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Nicotinic acetylcholine receptor knockout mice as animal models for studying receptor function

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Abstract

Nicotinic acetylcholine receptors are pentameric ligand-gated ion channels, which are involved in a wide range of neuronal functions. During the past decade, a large number of nicotinic acetylcholine receptor subunits have been cloned and showed a discreet yet overlapping distribution pattern. Recently, several groups have produced mutant mice lacking specific nicotinic acetylcholine receptor subunits. In this review, we focus on how the study of these knockout mouse models has advanced our understanding of the role individual nicotinic acetylcholine receptor subunits play in the function and composition of endogenous receptors and the diverse pharmacological actions of nicotine in the mammalian nervous system.

Keywords: Nicotinic acetylcholine receptor

1. Introduction

Nicotinic acetylcholine receptors are expressed in muscle, the central nervous system (CNS) and the peripheral nervous system. Specific agonists of these receptors, such as nicotine, exert diverse cellular and behavioural effects, which include addiction, cognitive enhancement, antinociception, and seizure induction. Pharmacological experiments with nicotinic receptor agonists and antagonists have helped to elucidate the function of acetylcholine and nicotinic acetylcholine receptors in the CNS and the peripheral nervous system. However, selective ligands for the multiple isoforms of neuronal nicotinic acetylcholine receptors are still scarce. A recent approach, which may overcome this limitation, involves genetic manipulations that result in ''knockout mice'' with genomic null mutations in the specific genes encoding for receptors, thus, in essence, providing a highly selective, albeit irreversible, ''antagonist''. Although there are some inherent problems with this approach (developmental requirements or compensatory effects, for example), these experiments provide new insight into the pharmacology and functional role of neuronal receptors in complex neurobiological systems. In this review, we focus on knockout mice lacking a nicotinic acetylcholine receptor subunit that have allowed a molecular dissection of nicotinic acetylcholine receptor subtypes in the CNS and that have led to the identification of particular nicotinic acetylcholine receptor subunits involved in nicotine-elicited behaviours in addition to being used as models of several human pathologies.

2. Using knockout mice as models

Since the pioneering work of Thomas and Capecchi (1987) on homologous recombination in mouse embryonic stem cells, the generation of knockout mice has become a powerful tool in the neuroscience field. This technology involves embryonic stem cells grown in vitro and genetically modified by the substitution of a nonfunctional copy of a given gene. These engineered embryonic stem cells are then injected into mouse blastocysts and re-implanted in a host mother's uterus. Some of the resulting chimeric mice contain the mutated embryonic stem cells in the germ cell layer, thus allowing the mutation to be passed onto the next generation. The resulting heterozygote mice can then be inter-bred to produce homozygous mutant knockout mice (Orr-Urtreger et al., in press; Capecchi, 1989).

In the past 5 years, the deletion of ligand-gated ion channels in mice has provided a model system for human pathologies, such as epilepsy ($GABA_A$ β3 receptor knockouts) (Homanics et al., 1997), or neuropsychiatric disorders, such as memory impairments (NMDA 1A receptor knockouts) (Tsien et al., 1996), learning defects (NMDA

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Table 1
Functional abnormalites following nicotinic acetylcholine receptor gene deletion
Abbreviations used: ACh; acetylcholine: SCG; superior cervical ganglion: DA; dopamine.

Nicotinic acetylcholine receptor subunit	Cellular/morphological phenotype	Behavioural phenotype	Survival	Refs.
α3	Autonomic nervous system defects; megacystis, mydriasis, and altered ACh responses in SCG neurons		1–8 weeks postnatal	Xu et al. (1999)
α4	Loss of high affinity nicotine binding	Reduced nicotine-elicited antinociception	Adult	Marubio et al. (1999)
α7	Lack of rapidly desensitizing nicotinic currents in hippocampal neurons	Normal baseline responses	Adult	Orr-Urtreger et al. (1997), Paylor et al. (1999)
α7 L247T	Slowly desensitizing nicotinic currents in hippocampal neurons		24-h postnatal	R. Broide (personal communication)
α9	Abnormal efferent innervation of hair cells in the cochlea	Suppression of cochlear responses	Adult	Vetter et al. (1999)
β2	Altered agonist sensitivity; Loss of presynaptic receptor activity in GABAergic terminals, high affinity nicotine binding, nicotine-induced DA release, and presynaptic receptor activity; Accelerated ageing	Enhanced passive avoidance responses; Loss of nicotine self-administration activity; Altered learning in aged animals; Reduced nicotine-elicited antinociception	Adult	Picciotto et al. (1995), Picciotto et al. (1998), Zoli et al. (1998), Lu et al. (1998)
β4	Reduced nicotine-elicited current in SCG neurones		Adult	Xu et al. (1999)
β2/β4	Autonomic nervous system defects: enlarged bladder and dilated ocular pupils		Increased mortality	Xu et al. (1999)
ε	Abnormal development of motor endplates		8–14 weeks postnatal	Witzemann et al. (1996), Missias et al. (1997)

2A receptor knockouts), or substance abuse (β2 nicotinic acetylcholine receptor knockouts) (Picciotto et al., 1998). In addition, the role a neurotransmitter receptor plays in cellular and pharmacological functions can be revealed, such as in the modification of long-term potentiation or depression in the hippocampus (NMDA 2B receptor knockouts) (Kutsuwada et al., 1996), (NMDA 2A receptor knockouts) (Sakimura et al., 1995). In some cases, the specificity of pharmacological compounds has been demonstrated (β2 nicotinic acetylcholine receptor knockouts) (Zoli et al., 1998), ($GABA_A$ β6 receptor knockouts) (Makela et al., 1997). Knockouts of nicotinic receptor subunits provide model systems for investigating the endogenous role of nicotinic acetylcholine receptors and the diverse effects of nicotine in the nervous system (Table 1).

3. Knockout of muscle nicotinic acetylcholine receptor subunits

In humans, congenital myasthenic syndromes are heterogeneous disorders associated with point mutations in muscle α1, β1, or ε nicotinic acetylcholine receptor subunits. Many of these mutations were found in the ε subunit resulting in either nonfunctional receptors or ones with altered allosteric properties. Symptomatic severe muscular weakness occurs only when a mutation affecting channel function or assembly is combined with a null mutation or when null mutations are expressed on both alleles (Ohno et al., 1996; Ohno et al., 1997). Thus, knockout mice that lack the ε nicotinic acetylcholine receptor subunit provide an animal model for congenital myasthenic syndrome.

During development, subunit composition changes occur at the neuromuscular junction, which are important for the structural and functional maturation of the synapse. A substitution of the adult ε subunit for the fetal γ subunit in the nicotinic acetylcholine receptor ($\alpha 2\beta\delta\gamma$ becomes $\alpha 2\beta\delta\varepsilon$) (Mishina et al., 1986; Gu and Hall, 1988) coincides with changes in the biophysical properties of the acetylcholine-gated channels at motor endplates: the mean channel conductance increases by approximately 50%, the Ca^{2+} permeability increases, and the mean open times of the channels decrease (Fischbach and Schuetze, 1980; Siegelbaum et al., 1984; Villarroel and Sakmann, 1996). At the transcriptional level, the ε subunit gene is activated postnatally while the γ subunit gene is down-regulated (Martinou and Merlie, 1991; Witzemann et al., 1996).

The functional significance of this developmental subunit switch was investigated using an ε nicotinic acetylcholine receptor subunit gene knockout. Similar to human patients, myasthenic symptoms were observed only in homozygous mutant mice. They appeared normal up to 1 month of age after which, unlike in their wild-type littermates, atrophy of skeletal muscle began concomitant with a general weakness and cessation of body weight gain.

At the molecular level, this developmental γ to ε subunit switch is partially responsible for the down-regulation of the γ subunit. In wild-type animals, the γ subunit is virtually undetectable 2 weeks after birth. In mutant mice, on the other hand, muscles retain the same levels of the γ subunit from P5 to P12 and, thereafter, there is a decrease in γ subunit labelling; yet, it is still immunodetectable until P74, the oldest age examined (Missias et al., 1997) The distribution of the decreased, albeit persistent, expression of receptors at the motor endplate was different in the knockout and wild-type mice. Motor endplates appear morphologically similar at low magnification, however, nicotinic acetylcholine receptors decrease in density as mutant endplates continue to grow without a net increase of receptor number (Witzemann et al., 1996; Missias et al., 1997). Electrophysiological recordings in muscle demonstrated early postnatal-like miniature endplate currents (mEPC) with longer time constants in 2-week-old mutant animals, whereas wild-type animals displayed adult-like mEPCs with shorter time constants. Accordingly, ε knockout mice maintain functional γ subunit-containing nicotinic acetylcholine receptors at the neuromuscular junction. The lack of adult ε subunit nicotinic acetylcholine receptors was partially, but not sufficiently, compensated by the prolonged maintenance of the fetal nicotinic acetylcholine receptor subunit demonstrating the requirement of the ε subunit to achieve a functional adult motor endplate.

4. The pharmacology of neuronal nicotinic acetylcholine receptors revealed using knockout mice

Neuronal nicotinic acetylcholine receptors are pentameric proteins encoded by a large multigene family consisting of at least seven α subunit (α2–8) and three β subunit (β2–4) genes, which are expressed in discreet yet overlapping patterns. In heterologous expression systems, such as *Xenopus* oocytes, neuronal receptors are capable of forming either functional homopentamers (α7 or α8), which are α-bungarotoxin (α-BTX)-sensitive or heteropentamers most likely comprised of two α subunits (α2, α3, α4, or α6) and three β subunits (β2 or β4) forming α-BTX-insensitive receptors (Couturier et al., 1990; Sargent, 1993; McGehee and Role, 1995) The α5 subunit can associate with α4/β2 or α3/β4 subunits and modify the functional properties of these receptor complexes (Conroy et al., 1992; Ramirez-Latorre et al., 1996; Wang et al., 1996) The β3 subunit is likely to function in a similar manner based on its sequence homology to the α5 subunit, but this has not yet been demonstrated (Le Novère et al., 1996). The endogenous subunit composition of nicotinic acetylcholine receptors is still not known. Patch clamp recordings in brain slices reveal a complex receptor pharmacology with often more than one type of receptor expressed in a single neuronal cell type (Alkondon and Albuquerque, 1993). In addition, both the rank order of potencies of nicotinic receptor agonists and the single channel conductance of nicotinic acetylcholine receptor in vivo do not always coincide with the values found in *Xenopus* oocytes (McGehee and Role, 1995).

An alternative approach to identify the diverse species of nicotinic acetylcholine receptors is to examine, using either electrophysiological or autoradiographic techniques, the nicotinic acetylcholine receptors that remain in knockout mice. Four groups of receptors have been identified in this way (Zoli et al., 1998) and have extended the existing classification described using other methods (Alkondon and Albuquerque, 1993) (Table 2).

Type 1 receptors are α-BTX-sensitive and are composed of α7 nicotinic acetylcholine receptor subunits in the mammalian CNS. Knockout mice deficient in the α7 nicotinic acetylcholine receptor subunit no longer contain [^{125}I]α-BTX binding sites and, in addition, have no differences in the high-affinity [^{3}H]Nicotine binding sites relative to wild-type littermates (Orr-Urtreger et al., 1997). Other subunits, such as the α4 and β2 nicotinic acetylcholine receptor subunits, do not appear to contribute to Type 1 receptors since neither β2 nor α4 knockout mice show differences in [^{125}I]-α-BTX binding when compared to their wild-type littermates (Zoli et al., 1998; Marubio et al., 1999). Furthermore, α7 mutant mice completely lack a detectable, inward, rapid, nicotine-elicited current in hippocampal cells (which in wild-type mice is blocked by methyllycaconitine), further implicating the α7 subunit's participation in the formation of Type 1 receptors in the CNS (Orr-Urtreger et al., 1997).

Type 2 receptors represent the majority of α-BTX-insensitive nicotinic acetylcholine receptors in the CNS and contain either α2/β2 or α4/β2 subunits. A high potency of epibatidine, a less potent response to nicotine and dimethylphenylpiperazinium and a weak effect of cytisine are the hallmark characteristics of these receptors initially described in cultured rat hippocampal neurons (Alkondon and Albuquerque, 1993). In wild-type mice autoradiography of brain slices incubated with [^{3}H]Epibatidine and [^{3}H]Nicotine reveal high-affinity sites in most brain regions with a high level of binding in the thalamus, a moderate level of binding in the cortex and a low level of binding in the hippocampus. In contrast, [^{3}H]Epibatidine and [^{3}H]Nicotine binding are no longer detectable in these regions in neither β2 nor α4 knockout mice (Zoli et al., 1998; Marubio et al., 1999). The comparison of binding data obtained in β2 and α4 knockout mice suggests that the vast majority of [^{3}H]Epibatidine and [^{3}H]Nicotine binding in the brain contains both α4 and β2. Some binding present in α4 but not β2 knockout mice demonstrate, indeed, that minor populations of non-α4/β2-containing receptors are present in the interpeduncular nucleus, the superior colliculus and in the substantia nigra. Based on in situ hybridization studies (Wada et al., 1989; Le Novère et

Table 2
Classes of nAChRs revealed using knockout and wild-type mice
α-BTX = α-bungarotoxin, MLA = methyllycaconitine, CNS = central nervous system, EPI = epibatidine, NIC = nicotine, CYT = cytisine, MCC = methylcarbamylcholine, ACH = acetylcholine, DHβE = dihydro-β-erythroidine, MCA = mecamylamine.
Reproduced with permission from Zoli et al. (1998).

Receptor class	Putative composition	Predominant localization in central nervous system	High affinity binding at equilibrium	Pharmacology in slices
Type 1	α7	Cortex and limbic areas	α-BTX	α-BTX and MLA-sensitive rapid desensitisation
Type 2	β2-α4-(α5?) β2-(α2?) β2-(α3?) β2-(α6-β3?)	Throughout the central nervous system Interpeduncular nucleus Hippocampus Catecholaminergic nuclei Superior colliculus	EPI > NIC = CYT = MCC = ACH	MLA-insensitive,
Type 3	β4-α3-(α5?)	Medial habenula, interpeduncular nucleus, dorsal medulla	EPI	MLA-insensitive, CYT = NIC, DHβE < MCA slow decay at 100 μM NIC
Type 4	(β4-α4?) (β4-α2?)	Lateral medial habenula Dorsal interpeduncular nucleus	EPI > CYT > MCC = ACH	MLA-insensitive, CYT = NIC, DHβE < MCA fast decay at 100 μM NIC

al., 1996), these binding sites are most likely formed by α2/β2-containing receptors in the interpeduncular nucleus and α3-or α6/β2-containing nicotinic acetylcholine receptors in the substantia nigra (Zoli et al., 1998; Marubio et al., 1999).

Autoradiography experiments also reveal Type 3 nicotinic acetylcholine receptors. This group does not contain α4, α7, or β2 subunits, and binds [^{3}H]Epibatidine with high affinity, but not cytisine or nicotine in equilibrium binding experiments. The interpeduncular nucleus, medial habenula, fasciculus retroflexus, area postrema, nucleus tractus solitarii, and dorsal motor nucleus of the vagus nerve all retain [^{3}H]Epibatidine binding sites in both the α4 and β2 homozygous mutant mice. Patch clamp recordings in the medial habenula and the dorsal motor nucleus of the vagus of β2 knockout mice showed an agonist rank order of potency of epibatidine ≫ nicotine = cytisine = dimethylphenylpiperazinium (Picciotto et al., 1998), which is consistent with α3β4 receptors expressed in *Xenopus* oocytes (Luetje and Patrick, 1991). Moreover, the distribution of Type 3 binding correlates well with the mRNA distribution of α3 and β4 subunits further suggesting an α3/β4 subunit composition for Type 3 receptors.

Type 4 receptors can be found in the dorsal cortex of the inferior colliculus, the dorsal tegmentum of the rostral medulla oblongata, the medial habenula, and the interpeduncular nucleus. Like Type 3 receptors, Type 4 receptors do not contain the β2 nicotinic acetylcholine receptor subunit, bind [^{3}H]Epibatidine with high affinity, and bind [^{3}H]Nicotine with low affinity. In contrast, they bind [^{3}H]Cytisine with a high affinity and desensitize faster than Type 3 receptors recorded in the medial habenula. The putative subunit composition of Type 4 receptors may be α4/β4 or α2/β4 (Wada et al., 1989; Dineley-Miller and Patrick, 1992; Zoli et al., 1998).

Therefore, the available data on nicotinic acetylcholine receptor subunit knockout mice confirm that at least four classes of neuronal nicotinic acetylcholine receptors in the mouse brain can be distinguished by their binding properties, distribution and cellular response to nicotinic receptor agonists. The precise contribution of other nicotinic acetylcholine receptor subunits, such as α2, α3, α5, α6, β3, and β4, remains to be elucidated with the appropriate knockout mice.

5. Behavioural analysis of knockout mice

Simple or complex behaviour in animals can be analyzed at several different levels: (1) the system level (sensory or motor, for example); (2) the network level concerning the interactions between individual neurons; and (3) the molecular level involving the molecules, which contribute to intercellular signalling. At any point along the way, the absence or modification of a critical component (in the absence of a compensatory element) results in

an alteration of behaviour. Thus, knockout mice provide useful models for investigating the contribution of a given molecule in a specific behaviour.

Nicotine administration improves information acquisition and memory retention in rodents and information processing in humans (Levin, 1992). Therefore, the cognitive effect of nicotine in the $\beta 2 -/-$ mice was examined using the passive avoidance test. This test measures an animal's latency to perform a highly probable response (in this case, entry from a well-lit chamber into an adjacent dark chamber) for which it had been previously punished during the training session. The β2 knockout mice tested for the retention of an avoidance response 24 h later showed marked differences from their wild-type littermates. Low doses of nicotine (0.01 mg/kg) increased the latency of entry into the dark chamber significantly in wild-type mice, but did not change the performance of $\beta 2 -/-$ mice. Thus, β2-subunit-containing receptors are an important component in mediating this effect of nicotine. Interestingly, the latency of entry into the dark chamber was significantly longer in vehicle-injected mutant mice than in their wild-type littermates, suggesting that β2-containing nicotinic acetylcholine receptors mediate the endogenous actions of acetylcholine in this behaviour.

6. Nicotine addiction

The reinforcing properties of many drugs of abuse, such as cocaine, ethanol, amphetamine and nicotine, are thought to be principally mediated by their interactions with the mesotelencephalic dopaminergic system. Nicotine, administered systemically, acts by binding to nicotinic acetylcholine receptors on either the cell soma in the substantia nigra and ventral tegmental area and/or nerve terminals in the dorsal and ventral (nucleus accumbens) striatum (Grady et al., 1992), therefore activating these cells and causing an increase in extracellular dopamine levels in the dorsal and ventral striatum (Pontieri et al., 1996). Many lines of evidence support this view. Systemic nicotine increases burst activity in vivo in midbrain dopaminergic neurons (Grenhoff et al., 1986), suggesting a burst-sequence-related release of dopamine (Gonon, 1988). Nicotine, administered systemically, preferentially increases dopamine release in the nucleus accumbens when compared to the dorsal striatum (Imperato et al., 1986). This release is blocked by locally administered nicotinic receptor antagonists in the somato-dendritic region of the ventral tegmental area dopaminergic neurons, but not in the nucleus accumbens (Nisell et al., 1994). Nicotine self-administration is attenuated by lesions of the mesolimbic dopamine neurons (Clarke et al., 1988; Corrigall et al., 1992) and by nicotinic receptor antagonists microinfused specifically into the ventral tegmental area (but not the nucleus accumbens) (Corrigall et al., 1994). At least six of the identified nicotinic acetylcholine receptor subunits are expressed in mesencephalic dopaminergic neurons (α3, α4, α5, α6, α7, β2, and β3) (Le Novère et al., 1996; Pidoplichko et al., 1997). In view of the lack of selective antagonists, knockout mice offer a unique opportunity to evaluate the contribution of nicotinic acetylcholine receptor isoforms to the reinforcing action of nicotine. The regulation of nicotine-elicited dopamine release and self-administration behaviour was investigated with the $\beta 2 -/-$ mutant mice.

In vivo microdialysis experiments, which detected dopamine levels in the striatum, showed a dose-dependent nicotine-elicited increase in dopamine levels in wild-type animals but not in knockout animals, implicating β2-subunit-containing nicotinic acetylcholine receptors in the pharmacological release of striatal dopamine. Moreover, the nicotine-elicited increase of discharge frequency of dopaminergic neurons of the substantia nigra and ventral tegmental area found in wild-type animals (at concentrations of nicotine similar to those found in the arterial blood of smokers during cigarette consumption) was absent in knockout mice.

Nicotine self-administration was also tested in mutant and wild-type mice. A catheter in the jugular vein was implanted that delivered either cocaine (during the training session) or low doses of nicotine (during the test period) in response to specific nose-poking activity. Both wild-type and knockout mice demonstrated self-administration activity during the training session, indicating that mutant mice are capable of learning this behaviour. After the switch to nicotine, however, knockout mice progressively ceased self-administration activity, while the wild-type mice continued for 5 days following the nicotine substitution, suggesting that the β2 nicotinic acetylcholine receptor subunit is an essential component to mediating the addictive effects of nicotine (Picciotto et al., 1998).

7. Aging, antinociception, and nicotinic receptors

A decrease in high affinity nicotine binding sites and other impairments in the cholinergic system are consistently found in human dementias and are thought to play a role in the cognitive deficits found in demented patients (Kasa et al., 1997). In rats, treatment with nicotinic receptor antagonists or lesions in the cholinergic system can impair performance on cognitive tasks (McGurk et al., 1989; McGurk et al., 1991; Elrod and Buccafusco, 1991). Moreover, recent studies suggest that smoking may delay the onset or reduce the incidence of Parkinson's and Alzheimer's disease via a neuroprotective effect (Levin and Simon, 1998). Furthermore, the activation of nicotinic acetylcholine receptors by in vitro nicotine administration has been shown to protect striatal neurons against the neurotoxicity mediated by the NMDA subtype of glutamate receptors (Marin et al., 1994) as well as the toxicity in cortical neurons caused by β-amyloid, the major component of senile plaques (Kihara et al., 1998). However, no

direct evidence for the impact of chronic loss of nicotinic acetylcholine receptors on neural tissue structure is available. Therefore, age-related neuroanatomical, biochemical, and cognitive parameters in mice lacking the β2 nicotinic acetylcholine receptor subunit during aging were examined (Zoli et al., 1999).

Two-year-old β2 knockout mice showed tissue hypotrophy, neuronal loss and astrogliosis and microgliosis in specific, localized cortical regions (Zoli et al., 1999), areas that are particularly vulnerable to the aging process (Coleman and Flood, 1987). The loss of the β2 subunit itself, however, was not sufficient to cause neuro-degeneration since areas like the thalamus had no detectable anatomical differences between knockout and wild-type mice, even though in wild-type mice, this area contains the highest concentration of β2 nicotinic acetylcholine receptor subunits. Thus, the absence of the β2 subunit may contribute to some of the degenerative processes that are ongoing in the mouse brain during aging and correlates with the loss of neurons, in particular, brain regions during pathological aging such as in Alzheimer's disease.

In accordance with this hypothesis, the loss of the β2 subunit and high affinity nicotine binding sites is not sufficient to cause deficits in spatial learning. In the Morris water maze, 1-year-old, adult knockout and control mice were able to localize a hidden platform equally well; however, 2-year-old, aged knockout mice learned at a significantly slower rate. A possible explanation for this phenomenon is that in young animals, other systems might compensate for a β2-containing nicotinic acetylcholine receptor deficit. An aged brain, however, might contain deficits that reveal the effects of the absence of β2-containing nicotinic acetylcholine receptors in a spatial learning task. This is consistent with recent experiments showing that lesions in cholinergic neurons cause little or no impairment in spatial learning (Chappell et al., 1998) unless other neurotransmitter systems are also impaired (Gallagher and Colombo, 1995).

Thus, β2 knockout mice have anatomical and behavioural deficits similar to those found in pathological aging; therefore, these mice may serve as a useful model for the study of dementias.

The antinociceptive properties of nicotine were first reported over 60 years ago by Davis et al. (1932); however, this effect was largely overlooked as the doses required for analgesia are just below seizure-eliciting concentrations. The discovery of new nicotinic receptor agonists, such as epibatidine and the creation of [(*R*)-5-(2-azetidinylmethoxy)-2-chloropyridine], more commonly known as ABT-594, (Spande et al., 1992; Bannon et al., 1998), once again cast light upon nicotinic antinociception. These compounds have been shown to be 100 times more potent than morphine in models of acute pain (Qian et al., 1993; Bannon et al., 1998). Nicotinic receptor agonists exert antinociceptive effects by interacting with one or more of the nicotinic acetylcholine receptors present in the pain pathway. The mechanism of action of antinociception was investigated using knockout mice (Marubio et al., 1999). Normal baseline responses to thermal stimuli with the hot-plate and tail-flick tests did not differ in α4 and β2 mutant mice vs. their wild-type littermate controls, suggesting that these two subunits are not essential in the perception of acute thermal nociception.

Using the hot-plate test, $\alpha4+/+$ mice showed a dose-dependent antinociceptive response to nicotine. In contrast to wild-type littermates, no dose of nicotine or epibatidine tested caused an antinociceptive response that was significantly different from saline in $\alpha4-/-$ mice. $\beta2-/-$ mice also exhibited a vastly reduced antinociceptive response at all doses of nicotine tested.

In contrast, nicotine was able to cause dose-dependent analgesia in the tail-flick test in $\alpha4+/+$ and $\alpha4-/-$ mice and $\beta2+/+$ and $\beta2-/-$ mice. However, in both cases, the dose–response curve for the mutant mice was shifted to the right signifying a decrease in sensitivity to nicotine in mutant mice. Thus, both the α4 and the β2 nicotinic acetylcholine receptor subunit contribute to the antinociceptive effects of nicotine, though to a larger extent in the hot-plate test, which primarily reflects activation of supraspinal regions.

The thalamus, raphe magnus, pedunculopontine tegmental nuclei and the dorsal horn of the spinal cord play an important role in the nicotinic antinociceptive pathways (Aceto et al., 1986; Iwamoto, 1991; Jurna et al., 1993; Bitner et al., 1998). Equilibrium binding experiments showed the loss of high-affinity nicotine and epibatidine binding sites in these areas in $\alpha4-/-$ and $\beta2-/-$ mice. Consistent with this finding, patch clamp recordings from serotonergic neurons in the raphe magnus (identified by their response to serotonin) and neurons in the thalamus demonstrated a loss of nicotine-elicited currents in $\alpha4-/-$ and $\beta2-/-$ mice, suggesting a contribution of the α4 and β2 nicotinic acetylcholine receptor subunits to functional receptors in areas implicated in supra-spinal nicotine-elicited antinociception. In contrast, neurons in the superficial layers (I–III) of the dorsal horn of the spinal cord of $\alpha4-/-$ and $\beta2-/-$ mice still displayed a nicotine-elicited, dose-dependent augmentation of the frequency of postsynaptic currents.

Disruption of the α4 and the β2 nicotinic acetylcholine receptor gene demonstrates that these receptor subunits are an important, though not exclusive, component of the nicotinic pain pathways and most likely cooperate to mediate the antinociceptive effect of nicotine. Specific pharmacological compounds, which target these receptors, may prove to be therapeutically useful for analgesia.

8. Conclusions and future directions

The differential contribution of single nicotinic acetylcholine receptor subunits in the various pharmacological

actions of nicotine has been difficult to assess. To date, knockout mouse technology has helped to elucidate the function of the ε subunit at the neuromuscular junction, the contribution of the α7, β2 and α4 subunits in the pharmacological profiles of subunit subtypes, and the α4 and β2 subunits in behaviour. Further studies investigating other effects of nicotine in these knockout mice are anticipated as well as knockouts of other subunits. New genetic technology provides the means to introduce specific mutations within a given gene and, thus, to test for the behavioural consequences of such mutations. Inducible knockout and knockin systems can be used if the deletion of a gene has lethal consequences or creates a developmental abnormality. In addition, region-specific knockouts and knockins might be used to investigate the specific role of a subunit in a defined brain structure. These advances will help shed light on the role of nicotinic acetylcholine receptors in brain function.

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References

Aceto, M.D., Bagley, R.S., Dewey, W.L., Fu, T.C., Martin, B.R., 1986. The spinal cord as a major site for the antinociceptive action of nicotine in the rat. Neuropharmacology 25, 1031–1036.

Alkondon, M., Albuquerque, E.X., 1993. Diversity of nicotinic acetylcholine receptors in rat hippocampal neurons: I. Pharmacological and functional evidence for distinct structural subtypes. J. Pharmacol. Exp. Ther. 265, 1455–1473.

Bannon, A.W., Decker, M.W., Holladay, M.W., Curzon, P., Donnelly-Roberts, D., Puttfarcken, P.S., Bitner, R.S., Diaz, A., Dickenson, A.H., Porsolt, R.D., Williams, M., Arneric, S.P., 1998. Broad-spectrum, non-opiod analgesic activity by selective modulation of neuronal nicotinic acetylcholine receptors. Science 279, 77–81.

Bitner, R.S., Nikkel, A.L., Curzon, P., Arneric, S.P., Bannon, W., Decker, M.W., 1998. Role of the nucleus raphe magnus in antinociception produced by ABT-594: immediate early gene responses possibly linked to neuronal nicotinic acetylcholine receptors on serotonergic neurons. J. Neurosci. 18, 5426–5432.

Capecchi, M.R., 1989. Altering the genome by homologous recombination. Science 244, 1288–1292.

Chappell, J., McMahan, R., Chiba, A., Gallagher, M., 1998. A re-examination of the role of basal forebrain cholinergic neurons in spatial working memory. Neuropharmacology 37, 481–487.

Clarke, P.B., Fu, D.S., Jakubovic, A., Fibiger, H.C., 1988. Evidence that mesolimbic dopaminergic activation underlies the locomotor stimulant action of nicotine in rats. J. Pharmacol. Exp. Ther. 246, 701–708.

Coleman, P.D., Flood, D.G., 1987. Neuron numbers and dendritic extent in normal aging and Alzheimer's disease. Neurobiol. Aging 8, 521–545.

Conroy, W.G., Vernallis, A.B., Berg, D.K., 1992. The alpha 5 gene product assembles with multiple acetylcholine receptor subunits to form distinctive receptor subtypes in brain. Neuron 9, 679–691.

Corrigall, W.A., Cohen, K.M., Adamson, K.L., 1994. Self-administered nicotine activates the mesolimbic dopamine system through the ventral tegmental area. Brain Res. 653, 278–284.

Corrigall, W.A., Franklin, K.B.J., Cohen, K.M., Clarke, P.B.S., 1992. The mesolimbic dopaminergic system is implicated in the reinforcing effects of nicotine. Psychopharmacology 107, 285–289.

Couturier, S., Bertrand, D., Matter, J.M., Hernandez, M.C., Bertrand, S., Millar, N., Valera, S., Barkas, T., Ballivet, M., 1990. A neuronal nicotinic acetylcholine receptor subunit (alpha 7) is blocked by alpha-BTX. Neuron 5, 847–856.

Davis, L., Pollock, L.J., Stone, T.T., 1932. Visceral pain. Surg. Gynecol. Obstet. 55, 418–427.

Dineley-Miller, K., Patrick, J., 1992. Gene transcripts for the nicotinic acetylcholine receptor subunit, beta4, are distributed in multiple areas of the rat central nervous system. Brain Res. Mol. Brain Res. 16, 339–344.

Elrod, K., Buccafusco, J.J., 1991. Correlation of the amnestic effects of nicotinic antagonists with inhibition of regional brain acetylcholine synthesis in rats. J. Pharmacol. Exp. Ther. 258, 403–409.

Fischbach, G.D., Schuetze, S.M., 1980. A post-natal decrease in acetylcholine channel open time at rat endplates. J. Physiol. 303, 125–137.

Gallagher, M., Colombo, P.J., 1995. Ageing: the cholinergic hypothesis of cognitive decline. Curr. Opin. Neurobiol. 5, 161–168.

Gonon, F.G., 1988. Nonlinear relationship between impulse flow and dopamine released by rat midbrain dopaminergic neurons as studied by in vivo electrochemistry. Neuroscience 24, 19–28.

Grady, S., Marks, M.J., Wonnacott, S., Collins, A.C., 1992. Characterization of nicotinic receptor-mediated [^{3}H]dopamine release from synaptosomes prepared from mouse striatum. J. Neurochem. 59, 848–856.

Grenhoff, J., Aston-Jones, G., Svensson, T.H., 1986. Nicotinic effects on the firing pattern of midbrain dopamine neurons. Acta Physiol. Scand. 128, 351–358.

Gu, Y., Hall, Z.W., 1988. Immunological evidence for a change in subunits of the acetylcholine receptor in developing and denervated rat muscle. Neuron 1, 117–125.

Homanics, G., DeLorey, T., Firestone, L., Quinlan, J., Handforth, A., Harrison, N., Krasowski, M., Rick, C., Korpi, E., Mäkelä, R., Brilliant, M., Hagiwara, N., Ferguson, C., Snyder, K., Olsen, R., 1997. Mice devoid of gamma-aminobutyrate type A receptor beta3 subunit have epilepsy, cleft palate, and hypersensitive behavior. Proc. Natl. Acad. Sci. U. S. A. 94, 4143–4148.

Imperato, A., Mulas, A., Di Chiara, G., 1986. Nicotine preferentially stimulates dopamine release in the limbic system of freely moving rats. Eur. J. Pharmacol. 132, 337–338.

Iwamoto, E.T., 1991. Characterization of the antinociception induced by nicotine in the pedunculopontine tegmental nucleus and the nucleus raphe magnus. J. Pharmacol. Exp. Ther. 257, 120–133.

Jurna, I., Krauss, P., Baldauf, J., 1993. Depression by nicotine of pain-related nociceptive activity in the rat thalamus and spinal cord. Clin. Pharmacol. 72, 65–73.

Kasa, P., Rakonczay, Z., Gulya, K., 1997. The cholinergic system in Alzheimer's disease. Prog. Neurobiol. 52, 511–535.

Kihara, T., Shimohama, S., Urushitani, M., Sawada, H., Kimura, J., Kume, T., Maeda, T., Akaike, A., 1998. Stimulation of alpha4beta2 nicotinic acetylcholine receptors inhibits beta-amyloid toxicity. Brain Res. 792, 331–334.

Kutsuwada, T., Sakimura, K., Manabe, T., Takayama, C., Katakura, N., Kushiya, E., Natsume, R., Watanabe, M., Inoue, Y., Yagi, T., Aizawa, S., Arakawa, M., Takahashi, T., Nakamura, Y., Mori, H., Mishina, M., 1996. Impairment of suckling response, trigeminal neuronal pat-

tern formation, and hippocampal LTD in NMDA receptor e2 subunit mutant mice. Neuron 16, 333–344.

Le Novère, N., Zoli, M., Changeux, J.P., 1996. Neuronal nicotinic receptor alpha 6 subunit mRNA is selectively concentrated in catecholaminergic nuclei of the rat brain. Eur. J. Neurosci. 8, 2428–2439.

Levin, E.D., 1992. Nicotinic systems and cognitive function. Psychopharmacology 108, 417–431.

Levin, E.D., Simon, B.B., 1998. Nicotinic acetylcholine involvement in cognitive function in animals. Psychopharmacology 138, 217–230.

Lu, Y., Grady, S., Marks, M.J., Picciotto, M., Changeux, J.P., Collins, A.C., 1998. Pharmacological characterization of nicotinic receptor-stimulated GABA release from mouse brain synaptosomes. J. Pharmacol. Exp. Ther. 287, 648–657.

Luetje, C.W., Patrick, J., 1991. Both alpha-and beta-subunits contribute to the agonist sensitivity of neuronal nicotinic acetylcholine receptors. J. Neurosci. 11, 837–845.

Makela, R., Uusi-Oukari, M., Homanics, G., Quinlan, J., Firestone, L., Wisden, W., Korpi, E., 1997. Cerebellar gamma-aminobutyric acid type A receptors: pharmacological subtypes revealed by mutant mouse lines. Mol. Pharmacol. 52, 380–388.

Marin, P., Maus, M., Desagher, S., Glowinski, J., Premont, J., 1994. Nicotine protects cultured striatal neurones against *N*-methyl-D-aspartate receptor-mediated neurotoxicity [see comments]. NeuroReport 5, 1977–1980.

Martinou, J.C., Merlie, J.P., 1991. Nerve-dependent modulation of acetylcholine receptor epsilon-subunit gene expression. J. Neurosci. 11, 1291–1299.

Marubio, L.M., Arroyo-Jimenez, M.M., Cordero-Erausquin, M., Léna, C., Le Novère, N., Huchet, M., Damaj, M.I., Changeux, J.P., 1999. Reduced antinociception in mice lacking neuronal nicotinic receptor subunits. Nature 398, 805–810.

McGehee, D.S., Role, L.W., 1995. Physiological diversity of nicotinic acetylcholine receptors expressed by vertebrate neurons. Annu. Rev. Physiol. 57, 521–546.

McGurk, S.R., Levin, E.D., Butcher, L.L., 1989. Radial-arm maze performance in rats is impaired by a combination of nicotinic-cholinergic and D2 dopaminergic antagonist drugs. Psychopharmacology 99, 371–373.

McGurk, S.R., Levin, E.D., Butcher, L.L., 1991. Impairment of radial-arm maze performance in rats following lesions involving the cholinergic medial pathway: reversal by arecoline and differential effects of muscarinic and nicotinic antagonists. Neuroscience 44, 137–147.

Mishina, M., Takai, T., Imoto, K., Noda, M., Takahashi, T., Numa, S., Methfessel, C., Sakmann, B., 1986. Molecular distinction between fetal and adult forms of muscle acetylcholine receptor. Nature 321, 406–411.

Missias, A.C., Mudd, J., Cunningham, J.M., Steinbach, J.H., Merlie, J.P., Sanes, J.R., 1997. Deficient development and maintenance of postsynaptic specializations in mutant mice lacking an 'adult' acetylcholine receptor subunit. Development 124, 5075–5086.

Nisell, M., Nomikos, G.G., Svesson, T.H., 1994. Infusion of nicotine in the ventral tegmental area of the nucleus accumbens of the rat differentially affects accumbal dopamine release. Pharmacol. Toxicol. 75, 348–352.

Ohno, K., Quiram, P., Milone, M., Wang, H., Harper, M., Pruitt, J., Brengman, J., Pao, L., Fischbeck, K., Crawford, T., Sine, S., Engel, A., 1997. Congenital myasthenic syndromes due to heteroallelic nonsense/missense mutations in the acetylcholine receptor epsilon subunit gene: identification and functional characterization of six new mutations. Hum. Mol. Genet. 6, 753–766.

Ohno, K., Wang, H., Milone, M., Bren, N., Brengman, J., Nakano, S., Quiram, P., Pruitt, J., Sine, S., Engel, A., 1996. Congenital myasthenic syndrome caused by decreased agonist binding affinity due to a mutation in the acetylcholine receptor epsilon subunit. Neuron 17, 157–170.

Orr-Urtreger, A., Goldner, F.M., Saeki, M., Lorenzo, I., Goldberg, L., De Biasi, M., Dani, J.A., Patrick, J.W., Beaudet, A.L., 1997. Mice deficient in the alpha7 neuronal nicotinic acetylcholine receptor lack alpha-bungarotoxin binding sites and hippocampal fast nicotinic currents. J. Neurosci. 17, 9165–9171.

Orr-Urtreger, A., Broide, R.S., Kasten, M.R., Dang, H., Dani, J.A., Beaudet, A.L., Patrick, J.W., in press. Mice homozygous for the L250T mutation in the alpha7 nicotinic acetylcholine receptor show increased neuronal apoptosis and die within one day of birth. J. Neurochem.

Paylor, R., Nguyen, M., Crawley, J.N., Patrick, J., Beaudet, A., Orr-Urtreger, A., 1998. Alpha7 nicotinic receptor subunits are not necessary for hippocampal-dependent learning or sensimotor gating: a behavioral characterization of acra7-deficient mice. Learning and Memory 5, 302–3016.

Piccioto, M., Zoli, M., Léna, C., Bessis, A., Lallemand, Y., Lenovere, N., Vincent, P., Merlo-Pich, E., Brulet, P., Changeux, J.-P., 1995. Abnormal avoidance learning in mice lacking functional-high affinity nicotine receptor in the brain. Nature 374, 65–67.

Picciotto, M.R., Zoli, M., Rimondini, R., Lena, C., Marubio, L.M., Pich, E.M., Fuxe, K., Changeux, J.P., 1998. Acetylcholine receptors containing the beta2 subunit are involved in the reinforcing properties of nicotine. Nature 391, 173–177.

Pidoplichko, V.I., DeBiasi, M., Williams, J.T., Dani, J.A., 1997. Nicotine activates and desensitizes midbrain dopamine neurons. Nature 390, 401–404.

Pontieri, F.E., Tanda, G., Orzi, F., Di Chiara, G., 1996. Effects of nicotine on the nucleus accumbens and similarity to those of addictive drugs. Nature 382, 255–257.

Qian, C., Li, T., Shen, T.Y., Libertine-Garahan, L., Eckman, J., Biftu, T., Ip, S., 1993. Epibatidine is a nicotinic analgesic. Eur. J. Pharmacol. 250, R13–R14.

Ramirez-Latorre, J., Yu, C.R., Qu, X., Perin, F., Karlin, A., Role, L., 1996. Functional contributions of alpha5 subunit to neuronal acetylcholine receptor channels. Nature 380, 347–351.

Sakimura, K., Kutsuwada, T., Ito, I., Manabe, T., Takayama, C., Kushiya, E., Yagi, T., Aizawa, S., Inoue, Y., Sugiyama, H., 1995. Reduced hippocampal LTP and spatial learning in mice lacking NMDA receptor epsilon 1 subunit. Nature 373, 151–155.

Sargent, P.B., 1993. The diversity of neuronal nicotinic acetylcholine receptors. Annu. Rev. Neurosci. 16, 403–443.

Siegelbaum, S.A., Trautmann, A., Koenig, J., 1984. Single acetylcholine-activated channel currents in developing muscle cells. Dev. Biol. 104, 366–379.

Spande, T.F., Garraffo, H.M., Edwards, M.W., Yeh, H.J.C., Pannel, L., Daly, J.W., 1992. Epibatidine: a novel (chloropyridyl)azabicycloheptaine with potent analgesic activity from an Ecuadoran poison frog. J. Am. Chem. Soc. 114, 3475–3478.

Tsien, J., Huerta, P., Tonegawa, S., 1996. The essential role of hippocampal CA1 NMDA receptor-dependent synaptic plasticity in spatial memory. Cell 87, 1327–1338.

Vetter, D.E., Liberman, M.C., Mann, J., Barhanin, J., Boulter, J., Brown, M.C., Saffiote-Kolman, J., Heinemann, S.F., Elgoyhen, A.B., 1999. Role of alpha9 nicotinic ACh receptor subunits in the development and function of cochlear efferent innervation. Neuron 23, 93–103.

Villarroel, A., Sakmann, B., 1996. Calcium permeability increase of endplate channels in rat muscle during postnatal development. J. Physiol. 496, 331–338.

Wada, E., Wada, K., Boulter, J., Deneris, E., Heinemann, S., Patrick, J., Swanson, L.W., 1989. Distribution of alpha 2, alpha 3, alpha 4, and beta 2 neuronal nicotinic receptor subunit mRNAs in the central nervous system: a hybridization histochemical study in the rat. J. Comp. Neurol. 284, 314–335.

Wang, F., Gerzanich, V., Wells, G.B., Anand, R., Peng, X., Keyser, K., Lindstrom, J., 1996. Assembly of human neuronal nicotinic receptor alpha5 subunits with alpha3, beta2, and beta4 subunits. J. Biol. Chem. 271, 17656–17665.

Witzemann, V., Schwarz, H., Koenen, M., Berberich, C., Villarroel, A., Wernig, A., Brenner, H.R., Sakmann, B., 1996. Acetylcholine recep-

tor epsilon-subunit deletion causes muscle weakness and atrophy in juvenile and adult mice. Proc. Natl. Acad. Sci. U.S.A. 93, 13286–13291.

Xu, W., Gelber, S., Orr-Urtreger, A., Armstrong, D., Lewis, R.A., Ou, C.N., Patrick, J., Role, L., De Biasi, M., Beaudet, A.L., 1999. Megacystis, mydriasis, and ion channel defect in mice lacking the alpha3 neuronal nicotinic acetylcholine receptor. Proc. Natl. Acad. Sci. U.S.A. 96, 5746–5751.

Zoli, M., Léna, C., Picciotto, M.R., Changeux, J.P., 1998. Identification of four classes of brain nicotinic receptors using beta-2 mutant mice. J. Neurosci. 18, 4461–4472.

Zoli, M., Picciotto, M., Ferrair, R., Cocchi, D., Changeux, J., 1999. Increased neurodegeneration during ageing in mice lacking high-affinity nicotine receptors. EMBO J. 18, 1235–1244.