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Review

Neuronal nicotinic receptors: insights gained from gene knockout and knockin mutant mice

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Abstract. Neuronal nicotinic acetylcholine receptors are ligand-gated ion channels that subserve a range of functions in the brain and peripheral nervous system. They are pentamers variously composed of α (α_2–α_{10}) and β subunits (β_2–β_4). Pharmacological and ligand-binding studies have shown that the different subunits vary in their distribution and channel properties, but precise delineation of the in vivo function of individual subunits has been hampered by lack of subunit-specific antagonists. The development of transgenic mice with targeted deletions of specific subunits (knockout mice) or mutations in critical receptor domains (knockin mice) has extended understanding of nicotinic receptors, revealing that some subunits are necessary for viability, whereas others mediate modulatory effects on learning and memory, locomotion, anxiety, nociception, dopaminergic neurotransmission, seizure threshold, development of the visual system and autonomic function. In some cases, studies of transgenic mice have confirmed expectations derived from pharmacological and expression studies, but in other cases, compensation by related subunits has revealed a degree of functional redundancy not predicted by previous approaches.

Key words. Acetylcholine; nicotinic; subunits; transgenic; knockin; knockout.

Introduction

Nicotinic acetylcholine receptors (nAChR) are expressed in muscle, autonomic ganglia, the central nervous system (CNS) and in some sensory organs. Nicotinic receptors are members of a gene superfamily of ligand-gated ion channels [1], which also includes glycine, γ-aminobutyric acid ($GABA_A$) and serotonin receptors [2]. In the brain, a number of discrete nuclei contain neurons that produce acetylcholine (ACh) as the principal neurotransmitter. Such nuclei, including the nucleus basalis of Meynert and the medial septal nucleus of the forebrain, as well as the pedunculopontine nucleus of the brainstem, send widespread projections to most brain areas where they interact with muscarinic and neuronal nAChRs (see fig. 1). Outside the CNS, neuronal nAChRs are found in autonomic ganglia where they regulate diverse functions in tissues under the control of the sympathetic and parasympathetic nervous system. Neuronal nAChRs are heteropentameric or homopentameric molecules variously composed of α subunits (α_2–α_{10}) and β subunits (β_2–β_4) [3–5]. One subgroup of neuronal nAChRs is heteromeric, composed of both α (α_{2-6}) and β (β_{2-4}) subunits, with the most common configuration in mam-

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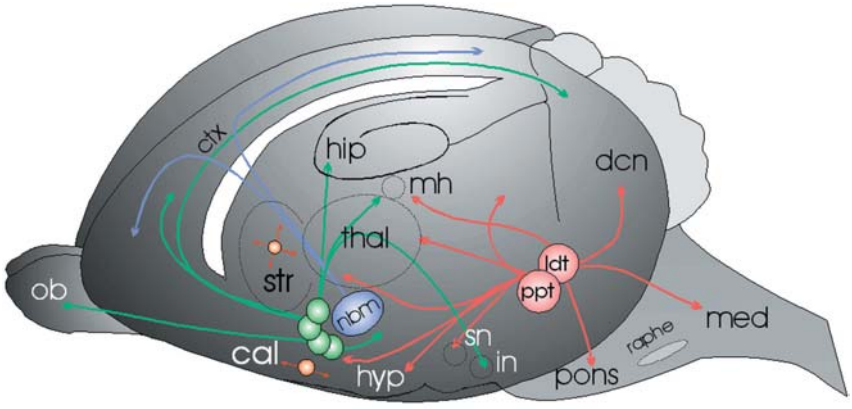

Figure 1. Schematic drawing of a mouse brain showing the location and projections of the major cholinergic nuclei (left hemisphere only). Not shown are the motor nuclei of the cranial nerves, which release acetylcholine onto muscle nicotinic receptors. Two main clusters of cholinergic nuclei project to neural structures. In the basal forebrain, the nucleus basalis of Meynert (nbm) sends projections throughout the isocortex (ctx). Anterior to the nucleus basalis lie four closely related nuclei (green circles, unlabelled): the medial septal nucleus, superiorly, followed by the ventral limb of the diagonal band, the horizontal limb of the diagonal band and, inferiorly, the substantia inominata. These nuclei project to the olfactory bulb (ob), the piriform, insular and entorhinal cortices, the amygdala, hippocampus (hip) and the habenulopeduncular tract, including the medial habenula (mh) and the interpeduncular nuclues (in). In turn, the forebrain nuclei receive cholinergic fibres from the pedunculopontine tegmental nucleus (ppt), which also innervates the lateral hypothalamus (hyp), substantia nigra of the midbrain (sn), pontine and medullary reticular nuclei (pons, med), the raphe magnus (raphe), as well as the thalamus (thal), vestibular nuclei and deep cerebellar nuclei (dcn). Closely associated with the pedunculopontine tegmental nucleus is the laterodorsal tegmental nucleus (ldt), which innervates the neighbouring dorsal raphe and locus coeruleus (not shown) and the medial habenula. Remaining cholinergic neurons include some interneurons of the striatum (str) and the islands of Calleja (cal).

malian brain containing α_4 and β_2 subunits. Another subgroup consists of homomeric receptors (α_{7-9}) [6], with the α_7 homopentamer being the most abundant example of this subgroup. The recently described α_{10} nAChR does not form functional homopentamers, but its coexpression has been shown to modify the activity of α_9 nAChR subunits [4]. Owing to the complex pharmacology of neuronal nAChRs and the lack of ligands with absolute receptor subtype specificity, the functional role of individual receptor subunits in vivo remains unclear. Attributing functions to individual subunits has been facilitated, however, by the development of genetically engineered mouse models. This review will focus on the use of gene knockout and knockin mutant mice as a tool for elucidating the in vivo role of individual neuronal nAChR subunits.

Structure of neuronal nAChRs

The nomenclature of neuronal nAChRs is borrowed from muscle nAChRs, to which they are closely related. In muscle, α and β nicotinic subunits (now designated α_1 and β_1) combine with δ and either γ or ε subunits to create the principal receptor of the neuromuscular junction [3, 6]. The first neuronal nAChR subunit was cloned based on predicted sequence homology to the α subunit of the mouse muscle AChR [7]. Since this first report, a gene family encoding 12 homologous genes of neuronal nAChR subunits have been identified and characterized in brain, autonomic ganglia and in sensory pathways of rodents, chicks and humans [4, 8–12]. The nine neuronal nAChR α subunits are designated α_2 to α_{10}, while the neuronal non-α subunits are designated as β_2 to β_4 [5, 13, 14]. All of the neuronal α nAChR subunits possess adjacent cysteines (analogous to cysteines 192–193 of the α subunit of the muscle AChR) that are believed to be intimately involved in acetylcholine binding [6, 15, 16]. The β subunits do not have these cysteines; however, they, too, contribute to the binding site and are important in conferring pharmacological properties to the receptor complex [17]. Early studies suggested that the functional neuronal nAChR is a heteropentamer made up by assembly of α and β subunits, with a putative $2\alpha:3\beta$ subunit stoichiometry [18, 19]. However, subsequent studies revealed the existence of so-called triplet receptors (which are, nonetheless, pentameric); these contain more than one α or β subunit [20–23]. Furthermore, expression studies in oocytes showed that homomeric receptor configurations were functional [24–26].

Early drug binding studies distinguished two principal groups of neural nAChRs on the basis of their high affinity for either α-bungarotoxin (type 1 nAChRs) or nicotine (type 2 nAChRs); these are now known to correspond to the structural subdivision of nAChRs into homopentameric and heteropentameric subgroups [27]. The ligand α-bungarotoxin (αBGT) has a high affinity for α_7 monomeric receptors and reveals this subunit to be the dominant type 1 receptor, widely distributed in most brain regions. These receptors rapidly desensitise on ex-

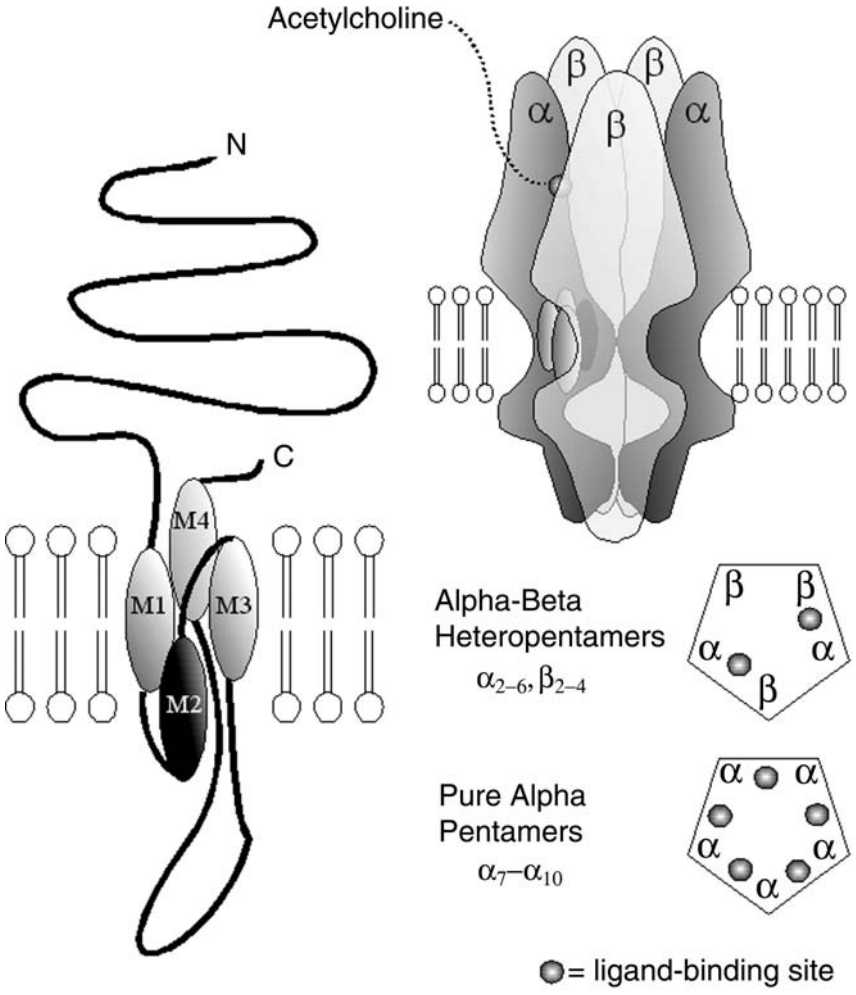

Figure 2. Putative structure of a generic neuronal nicotinic receptor subunit (left), showing four transmembrane domains (M1–M4) and a large intracytoplasmic loop between M3 and M4. Site-directed mutagenesis studies indicate that the M2 domain lines the ion channel pore. Functional heteromeric receptors can be obtained by expressing α subunits (α_2–α_6) and β subunits (β_2–β_4) together, and the presumed stoichiometry is $2\alpha:3\beta$. Homomeric receptors can be produced by expression of α_7, α_8 or α_9 subunits alone. The newly described α_{10} subunit can form functional heteromeric complexes with α_9 subunits but not with β subunits; α_9 and α_{10} subunits are therefore, by convention, grouped with the α_7 homopentamers.

posure to nicotinic agonists and are involved in phasic synaptic responses [27–29]. In contrast, most high-affinity nicotine binding is accounted for by heteromeric nAChRs containing α_4 and β_2 subunits. These receptors also bind the endogenous ligand ACh with high affinity, and unlike the α_7 receptors, they desensitise slowly. This simple classification of brain nicotinic binding sites has since been expanded into four main classes based on binding studies conducted in nAChR subunit knockout mice [30].

Amino acid sequences of various cloned nAChR subunits appear to be strongly homologous and show similar hydrophobicity profiles [31, 32]. The genes encode four principal domains: a large hydrophilic amino-terminal domain (210–220 amino acids); a compact hydrophobic region (70 amino acids, further subdivided into three transmembrane segments of 19–28 uncharged amino acids, M1, M2 and M3); a short hydrophilic cytoplasmic domain of variable length; and a transmembrane carboxyl-terminal segment of about 20 hydrophobic amino acids (M4). The hydrophilic amino-terminal domain carries the ACh binding site and faces the synaptic cleft [15, 33]. The subunits are arranged around a central channel, and experiments using site-directed mutagenesis have provided evidence that the M2 membrane-spanning region lines the ion channel pore of the receptor [34, 35] (see fig. 2).

Activation of presynaptic nAChRs is known to facilitate the release of several neurotransmitters, including GABA, glutamate, dopamine and ACh itself [36–38]. Studies of GABAergic facilitation have primarily implicated $\alpha_4\beta_2$ nAChRs [37, 39]. Glutamate facilitation appears to be mediated by α_7 nAChRs in many brain regions [40, 41] but may also involve $\alpha_4\beta_2$ nAChRs in some con-

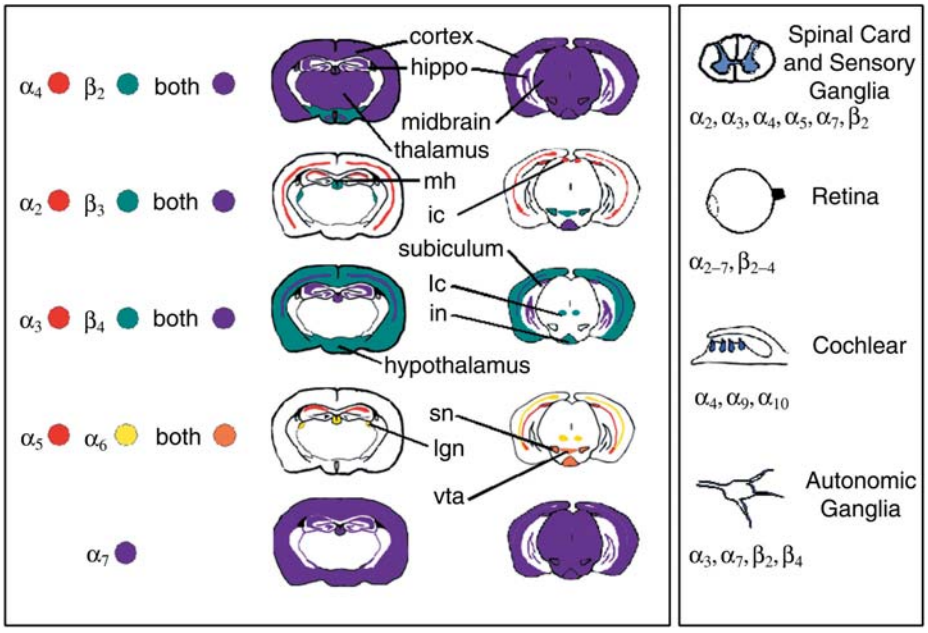

Figure 3. Schematic diagram showing the distribution of nicotinic receptor subtypes in brain, spinal cord, retina, cochlear and autonomic ganglia. The left column illustrates coronal brain slices taken at the level of the hypothalamus, thalamus and hippocampus; brain slices in the right column are taken through the junction of the brainstem and the peduncular region and include the posterior hippocampus (see fig. 1). Additional nuclei outside the planes of section have been included for illustrative purposes. The α_4 and β_2 subunits are widely co-expressed, indicating the predominance of $\alpha_4\beta_2$ receptors in the mammalian brain, and corresponding to areas of high-affinity nicotine binding. The α_7 homopentamer is also widespread and corresponds with regions of α-bungarotoxin binding; note that α_7 expression is largely or wholly absent from the thalamus, although it is expressed in the colliculi. Other subunits have a more limited expression. The α_2 subunit is largely confined to the interpeduncular nucleus but it is also found in the stratum oriens of the hippocampus. The β_3 subunit is predominantly expressed in the medial habenula and some tegmental structures; it is also detected at low levels in other regions, including the cortex. The α_3 and β_4 subunits are often paired in the autonomic nervous system, but studies of knockout mice have shown that β_2 may substitute for β_4 when β_4 is unavailable. Although α_3 is detectable in the hippocampus, levels of expression are low; it has also been detected at low levels in the thalamus (not shown). The α_5 and α_6 subunits are both expressed in tegmental structures; they may combine with the ubiquitous β_2 subunit but may also form combinations involving the β_3 subunit. In particular, the distributions of α_6 and β_3 are very similar and correspond to regions of MII α-conotoxin binding. The α_6 subunit is found in the superior colliculus and lateral geniculate nucleus but not elsewhere in the thalamus. The α_6 and β_4 subunits are also expressed in the locus coeruleus. Although many subunits are detectable in the retina, the most important appears to be the β_2 subunit, probably in combination with α_3 and α_6. Note that transcripts for each subunit may also detected at low levels in additional areas, not shown. Abbreviations: hippo, hippocampus; mh, medial habenula; ic, inferior colliculus; lc, locus coeruleus; in, interpeduncular nucleus; sn, substantia nigra pars compacta; lgn, lateral geniculate nucleus; vta, ventral tegmental area.

texts [42–44]. The subunit composition of nAChRs modulating dopamine release is uncertain; initial studies implicating $\alpha_4\beta_2$ nAChRs in this role have been questioned, and a contribution from α_3, β_4, and α_6 has been proposed [36, 38, 40, 45]. In addition, postsynaptic nAChRs have also been demonstrated in a variety of brain regions, and application of nicotinic agonists in vitro can elicit cellular depolarisation and action potentials in neurons bearing nAChRs [46–48]. There is also in vitro evidence implicating nAChRs in fast synaptic transmission in the hippocampus [49].

Expression profile of the nAChR subunits

In situ hybridization has been used to characterize the regional distribution of the various subunit messenger RNAs (mRNAs) in the central and peripheral nervous system in rat [5, 28, 50–53] and mouse [54, 55] (see fig. 3). These experiments have demonstrated that the nAChR subunit distribution varies between subunits. The α_2 mRNA has a limited expression pattern, with moderate signal detected in the interpeduncular nucleus, levels detected in the dorsal tegmental nucleus, inferior colliculus, hippocampus and subiculum and even lower levels in the cortex [52]. The α_3 mRNA was expressed in the medial habenula, entorhinal cortex, thalamus, hypothalamus, autonomic ganglia, hippocampus, cortex and ventral tegmental area and only a weak signal in the cerebellum [52]. A number of studies have failed, however, to confirm the presence of α_3 mRNA in brainstem dopaminergic neurons [45, 51, 54] and thalamus [45, 54]. Of the α_3 mRNA expression studies, the study of Wada et al. [52] used riboprobes to detect transcripts rather than the less sensitive method of labelled oligonucleotides. Subunit α_3 mRNA was, however, identified in the retina and

autonomic ganglia [56]. The α_4 mRNA is more widely expressed, with high levels detected in the thalamus, medial habenula, substantia nigra pars compacta, ventral tegmental area, piriform cortex, endopiriform nucleus, amygdala, subicular complex, septum, interpeduncular nucleus, somatosensory cortex and the caudal linear raphe. Moderate levels of α_4 mRNA were also detected in other regions of the cerebral cortex and the hypothalamus and low levels in the striatum. Expression of α_4 has also been detected in the cochlear and vestibular ganglia [52]. The α_5 subunit mRNA has been detected in a small number of localized sites, with high levels detected in the subiculum, parasubiculum, substantia nigra pars compacta, ventral tegmental area and interpeduncular nucleus [57]. Lower levels were detected in the cortex [57]. The α_6 nAChR subunit is expressed at high levels in some subcortical structures: the substantia nigra pars compacta, ventral tegmental area, locus ceruleus, medial habenula, the interpeduncular nucleus and the target fields of the retinal ganglion cells in the thalamus [51].

Of the homomeric nAChRs, transcripts encoding the α_7 nAChR subunit are the most abundant and have been found in discrete neuronal populations throughout the rat brain [28]. High levels were detected in the hippocampus, hyopothalamus, amygdala, olfactory areas, endopiriform nucleus, claustrum and in the isocortex. Moderate levels were detected in the medial habenula, interpeduncular nucleus and in the superior and inferior colliculi. In the brainstem, moderate signals were expressed in the central gray, dorsal and median raphe nuclei, tegmental nuclei and in the lateral lemniscus. A weak signal was also detected in the cerebellum [28]. Transcripts of the α_8 subunit have only ever been purified from whole chick brain [58] and chick retina [58], so its precise regional distribution is unknown. The α_9 subunit has a very limited expression in the brain, with transcripts detected in the pars tuberalis of the adenohypophysis. Messenger RNA for α_9 was also detected in the nasal epithelium, the hair cells of the cochlear and the skeletal muscle of the tongue [5]. The α_{10} subunit was the most recently cloned member of the nAChR family [4]. It is structurally related to the rat α_9 subunit and is also expressed in cochlear hair cells. The α_{10} subunit forms functional heteromeric receptors with the α_9 subunit, but it does not form homomeric complexes, nor does it form functional complexes with α_2–α_6 or β_2–β_4 nAChR subunits [4].

Like the α_4 subunit, the β_2 subunit showed widespread expression in the nervous system, with the highest levels in the thalamus, substantia nigra pars compacta, ventral tegmental area, piriform cortex, entorhinal cortex, and in the somatosensory and motor areas of the brainstem [52], Moderate levels were detected in the medial habenula [52]. In contrast, β_3 mRNA has a more limited expression pattern, with high levels detected in the medial habenula, substantia nigra pars compacta, ventral tegmental area, thalamus and the mesencephalic nucleus of the trigeminal nerve [50]. Transcripts encoding the β_4 subunit were detected in a diverse number of loci, with the medial habenula having the greatest signal, but moderate signal was also detected in the cortex, olfactory regions, hippocampus, hypothalamus, locus coeruleus, pontine nuclei and the cerebellum [53]. There was also isolated but intense hybridisation for β_4 in the interpeduncular nucleus and in the motor nucleus of the trigeminal nerve [53].

In summary, it appears that the α_4, β_2 and α_7 subunits are most widely expressed in the rodent brain, whereas α_2, α_3, α_5, α_6, β_3 and β_4 subunits show a more limited expression profile. The distribution of α_4 mRNA overlaps that of β_2 mRNA [52]. In addition, it was demonstrated that more than one type of α subunit is expressed in some brain regions. The thalamus, for instance, contains both α_3 and α_4 mRNAs [52], and a variety of subunits (including α_4, α_5, α_6, β_2 and β_3) [50–52, 57] are expressed in the substantia nigra pars compacta, suggesting, that nicotinic receptors are likely to play a major role in the regulation of dopaminergic neurotransmission.

Phenotype of β_2 nAChR knockout mice

The β_2 nAChR subunit was the first of the neuronal nicotinic receptors to be targeted in knockout experiments [59]. A large number of studies have used this knockout model line to examine the role of this subunit in drug reinforcement, learning and memory, neurodegeneration, nociception and development of the visual system [6, 27, 60, 61]. The first publication on this line of mice [59] suggested that the β_2 nAChR subunit is required for nicotine-mediated enhancement of passive avoidance, a paradigm that is thought to model learning and memory function. Given a choice between a well-illuminated and a dark cage, mice will stay in the dark. In this paradigm, mice are punished for entry into a dark region of the cage and then retested to assess the latency to reenter the dark region. Knockout mice exhibited an increased latency to reenter the dark region on subsequent occasions, compared with wild-type mice subjected to the same conditioning stimulus. This result was interpreted as showing that the learning of negative associations was enhanced in mutant mice compared with controls (at least within the context of a passive avoidance paradigm). This unexpected finding needs to be interpreted with caution, however. The experiment was undertaken on a small number of mice with a mixed genetic background, and the result has not been replicated in an independently generated line of β_2 knockout mice. The same investigators found no improvement in the learning capacity of β_2 knockouts in the Morris water maze, which assesses the acquisition of spatial information [59]. Furthermore, a detailed be-

havioral study undertaken on the same line of β_2 knockout mice backcrossed 10 generations to the C57BL6 strain showed that young knockout and control mice did not differ in the acquisition of either contextual or tone-conditioned fear responses; older mutant males actually showed learning impairment [62]. Further examination of aged β_2 knockout mice has shown impairment in spatial learning and evidence of neocortical atrophy, with loss of pyramidal neurons and an astroglial and microglial response [63].

A subsequent study of β_2 knockout mice focused on dissecting the pathways in the mesolimbic dopamine system that are thought to mediate the reinforcing effect of nicotine [64]. Nicotine was found to stimulate dopamine release in the ventral striatum of wild-type mice but not in knockout mice. Furthermore, β_2 knockout mice did not learn to self-administer nicotine even though they learned to self-administer a different reinforcing agent, cocaine, whereas wild-type controls self-administered both drugs. Patch-clamp studies demonstrated that mesencephalic dopaminergic neurons derived from normal mice responded to nicotine with an increase in firing rate and dose-dependent inward cationic currents, whereas neurons from knockout mice were insensitive to nicotine, suggesting that the β_2 nAChR subunit is an essential component of the nicotinic receptor complex on dopaminergic neurons. A complementary study confirmed the pivotal role of this subunit in mediating nicotine-stimulated dopamine release from synaptosomes [65]. Using a combination of nicotinic antagonists and knockout mice lacking β_2, α_4 or α_7 subunits, Klink et al. [66] demonstrated four types of nAChR currents in dopaminergic and GABAergic neurons of the substantia nigra and ventral tegmental area, mediated by nAChRs with putative compositions of $\alpha_4\alpha_6\alpha_5(\beta_2)_2$ for the first subtype, $(\alpha_4)_2\alpha_5(\beta_2)_2$ for the second subtype, $(\alpha_4)_2(\beta_2)_3$ or $\alpha_4\alpha_6\alpha_5(\beta_2)_2$ for the third subtype and $(\alpha_7)_5$ for a fourth, less prevalent subtype. These studies were subsequently extended to examine the response of β_2 nAChR subunit knockout mice to cocaine [67], a drug known to condition place preference in mice [68]. In normal mice, the conditioning effect of a low dose of cocaine (5 mg/kg) could be blocked by the nonselective nicotinic antagonist mecamylamine, and the effects were attenuated though not abolished in β_2 knockout mice. Knockout mice also failed to show modulations in dopamine metabolism or upregulation of fos-related antigens in the basal ganglia in response to low-dose cocaine, although these changes were readily demonstrated in normal mice. Overall, these studies reveal that nAChRs containing the β_2 subunit subserve the reinforcing effect of nicotine and augment the response to cocaine; they are also located on dopaminergic neurons, where they facilitate the release of dopamine. By modulation of dopamine release, nicotine receptors are likely to be major players in regulating addictive behavior.

Given that nicotine has antinociceptive and anxiolytic properties and that these same properties are also found in many other addictive substances, it might be expected that β_2 knockout mice, lacking high-affinity nicotine receptors, would show alterations in antinociception and anxiety-like behaviour. Indeed, as discussed below, the antinociceptive properties of nicotine are attenuated in both β_2 knockout mice and α_4 knockout mice, indicating the likely dominant role of $\alpha_4\beta_2$ nAChRs in mediating this effect. In contrast to studies undertaken in α_4 knockouts [54], behavioural analysis of β_2 knockout mice has not shown any alteration in anxiety-like behaviour [59, 69].

Studies of β_2 knockout mice have also clarified some of the mechanisms underlying development of visual pathways, although here the evidence strongly suggests that nAChRs other than the ubiquitous $\alpha_4\beta_2$ nAChRs are primarily involved. In normal mice, self-propagating waves of excitation may be visualised on the retina with calcium-sensitive dyes in late embryonic and early neonatal life. These retinal waves are initially sensitive to blockade by nicotine receptor antagonists, but from about the 10th postnatal day (P10), retinal wave activity becomes resistant to nicotinic antagonists and is instead sensitive to glutamate receptor blockers that preferentially block α-amino-3-hydroxy-5-methyl-4-isoxagolepropionate (AMPA)-type receptors, indicating a shift to glutamatergic mechanisms. In studies of β_2 knockout mice, retinal waves were absent between P1 and P7, and glutamate-mediated waves appeared precociously at P8 [70]. There was a corresponding failure of development of eye-specific layers in the terminal fields of retinal ganglion cells within the lateral geniculate nucleus and superior colliculus, with altered cortical architecture and reduced visual acuity [71]. By contrast, α_4 knockout mice showed normal layering of postretinal visual nuclei, and α_3 knockout mice showed only subtle changes in the spatiotemporal properties of their retinal waves; the waves remained sensitive to nicotinic antagonists [70, 71]. This indicates that nAChRs containing β_2 subunits mediate early postnatal retinal waves and that the likely partners for the β_2 subunit include the α_3 subunit but not the α_4 subunit, with a probable contribution from other subunits such as α_6.

Phenotype of α_4 nAChR knockout mice

The first α_4 nAChR knockout line was generated by Marubio et al. [60] and was used to investigate the identity of subunits involved in nicotine-mediated antinociception. Both α_4 and β_2 knockout lines displayed a dramatically reduced antinociceptive effect of nicotine on the hot-plate test, a test of supraspinal pain pathways. By contrast, the response to nicotine in the tail-flick assay, a test of spinal pain pathways, was only minimally abnor-

mal in knockout mice. This differential effect was also reflected in the finding that neurons from the raphe magnus and thalamus failed to respond to nicotine in patch-clamp recordings, whereas spinal dorsal horn sensory neurons from knockout mice respond to nicotine with a dose-dependent increase in the frequency of post-synaptic currents. This preservation of nicotinic responses in spinal sensory neurons was thought to be attributable to persistent nAChRs composed of α_3 and β_4 subunits.

An independently generated line of α_4 nAChR knockout mice was assessed from a behavioural perspective [54]. Examination of spontaneous motor behaviour in α_4 knockouts revealed significant increases in several components of the behavioural ethogram that characterise normal rodent behaviour during habituation to a novel environment. Relative to wild-type control mice, α_4 knockout mice showed an increase in locomotion, sniffing and total rearing. Furthermore, the behaviour of knockout mice in the elevated plus-maze assay was consistent with increased basal levels of anxiety; the knockout mice made a reduced number of entries into the open, exposed arms of the maze and also spent proportionally less time in the open arms. In response to nicotine, genetically normal mice exhibited early reductions in a number of behaviours (within 15 min of administration); conversely, heightened levels of motor activity in knockout mice were reduced by nicotine significantly later (45 min after nicotine administration). The persistence of nicotine-induced behavioural changes in the absence of the major high-affinity nicotine receptor ($\alpha_4\beta_2$ nAChRs) suggests that the remaining high-affinity nicotine binding sites in the habenulo-interpeduncular system are sufficient to modulate motor activity in actively exploring mice [54].

The same line of α_4 knockout mice has been used to examine the role of nAChR receptors in modifying susceptibility to seizures. Autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE) is an inherited form of epilepsy in humans, characterised by brief seizures during sleep. To date, three mutations in the α_4 nAChR gene (α_4 S248F, α_4 259insL, α_4 S252L) and two mutations in the β_2 nAChR gene (β_2 V287M, β_2 V287L) have been identified in different ADNFLE kindreds [72–77]. All five mutations affect the second transmembrane domain of the involved subunit, suggesting a disruption of channel function, but studies in oocyte expression systems have produced conflicting impressions of the likely effect of these mutations in vivo. When mutated α_4 subunits have been coexpressed with normal β_2 subunits, the resultant receptors have generally shown reduced current flow, decreased sensitivity to ACh, increased desensitisation or combinations of these defects, leading to overall hypofunction of the receptors [72, 73, 78–80]. Figl et al. [81], however, reported use-dependent potentiation of the nicotinic response with two of the α_4 mutations (α4 S248F, α_4 259insL), and characterisation of the two known β_2 mutations in oocyte systems has showed increased affinity for ACh, in the case of V287M, and delayed desensitisation, in the case of V287L, leading to increased current flow [76, 77]. Thus, it remains unclear whether ADNFLE is associated with hypofunction or hyperfunction of $\alpha_4\beta_2$ nAChRs in vivo. Given that $\alpha_4\beta_2$ nAChRs have been implicated in the facilitation of a number of neurotransmitters, plausible hypotheses can be generated to support either of these two possibilities: hypofunction of $\alpha_4\beta_2$ receptors could lead to a deficit in the usual facilitation of GABA release, while hyperfunction of these receptors could inappropriately augment glutamate release.

In view of these observations, α_4 knockout mice were observed for signs of spontaneous seizures and were compared with wild-type controls in response to a range of proconvulsants, including pentylenetetrazole, bicuculline, kainic acid, strychnine and 4-aminopyridine. Despite prolonged observation of α_4 knockout mice, with and without electroencephalographic monitoring [82], no spontaneous seizures were observed. Following injection with the GABA receptor antagonists pentylenetetrazole or bicuculline, however, α_4 knockout mice showed a significant excess of major motor seizures and death, relative to wild-type controls [83], and a shift in their electroencephalographic phenotype away from hypokinetic, absence-like seizures towards more severe convulsive seizures [82]. Mutant mice also had an increase in kainic acid and strychnine-related minor motor seizures, but, paradoxically, the seizure response to 4-aminopyridine was reduced [83]. This agent causes seizures by stimulating the release of endogenous glutamate [84], and the decreased response in α_4 knockouts suggests that the mutation causes a compensatory downregulation of glutamatergic neurotransmission [83]. Overall, these studies suggest that in mice, intact α_4 subunits provide partial protection against seizures induced by the GABA receptor blocking agents pentylenetetrazole or bicuculline. Although this finding supports the hypothesis that $\alpha_4\beta_2$ hypofunction might predispose to seizures, a recently published functional study undertaken on mutated nAChR subunits expressed in *Xenopus* oocytes [85] suggested that the single common denominator in ADNFLE mutations might be hyperfunction of the $\alpha_4\beta_2$ receptor. In this study, mutations identified in the α_4 nAChR subunit (S248F, L776ins, S252L) and β_2 nAChR subunit (V287M) of ADNFLE patients were coexpressed with control DNA. All mutations were associated with increased sensitivity to the endogenous ligand, acetylcholine, although an increase in rapid desensitisation was also seen for some mutations. This study represents a refinement of previous approaches because expression of the normal and mutant allele in the same cell more accurately reproduces the heterozygous state seen in ADNFLE patients. Unlike previous expression studies that provided conflicting results, this data suggests that hy-

perfunction of the receptor may be responsible for ADNFLE, although the definitive experiment in which the human mutations are introduced into an animal model has yet to be published. Only transgenic mice can hope to reproduce the complex subunit interactions that most likely exist in the brain of sufferers of ADNFLE.

This line of α_4 nAChR knockout mice has also been used to examine the potential role of the α_4 nAChR subunit in nicotine-mediated neuroprotection in an animal model of Parkinson's disease [86]. The potential for such a neuroprotective effect has been suggested by epidemiological evidence that cigarette smoking may be protective for Parkinson's disease [87, 88]. The study examined the effects of acute nicotine pretreatment upon the extent of nigrostriatal neurodegeneration induced by methamphetamine in wild-type and α_4 knockout mice. The loss of dopaminergic nerve terminals was assessed by autoradiography with [^{3}H]-mazindol, a molecule that binds to dopamine transporter sites on dopaminergic terminals in the striatum. In wild-type mice but not knockout mice, acute nicotine treatment produced significant inhibition of methamphetamine-induced neurodegeneration. The same study confirmed the neuroprotective effects of nicotine in a rat 6-hydroxydopamine model of nigrostriatal degeneration. The results suggest that receptors containing the α_4 nAChR subunit may mediate the observed neuroprotective effect of nicotine upon Parkinsonian-like damage in vivo.

The interaction between the α_4 subunit and dopaminergic systems has also been explored by other investigators, using transgenic mice with a leucine-to-serine point mutation in a critical residue within the second transmembrane domain of the α_4 nAChR subunit (L9'S knockin). This mutation results in increased sensitivity of $\alpha_4\beta_2$ receptors to agonists. Even in the hemizygous state the knockin mutation results in dramatic late embryonic loss of midbrain dopaminergic neurons [89]. These mutants fail to feed and die in the first postnatal day. The cell death was possibly due to the persistent activation of nAChRs by circulating choline, which in low concentrations was shown to be an agonist at mutant $\alpha_4\beta_2$ nAChRs but not at normal $\alpha_4\beta_2$ receptors. A related strain of mice that retained the neomycin resistance cassette within an upstream intron had reduced expression of the mutated receptor and heterozygotes of this strain were viable, though homozygote mice died within 24 h of birth. Detailed behavioural examination of the neo-intact heterozygous mice revealed transient hyperactivity in a novel environment. This hyperactivity was decreased by doses of nicotine that had no effects on wild-type mice, consistent with the heightened sensitivity of the mutated receptors. Heterozygous neo-intact mutants also failed to show normal improvement in a rotarod test of motor learning, and they displayed increased anxiety-like behaviour, as assessed by a reduction in the number of entries and time spent in the open arms of the elevated plus maze. The mirrored chamber test also revealed an excess of anxiety-like behaviour. These findings are difficult to reconcile with previous observations that complete absence of α4 subunits also results in heightened anxiety-like behaviour [54], but these disparate results suggest that there may be a narrow range of activity of $\alpha_4\beta_2$ receptors that is appropriate for regulation of behaviour and that deviations to either side of this range may produce similar deficits.

Heterozygous neo-intact α_4 L9'S mutants were challenged with low-dose amphetamine (5 mg/kg) at 3 and 11 months [89]. Mutants at 11 months showed a significant reduction in amphetamine-induced locomotion compared with younger mice of the same genotype, whereas wild-type mice showed a lesser reduction with advancing age. Although the authors attributed the locomoter slowing to a reduction in nigrostriatal functioning with age, direct evidence in the form of nigral cell counts was not provided. Coupled with the observed degeneration of dopaminergic neurons in neo-deleted L9'S mice, however, a compelling case can be made that overactivity of the α_4 nAChR subunit is detrimental to dopaminergic neurons. In summary, it appears that activation of the α_4 nAChR subunit with exogenous agonists can prevent nigral cell loss, as seen in Parkinson's disease, but excessive nonphysiological levels of activation of α_4 nAChRs can also be damaging. Again, as suggested by anxiety-like behaviour in α_4 knockout mice and by conflicting theories of the basis of ADNFLE, it appears that there may be an optimal range of $\alpha_4\beta_2$ activity and that deviations outside this range in either direction are detrimental to neural function.

Phenotype of α_3 nAChR knockout mice

Mice lacking the α_3 nAChR subunit have been generated by targeted deletion of exon 5 [90], and Northern blot experiments confirmed the lack of α_3 nAChR subunit transcripts. The null mutation was not associated with increased intrauterine mortality, but about 40% of knockout mice died in the first 3 days of life, with the remainder dying within 2 months of weaning. The precise cause of death was not known, but knockout mice had enlarged urinary bladders, with evidence of urinary sepsis, bladder calculi and chronic urinary dribbling. Physiological studies on bladder strips derived from knockout mice demonstrated a lack of contractility in response to nicotine, whereas the muscarinic agonist carbamoylcholine (coadministered with the ganglionic blocker hexamethonium given to block intramural parasympathetic ganglia) caused a dose-dependent increase in contractility, confirming that the postganglionic neuron and neuromuscular synapse was normal. Knockout mice also showed widely dilated, nonreactive

pupils. Patch-clamp recordings on superior cervical ganglia from neonatal mice (a sympathetic ganglion that mediates pupillary dilation) confirmed that the autonomic ganglia in these mice showed a defective response to nicotine, although it might have been more relevant to study the ciliary ganglion, as this ganglion mediates pupillary constriction and the knockout phenotype suggests a failure of ciliary function. The study was significant in that it confirmed the pivotal role played by the α_3 nAChR subunit in transmission at autonomic ganglia. The megacystis-microcolon-intestinal hypoperistalsis syndrome is a rare disease of childhood that presents early with impaired intestinal peristalsis, hydronephrosis and hydroureters. Although readily detectible in control tissues, α_3 nAChR subunit mRNA was not found in tissues taken from patients with the clinical syndrome [91]; however, unlike the α_3 nAChR subunit knockout mice, patients with this syndrome do not show a failure of pupillary constriction.

Detailed autoradiographic studies undertaken in α_3 nAChR subunit knockout mice identified the brain regions where this subunit is likely to make contributions to functional nicotinic receptors [92]. On the basis of heterologous expression studies, α-conotoxin MII (αCtxMII) was previously thought to bind preferentially to native $\alpha_3\beta_2$ receptors, and A855380-resistant epibatidine binding is thought to identify native $\alpha_3\beta_4$ receptors [30, 38]. Surprisingly, binding of [^{125}I] αCtxMII in α_3 knockout mice was largely preserved, although reductions were found in the habenulointerpeduncular tract, suggesting that this is a major site of $\alpha_3\beta_2$ receptors and that other α subunits must contribute to αCtxMII binding elsewhere. Indeed, as discussed below, α_6 knockout mice have been used to show that α_6 nAChRs subunits make a substantial contribution to αCtxMII binding [45]. As expected, A855380-resistant [^{125}I] epibatidine binding was severely reduced in α_3 knockout mice, with major reductions seen in the habenulointerpeduncular tract, the inferior colliculus, the medial vestibular nucleus and the prepositus hypoglossal nucleus.

Phenotype of β_4 knockout and β_2/β_4 nAChR double-knockout mice

Like β_2 nAChR subunit knockout mice, β_4 knockout mice grow to adulthood with no gross behavioural or anatomical abnormalities. Minor histological abnormalities, in the form of focal mucosal hyperplasia or dysplasia with an increase in the incidence of mitotic figures, were seen in a small number of β_4 knockout mice examined [93]. In contrast, β_2/β_4 nAChR double-knockout mice had a phenotype similar to the α_3 nAChR knockout, with impaired growth and increased perinatal mortality. They also had enlarged bladders, dribbling urination, bladder stones and chronic urinary infection with both Gram-positive and Gram-negative bacteria. Their pupils were dilated and failed to constrict to light. ACh-activated whole-cell currents were not detectible in superior cervical ganglion neurons from β_2/β_4 nAChR double-knockout mice and were reduced in β_4 nAChR knockout mice. A defect in nicotinic transmission was confirmed in parasympathetic intramural ganglia by examination of bladder strips. As for α_3 nAChR knockouts, bladder strips from double-knockout mice were still able to contract when stimulated with a muscarinic agonist or an electric field, indicating a selective nicotinic deficit rather than a loss of intrinsic contractility. In summary, major defects were seen in both sympathetic and parasympathetic autonomic ganglia in β_2/β_4 nAChR double-knockout mice. Although careful studies demonstrated subtle physiological deficits in β_4 nAChR single-knockout mice, the presence of a functional β_2 nAChR allele was able to prevent pathologically significant effects. Recalling the subtle phenotype of β_2 single-knockout mice, which was characterised by alterations in learning and in nicotine-mediated antinociception but not by altered viability, it appears that intact genes for either the β_2 or β_4 subunit are sufficient to produce viable mice with essentially normal autonomic function, suggesting a degree of functional redundancy in β subunits expressed in autonomic ganglia.

Phenotype of α_6 nAChR knockout mice

Mice homozygous for the targeted deletion of the α_6 nAChR allele were neurologically normal, with no apparent changes in body size, fertility, locomotion or brain histology of major nuclei [45]. Immunohistochemistry, in situ hybridisation and ligand autoradiography for a large number of dopaminergic markers in the striatum and substantia nigra were also normal. In addition, no changes were seen in the steady-state levels of α_3, α_4, α_5, α_7, β_2 and β_4 mRNA. The most significant finding in the brain of α_6 knockout mice was a global loss of high-affinity [^{125}I] αCtxMII binding and a decrease in high-affinity binding for [^{3}H] nicotine, [^{3}H] epibatidine and [^{3}H] cytisine in the superior colliculus and lateral geniculate nucleus, sites constituting retinal ganglion cell relay nuclei. [^{125}I] αCtxMII binds strongly to structures in the visual system (retina, superior colliculus, olivary pretectal nucleus, geniculate nucleus) [92, 94], the dopaminergic system (including the substantia nigra and its projections in the nucleus accumbens, caudate-putamen and lateral habenula) and the habenulo-interpeduncular system. This result, together with a lack of major changes in [^{125}I] αCtxMII binding identified in α_3 nAChR knockout mice [92] and prior studies showing that αCtxMII partially inhibits nicotine-induced dopamine release in striatal synaptosomes [94–97] and

nicotinic currents in dopaminergic neurons [66], provides strong support for the notion that α_6 rather than α_3 preferentially combines with β_2 in dopaminergic neurons, and it is this receptor configuration that may play a major role in nicotinic modulation of dopaminergic neurotransmission.

Phenotype of α_7 nAChR knockout mice

Mice lacking the α_7 nAChR subunit were found to be viable and fertile, with no obvious physical or neurological deficit [98]. As expected, knockout mice had normal high-affinity [^{3}H] nicotine binding but lacked [^{125}I] α-bungarotoxin binding, which is prominent in the hippocampus, amygdala and neocortex of control mice. The brain of α_7 knockouts appeared to be structurally normal, with no histological deficits encountered in the hippocampus or primary somatosensory cortex and no evidence of glial cell proliferation to suggest that neurodegeneration was occurring. Electrophysiological evaluation of cultured neonatal hippocampal cells from normal mice demonstrated methyllycaconitine-sensitive, rapidly desensitising nicotine-evoked currents, whereas application of high concentrations of nicotine failed to elicit a response in cells derived from α_7 knockouts.

At high doses, nicotine produces seizures, and previous studies, undertaken in inbred strains of mice, have suggested that sensitivity to nicotine-induced seizures may relate to the density of α-bungarotoxin binding sites in the hippocampus. Given that most α-bungarotoxin binding corresponds to homomeric α_7 pentamers, it was hypothesised that this subunit might be an important mediator in nicotine-induced seizures. Surprisingly, however, when this hypothesis was tested in α_7-knockout mice, there was no change in the dose of nicotine required to produce seizures [99]. This observation was not explained by compensatory changes in steady-state levels of α_4, α_5, α_6, α_7, β_2 and β_4 transcripts.

Findings in another mutant strain, however, have suggested a potential role of the α_7 subunit in nicotine-induced seizures after all [100]. Although a lack of α_7 nAChR subunits does not protect against nicotine-induced seizures, a hyperfunctioning mutation appears to increase sensitivity to nicotine-induced seizures [100]. A gain-of-function mutant was produced with a leucine-to-threonine missense mutation at position 250 of the channel domain (α_7 L250T). Mice homozygous for this mutation die shortly after birth, whereas heterozygous mice grow to adulthood and appear to have normal behaviour under basal conditions. In response to nicotine, however, the heterozygous α_7 L250T mice showed a significantly greater number of generalised tonic clonic seizures than control mice. They were also reported as showing unique, stereotyped movements in response to nicotine, consisting of head bobbing and paw tapping, which were never seen in wild-type mice. Although these movements were described as stereotypical in nature and interpreted by the authors as possibly showing a role of the α_7 subunit in locomotion, electroencephalographic characterisation would be required to rule out the possibility that they are a form of seizure activity.

The cause of neonatal death in homozygous L250T knockin mice (T/T) is not clear. Homozygous T/T mice showed a marked reduction in α_7 nAChR protein levels and extensive apoptotic cell death throughout the somatosensory cortex [98]. Apoptosis was thought to be due to increased Ca2+ influx, which is plausible given that the α_7 homopentamer is normally more permeable to calcium than the heteromeric nAChRs [6]. It was noteworthy that there was no correlation between the sites of normal high expression of the α_7 nAChR protein and the sites of apoptosis. The hippocampus and olfactory bulb of T/T mice, which exhibit high levels of α_7 nAChR transcripts and [^{125}I] α-bungarotoxin binding in normal mice, showed lower levels of apoptosis compared with the somatosensory cortex. This suggests that compensatory developmental factors unrelated to absolute levels of α_7 nAChR transcription may be at play.

The role of the α_7 nAChR subunit in autonomic control has also been studied using α_7 knockouts [101]. Autonomic ganglia express a number of nAChR subunits [102, 103]. The α_7 knockout mice had impaired baroreceptor-mediated sympathetic responses (measured as an increase in heart rate) to sodium nitroprusside-induced vasodilatation, and there was corresponding evidence of denervation supersensitivity when sympathetic agonists were applied to isolated cardiac muscle. The defect in sympathetic activity was found not to be due to impaired availability of noradrenaline in sympathetic nerve terminals. Further control studies showed that parasympathetic function (measured as a bradycardic response to vasoconstrictor drugs) was normal in knockout mice. This study suggests that functional α_7 nAChR subunits containing monomeric receptors are likely to be major players in the regulation of sympathetic activity but not of parasympathetic activity in the heart.

Phenotype of α_9 nAChR knockout mice

Cochlear hair cells express the α_9 nAChR subunit [5] and are innervated by cholinergic efferent fibres originating from the brainstem superior olivary complex. Targeted deletion of the α_9 nAChR subunit gene resulted in mice with abnormal efferent connections to the cochlea [104]. In knockout mice, a single large terminal instead of multiple smaller synapses innervated individual hair cells. Furthermore, efferent fibre stimulation in knockout mice failed to cause suppression of cochlear responses, con-

firming that a functional α_9 nAChR gene is essential for brainstem regulation of cochlear sensory input. Despite these anatomical changes, the α_9 nAChR subunit knockout mice had no obvious abnormalities in audition, balance or coordinated locomotion.

Conclusion

Recent developments in transgenic technology have resulted in the generation of knockout and knockin mice, which have greatly advanced understanding of the functional role of neuronal nicotinic receptors. Although functional forms of some subunits or subunit combinations are vital for survival, the majority of others appear to mediate modulatory effects on a number of processes, including learning and memory, locomotor behaviour, anxiety, nociception, dopaminergic neurotransmission, seizure threshold, development of the visual system and autonomic function. As subunit-specific agonists and antagonists are not available, these transgenic lines have been pivotal in clarifying the in vivo role played by a large number of nicotinic receptor subunits. Despite the strengths of this approach, it should also be noted that some caution is required in interpreting the results of studies in transgenic animals. As many of the subunits are expressed during embryogenesis, there is a possibility that developmental compensation may occur either in the expression of other nAChR subunits or in related neurotransmitters. Compensatory changes are seen in dopamine receptor and dopamine transporter knockout mice [105]. It is interesting to note, however, that no changes were seen, at least at the level of transcription, in the expression of other nAChR subunits in α_4 nAChR [54, 60], β_2 nAChR knockout mice [59] and α_6 nAChR [45]. The relative expression of individual subunits needs to be reevaluated at the protein level. In addition, there are acknowledged behavioural differences between mouse strains [106]. This is an important caveat in interpretating the behavioural phenotype of individual knockout lines in general and is especially relevant for experiments described in this review, as there was a broad spectrum of background strains and ancestral embryonic stem cells used in generating the transgenic models. Another practical limitation that arises when transgenic animals are used to study receptor function is that mutations, unlike receptor agonists and antagonists, cannot be washed in and out of an in vitro physiological preparation to provide a direct before-and-after comparison within the same monitored cell. Nonetheless, transgenic animals offer a unique opportunity to observe the function of specific genes in the complex environment of a living organism, and transgenic techniques usefully complement traditional approaches. Future research trends will involve the generation of transgenic mice with multiple nAChR subunit gene ablations and inducible knockouts. The ability to inactivate genes in specific locations at specific times will greatly advance our understanding of the in vivo role of individual nAChR subunits.

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