-loop of agonist binding site
α4L9′S	Amino acid change Leu142Ser in exon 5 of Chrna4	M2 transmembrane region, channel domain
α4L9′A	Amino acid change Leu142Ala in exon 5 of Chrna4	M2 transmembrane region, channel domain
α4S248F	Amino acid change Ser248Phe in exon 5 of Chrna4	M2 transmembrane region, channel domain
α4T529A	Amino acid change Thr529Ala in exon 5 of Chrna4	Large intracellular loop between M3 and M4
α6L9′S	Amino acid change Leu280Ser in exon 5 of Chrna6	M2 transmembrane region, channel domain
α7L250T	Amino acid change Leu250Thr in exon 8 of Chrna7	M2 transmembrane region, channel domain
β2V287L	Amino acid change Val287Leu in exon 5 of Chrnb2	M2 transmembrane region, channel domain

of this mouse polymorphism in nicotine sensitivity and nAChR function has not been reported to cause cell death.

Although it appears that the α4L9′S gain-of-function mutation is the only α4 mutation introduced into mice that is neurotoxic, a gain-of-function mutation in *Chrna7*, the gene that codes for the nAChR α7 subunit, also is neurotoxic. Heterozygous and homozygous α7L250T knock-in mice with a Leu to Thr substitution in the amino acid 250 of M2 region [corresponds to chicken α7L247T [66] and human α7L248T [67] polymorphisms] show increased apoptosis-related terminal deoxytransferase-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 α6L9′S knock-in mice with a Leu to Ser substitution in the 9′ position of M2 region [54], and the β2V287L 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 α4L9′S and α4L9′A knock-in mice have less α4* 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 α4L9′S and α4L9′A constructs. [^{125}I]-α-BTX binding assays and western blots also have shown that α7L250T knock-in mice express less α7 receptors than do wild-type mice [68,70]. In contrast, the α4S248F [64], α4T529A [65] and β2V287L [51] knock-ins have little effect on [^{125}I]-epibatidine binding, although homozygous α4S248F knock-ins show enhanced binding in a few brain areas [64], and α4T529A knock-ins decreased binding in thalamus [65]. Likewise, location and intensity of [^{125}I]-α-conotoxin binding is intact in α6L9′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 α6L9′S knock-in animals. Fast-scan cyclic voltammetry in brain slices confirmed that after burst stimulation dopamine overflow is increased in α6L9′S knock-in mice but not in α4 knock-out/α6L9′S knock-in mice. Unlike wild-type slices, α6L9′S brain slices do not exhibit synaptic depression for one pulse stimulation. Removal of α4 subunits re-establishes synaptic depression in α6L9′S slices. Furthermore, part of the α6L9′S* nAChR channels seem to be tonically active, which affects dopamine release and turnover patterns. However, the pacemaker firing is intact in α6L9′S dopaminergic neurons.

Functional experiments showed that thalamic, cortical or striatal synaptosomes prepared of adult heterozygous α4L9′S [69], homozygous α4L9′A [61], and α4S248F [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 α4L9′S and α4L9′A mice this effect may be due to decreased nAChR number [61,69]. $^{86}Rb^{+}$ efflux studies in α4A529T knock-in midbrain synaptosomes have also revealed decreased receptor function in comparison to wild-type α4A529T* receptors, with larger percentage of response derived from the high-affinity α4β2 component [65]. Furthermore, synaptosomes obtained from striatum and olfactory tubercle of α6L9′S mice release more [^{3}H]-dopamine, but not [^{3}H]-GABA than those obtained from wild-type animals [54]. Elimination of α4 subunits dramatically decreases the sensitivity of α6L9′S* receptors, which is seen as wild-type level [^{3}H]-dopamine release from striatal and olfactory tubercle synaptosomes [71].

At present, there are no published results of electrophysiology or β2* receptor function in β2V287L knock-in mice. In *Xenopus* oocytes, it has been found that α4β2V287L mutant receptors are more sensitive to acetylcholine than wild-type α4β2 receptors [72,73]. Like α4S248F and α4+L264 mutations, β2V287L 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 α3[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 α3[5] mice to an average of five months.

The α4L9′S and α7L250T 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 α4L9′S protein is enhanced by deleting the *Neo* cassette. In *Neo*-deleted α4L9′S line even heterozygous pups die within the first postnatal day. In contrast, α4L9′A knock-in mice are viable and fertile [50] while the β2V287L 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, α4L9′S heterozygotes [49], α6L9′S homozygotes [54] and β2V287L knock-in mice [52] show hyperactivity. α4L9′S heterozygotes also show impaired motor learning [49] and α6L9′S [71] as well as β2V287L mice [52] habituate poorly to a novel environment. α4 knock-out/α6L9′S knock-in mice show wild-type behavior, suggesting that α4α6* receptors mediate the hyperactivity observed in α6L9′S knock-in mice.

Due to the loss and dysfunction of dopaminergic cells, heterozygous α4L9′S knock-in mice exhibit altered locomotor response to amphetamine. A response to 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 α4L9′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 α4L9′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].

β2V287L knock-in mice created by Xu et al. [52] show an interesting behavioral phenotype. 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 α4L9′A knock-in mice [61]. Homozygous β2V287L mice do not develop avid running-wheel turning behavior, and their diurnal activity counts are lower than in wild-type mice. However, the homozygous β2V287L 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 α4L9′Sβ2 receptors were 30-fold [49], α4L9′Aβ2 receptors roughly 15-fold [61] and α7L250T* 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 α4L9′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 α4L9′S, α4L9′A and α6L9′S mutant midbrain neurons at doses that either activate only slightly, do not activate, or reduce activity in wild-type neurons [44,47,51,60]. Zhao-Shea et al. [45] also demonstrated that in α4L9′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. Pyramidal cells obtained from α4S248F or α4+L264 knock-in mice [64] as well as GABAergic interneurons, pyramidal neurons, granule cells and hippocampal slices from α7L250T knock-in mice [69,71] 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 α4* or α7 nAChRs. α4L9′A mutation increases nicotine-induced desensitization of α4*-containing channels when measured in synaptosomes from mouse thalamus and cortex [62]. α4L9′A* receptors are also hypersensitive to functional upregulation caused by chronic nicotine. When expressed in *Xenopus* oocytes, α4S248F, α4+L264 and β2V287L mutations have also been found to enhance nicotine-induced desensitization [65,75,76] and α4+L264 also increases the probability of receptor transition to the active state [76]. Desensitization properties of α7L250T receptors may depend on the tissue sample as well as genotype: in homozygous α7L250T knock-in hippocampal neuronal cultures nicotine produces a fast, rapidly desensitizing current [69], whereas hippocampal slices from heterozygous α7L250T knock-in mice show slower nicotine-induced desensitization than wild-type slices [71]. Studies in *Xenopus* oocytes support faster desensitization rate in α7L250T channels [77].

α4L9′A and interestingly also α4A529T knock-in mice are more sensitive to the nicotine-induced hypothermia than the wild-type mice [51,54,66]. Homozygous α4L9′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 [54], but sensitization to nicotine-induced hyperactivity more efficiently than wild-type animals [51,54]. Nicotine-induced motor response is intact in α4A529T knock-in mice [66]. Contrary to α4 gain-of-function mutants, α6L9′S knock-in mice are hypersensitive to nicotine-induced locomotor activation but not hypothermia [55,72] and they do not develop tolerance or sensitization to the nicotine-induced activation [72].

Heterozygous α7L250T knock-in mice show unique stereotypic behavior in response to low doses of nicotine [71]. 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 α4L9′S heterozygotes is intact but they are six times more sensitive to the analgesic effect of nicotine than are wild-type mice [78]. This effect was only observed in hot plate test, not in tail flick test, indicating that α4* 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 [79]. 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 *CHRNB2* that cause ADNFL epilepsy have been introduced into mice: α4S248F (in mouse gene; S252F in rat *Chrna4* gene) [64,65], α4+L264 [64] and β2V287L [52,53]. The α4S248F and α4+L264 ADNFLE mutations reported by Klaassen et al. [64] as well as β2V287L ADNFLE mutation reported by Manfredi et al. [52] 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 α4S248F mice by Klaassen et al. [64] 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 [80].

The phenotypes of α4S248F knock-in mice developed by Teper et al. [65] and β2V287L knock-in mice generated by Xu et al. [53] are more moderate. Spontaneous seizures are rare in these animals [53,65] and epileptiform changes in EEG have not been found in α4S248F mice [65]. When given nicotine, α4S248F mice develop distinct two-phase stereotypic behavior called dystonic arousal complex [65]. 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 α4S248F knock-in mice are less sensitive to severe nicotine-induced seizures than wild-type animals. However, β2V287L knock-in mice show increased sensitivity to nicotine-induced seizures [53].

Considerable differences in the two α4S248F 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. [64], CD1 in Teper et al. [65]] and also different embryonic stem cells [129S4/SvJae in Klaassen et al. [64] vs.129Sv W9.5 in Teper et al. [65]] used in the generation of these lines.

The α4L9′S and α4L9′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. α4L9′S and α4L9′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 [62,70]. 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 [62]. Another seizure-like phenomenon in α4L9′A mice is nicotine-sensitivity of neurons in hypoglossal nucleus [81]. 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 α4L9′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 α4L9′A knock-in mice but not in wild-type mice.

α7L250T knock-in mice are another, non-ADNFLE related mouse line that shows increases sensitivity to nicotine-induced seizures [71,82]. The latency to seizure is markedly shorter and the behavioral scores are higher in the knock-in animals than in their wild-type littermates [71]. The nicotine-induced seizures are accompanied by high-amplitude rhythmic discharges in EEG recordings [82]. Interestingly, nicotine-induced seizures affect gene expression differently in wild-type and α7L250T knock-in mice [83]. In wild-type mice, nicotine-induced seizures altered the expression of 559 genes, whereas in α7L250T 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 α4 knock-in mouse lines. As can be expected of a gain-of-function mutation, α4L9′A mice develop a place preference for nicotine at a 50-fold lower dose than wild-type mice [51]. Cahir et al. [84] also demonstrated that the gain of function α4 S248F knock-in mouse self-administer more nicotine than controls at a low dose of nicotine. Since the α4 knock-in mice highlight the role of α4* receptors, it can be concluded that their selective activation is sufficient for the reinforcing effects of nicotine. As for the α4T529A 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 [66]. They also do not develop nicotine-induced conditioned place preference, unlike control animals.

Studies with knock-in mice also indicate that α4* receptors also are involved in the reinforcing properties of other addictive compounds. Varenicline, an nAChR agonist [85,86] that is used as a smoking cessation aid [87,88], has been recently found to reduce voluntary ethanol consumption in mice and rats [89,90]. The varenicline-induced reduction in alcohol consumption involves α4* nAChRs as α4L9′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 [91,92]. Local infusions showed that α4* receptors in posterior, but not anterior, VTA are necessary for this effect [45,91].

3.6. Other findings

When treated with dopamine D2 receptor agonist, quinpirole, α4L9′A knock-in mice develop a severe motor dysfunction characterized by rigidity, catalepsy, akinesia and tremor [63]. 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–α4* 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 [93,94], long-term stable transgene expression, stable integration into both dividing and nondividing cells, including adult neurons, as well as capacity to carry large inserts [95]. 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 [96,97].

4.1. α5 Knock-out mice re-expressing α5 subunit in medial habenula

The expression of the α5 subunit is restricted in the brain with the highest levels of α5 subunit RNA located in the habenulo-interpeduncular pathway, cortex, and hippocampus, and lower expression levels in VTA and SN [98]. Fowler et al. reported that α5 knock-out mice self-administer significantly more nicotine at high dose levels than do their wild-type littermates [99]. However, at low doses nicotine intake is not different from controls suggesting that the motivation that controls nicotine is not altered in α5 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 α5 nAChRs in the high dose nicotine intake observed in α5 knock-out mice, Fowler et al. re-expressed the α5 subunit in medial habenula (MHb) neurons. Re-expression of the α5 subunit in the habenulo-interpeduncular pathway reduces nicotine intake to control levels demonstrating the role of habenulo-interpeduncular α5 nAChRs in regulating drug intake at high doses of nicotine.

Functional studies in synaptosomes have shown that agonist-induced $^{86}Rb^{+}$ efflux is attenuated in α5 knock-out mouse interpeduncular nucleus (IPN) [99]. Lentiviral re-expression of α5 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 α5 knock-outs and again rescued in α5 re-expressing mice. Collectively, these findings suggest that the habenulo-interpeduncular pathway is involved in regulating nicotine intake.

4.2. β2 Knock-out mice re-expressing β2 subunit in ventral tegmental area or substantia nigra pars compacta

The genetically rescued β2 knock-out mice generated by Maskos et al. [100] re-express β2 subunit in VTA. This brain area was chosen because the nAChRs in this region are involved in mediating nicotine's effects on cognition and reinforcement [101]. Later, β2 subunit has also been reintroduced into the substantia nigra pars compacta (SNpc) of β2 knock-out mice [102]. SN is part of the nigrostriatal dopaminergic pathway that is essentially involved in motor planning and motor learning [103]. Thus, together these mouse models allow comparing the roles of β2* nAChRs in VTA and SN. Re-expression of β2 subunit in VTA is sufficient to restore the spontaneous firing pattern of dopaminergic neurons that is disturbed in β2 knock-out mice [104] as well as the effect of nicotine on firing of VTA dopaminergic neurons [100]. However, only firing frequency was restored, while bursting activity remained deficient [104]. In addition, the effect of nicotine was shorter lasting in β2 re-expressing mice than in wild-type animals suggesting that sustained activity depends on β2* receptors on other, possibly glutamatergic, structures [100].

In vivo microdialysis showed that local re-expression of β2 subunit in VTA reinstate nicotine-induced dopamine release in nucleus accumbens shell [100,105]. β2 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 [105]. However, basal extracellular dopamine levels are not affected by either β2 knock-out or re-expression. In striatum, β2 knock-out mice have lower basal extracellular dopamine level than wild-type mice [102]. Re-expression of β2 in SNpc restores dopamine concentration as well as nicotine response to wild-type level.

β2 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 [102]. Re-expression of β2 in SNpc reduced distance travelled and fast movements to wild-type levels [103]. However, slow movements were not restored. Re-expression of β2 in VTA does not affect distance travelled or fast movements but it restores slow exploratory movements [100,102].

In an intra-VTA nicotine self-administration paradigm, wild-type mice learn rapidly to seek and self-administer nicotine [100]. This behavior is abolished in β2 knock-out mice, but lentiviral rescue of VTA β2 subunits re-establishes self-administration [100,106], although β2 re-expressing mice acquire self-administration more slowly than wild-type animals. Re-expression of β2 in SN does not rescue nicotine self-administration behavior [106]. Pons et al. [106] also reported that the lack of nicotine self-administration in α4 and α6 knock-out mice is corrected by re-expression of α4 and α6 in VTA.

In social interaction task, β2 knock-out mice spend increased time in social contact and overemphasized approach attempts towards other, previously unfamiliar mice [107]. 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 β2 subunit gene. Re-expression of β2 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 [108]. 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. [109]. 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 [110]. 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 [111,112]. Maskos et al. [110] in 2008 showed development of fiber-optic imaging of the intact mouse brain using these transgenic GCaMP lines. 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 [113]. 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 [114]. 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 (e.g. 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 [115] for a summary of the technique).

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.

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