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Insights into the Neurobiology of the Nicotinic Cholinergic System and Nicotine Addiction from Mice Expressing Nicotinic Receptors Harboring Gain-of-Function Mutations

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Abstract—Nicotinic acetylcholine receptors (nAChRs) are ligand-gated, cation-selective ion channels expressed throughout the brain. Although these channels have been investigated for several decades, it is still challenging 1) to identify the important nAChR subunits in cholinergic transmission and nicotine dependence and 2) to develop nAChR subtype-specific ligands. To overcome these challenges, we and others have studied mice expressing mutant, gain-of-function nAChR subunits. In this review, we discuss this research approach and the results it has yielded to date. Gain-of-function mutations, including those in nAChR subunits, provide an approach that is complementary to loss-of-function studies such as gene knockouts; the former allows one to answer questions of sufficiency and the latter addresses questions of necessity. Mutant mice expressing gain-of-

function nAChR subunits are commonly produced using traditional gene targeting in embryonic stem cells, but novel approaches such as bacterial artificial chromosome transgenesis have yielded important insights as well. $\alpha 7$ nAChRs were the first nAChRs to be targeted with a gain-of-function mutation, followed by a pair of $\alpha 4$ nAChR gain-of-function mutant mice. These $\alpha 4$ nAChR gain-of-function mice ($\alpha 4$ L9'S mice, followed by $\alpha 4$ L9'A mice) provided an important system to probe $\alpha 4$ nAChR function in vivo, particularly in the dopamine reward system. $\alpha 6$ nAChR gain-of-function mice provided the first robust system allowing specific manipulation of this receptor subtype. Other targeted mutations in various nAChR subunits have also been produced and have yielded important insights into nicotinic cholinergic biology. As nAChR research advances and more details associated with nAChR expression and function emerge, we expect that existing and new mouse lines expressing gain-of-function nAChR subunits will continue to provide new insights.

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I. Introduction

Nicotinic acetylcholine receptors (nAChRs¹) are pentameric ligand-gated ion channels (Itier and Bertrand, 2001). These protein complexes are widely expressed in muscle and neuronal tissues, and their potential utility as drug targets for various therapeutic interventions has been increasingly recognized in recent years (Aubin et al., 2011; Wallace and Porter, 2011; Williams et al., 2011). Heteromeric nAChRs are composed of α and β subunits, which assemble around and operate a central, cation-conducting pore domain (Keramidas et al., 2004; Absalom et al., 2009; Zouridakis et al., 2009). Muscle-type nAChRs, including their subunit stoichiometry and role as postsynaptic ACh receptors at the neuromuscular junction (Changeux, 2010a), are well understood. In contrast, neuronal-type nAChRs comprise a larger group of receptor subtypes with a subcellular expression profile and functional role that is not as well understood. Interest in neuronal nAChRs arises from 1) their important natural role in the nicotinic cholinergic system (Maskos, 2010) as well as 2) their activation and/or desensitization by nicotine in cigarette smoke (Picciotto et al., 2008). These two overlapping roles in the brain continue to drive nAChR basic research and drug development efforts involving these receptors.

Research into nAChR expression and function has been hampered by two critical challenges: 1) the diversity of nAChR stoichiometries and expression patterns in the brain, and 2) selectivity, or lack thereof, of specific agonists or antagonists capable of functionally isolating specific nAChR subtypes *in vivo*. Aside from homomeric-type $\alpha 7$ or $\alpha 8$ nAChRs, there are three neuronal β subunits ($\beta 2$ - $\beta 4$) and at least seven neuronal α subunits ($\alpha 2$ - $\alpha 6$, $\alpha 9$, $\alpha 10$) that assemble to form heteromeric nAChR pentamers (Keramidas et al., 2004; Dani and Bertrand, 2007; Absalom et al., 2009; Zouridakis et al., 2009). Each of these subunits is expressed in multiple brain areas, often with specific subcellular localization and developmental expression patterns (Marks et al., 1992; Azam et al., 2002). Thus, the "diversity" challenge arises from both 1) the large number of possible subunit combinations and 2) the poorly understood anatomical, subcellular, and developmental expression patterns of each of these subunits. Medium- or high-throughput screening assays in heterologous expression systems are required for discovering subtype-selective therapeutic neuronal nAChR compounds (Lester, 1988), but this problem of diversity has vitiated learning the appropriate subunit combination from the appropriate neuronal type. Even when the relevant subunit combination is known, challenges also arise in functional reconstitution

of the particular nAChR subtype in ectopic expression systems (Drenan et al., 2008b).

During the past 10 to 15 years, approaches in mouse genetics have enabled investigators to address and, in some cases, overcome the challenges noted above. Gene-targeting approaches, used mainly to produce nAChR knockout (KO) mice, have provided important new details into nAChR neurobiology (Zhang, 2006; Fowler et al., 2008; Mineur and Picciotto, 2008; Changeux, 2010b). Mice lacking $\alpha 7$ nAChR subunits have subtle phenotypes, such as decrements in sustained attention (Young et al., 2004) and impairments in passive avoidance learning (Marubio and Paylor, 2004). Although $\alpha 7$ KO mice show alterations in DA neuron firing rates in response to endogenous ACh (Mameli-Engvall et al., 2006), they are largely normal with respect to nicotine and the DA reinforcement pathway (Naylor et al., 2005; Walters et al., 2006).

$\alpha 3$ and $\beta 4$ nAChR subunits are often coexpressed in the same nAChR pentamer, in both central neurons (Zoli et al., 1999) and peripheral nerves (Yeh et al., 2001). $\alpha 3$ KO mice die perinatally, possibly because of autonomic dysfunction (Xu et al., 1999a). $\beta 4$ KO mice, however, are grossly normal and may exhibit some compensation by $\beta 2$ nAChR subunits (Xu et al., 1999b). These mice have decreased somatic signs of nicotine withdrawal (Salas et al., 2004), which was an important initial observation leading to the hypothesis that $\beta 4$ (Frahm et al., 2011) and/or $\alpha 5^*$ (* denotes the possibility that other nAChR subunits may be present in the pentameric receptor) nAChRs (Fowler et al., 2011) in the medial habenula (MHb)/interpeduncular nucleus (IPN) pathway mediate the aversive properties of nicotine. $\alpha 2$ and $\alpha 5$ KO mice also point to a critical role for these nAChR subunits in nicotine withdrawal (Salas et al., 2009). Fowler et al. (2011) demonstrated that $\alpha 5^*$ nAChRs in MHb and/or IPN are critical regulators of nicotine intake. These results, along with those of Frahm et al. (2011), support the idea that human genetic polymorphisms in the $\alpha 5$ nAChR subunit gene (Saccone et al., 2009) may partially underlie susceptibility to nicotine dependence.

$\beta 2$ nAChR subunits are essential components of most heteromeric receptors with high affinity for nicotine, and $\beta 2$ KO mice exhibit a number of behavioral and physiological phenotypes that reflect this fact. $\beta 2$ KO mice are grossly normal (Picciotto et al., 1995), but nicotine-induced firing of DA neurons and nicotine-stimulated DA release is largely abolished (Picciotto et al., 1998; Grady et al., 2011). Consequently, $\beta 2$ KO mice do not self-administer nicotine (Picciotto et al., 1998), and nicotine is unable to condition a place preference (Walters et al., 2006) or activate locomotion in these mice (King et al., 2004; Villégier et al., 2006). Most $\alpha 4$ nAChRs also contain $\beta 2$ subunits, and $\alpha 4$ KO mice recapitulate many phenotypes of $\beta 2$ KO mice. $\alpha 4$ KO mice have blunted nicotine-elicited DA release (Marubio et al., 2003), nicotine-stimulated locomotion is altered

¹Abbreviations: ACh, acetylcholine; ADNFLE, autosomal-dominant nocturnal frontal-lobe epilepsy; BAC, bacterial artificial chromosome; CPP, conditioned place preference; DA, dopamine; ES, embryonic stem; IPN, interpeduncular nucleus; KO, knockout; MHb, medial habenula; nAChR, nicotinic acetylcholine receptor; neo, neomycin; SNc, substantia nigra pars compacta; VTA, ventral tegmental area; WT, wild type.

(Marubio et al., 2003), and nicotine self-administration is abolished in these mice (Pons et al., 2008; Exley et al., 2011).

Interest in $\alpha 6^*$ and $\beta 3^*$ nAChRs has been strong since the demonstration that these subunits exhibit an expression pattern restricted mainly to catecholaminergic and visual system neurons (Deneris et al., 1989; Le Novère et al., 1996; Forsayeth and Kobrin, 1997; Léna et al., 1999; Vailati et al., 2000; Whiteaker et al., 2000; Azam et al., 2002; Champtiaux et al., 2002; Zoli et al., 2002). $\alpha 6$ and $\beta 3$ KO mice have reduced nicotine-stimulated DA release in striatum (Champtiaux et al., 2002, 2003; Cui et al., 2003). $\beta 3$ nAChR subunits are important for $\alpha 6^*$ nAChR biogenesis in the DA system, because $\alpha 6^*$ nAChR binding and functional expression is dramatically reduced in $\beta 3$ KO mice (Cui et al., 2003). Acute intravenous nicotine self-administration is eliminated in $\alpha 6$ KO mice and is restored when $\alpha 6$ subunits are selectively re-expressed in the VTA (Pons et al., 2008). In intracranial nicotine self-administration experiments where learning is required, $\alpha 6$ KO mice show a trend (though not a significant difference) toward reduced self-administration compared with control mice (Exley et al., 2011). $\beta 3^*$ and/or $\alpha 6^*$ nAChRs may be involved in anxiety as well, because $\beta 3$ KO mice exhibit reduced anxiety (Booker et al., 2007).

Heteromeric nAChRs containing $\alpha 9$ and $\alpha 10$ subunits are specialized nAChRs with high Ca^{2+} permeability in auditory hair cells (Elgoyhen and Katz, 2012). $\alpha 9\alpha 10$ nAChRs are present at the medial olivocochlear (MOC)-hair cell synapse, and $\alpha 9\alpha 10$ nAChR activation by ACh causes Ca^{2+} -induced SK K^+ channel activation and hyperpolarization of the hair cell membrane (Elgoyhen and Katz, 2012). $\alpha 9$ KO mice have disturbed cochlear responses, and improper synaptic formation in the cochlea (Vetter et al., 1999; Murthy et al., 2009). Work with $\alpha 10$ KO mice demonstrated the essential nature of these subunits to the hair cell nAChR (Vetter et al., 2007).

nAChR KO mouse strains, although an essential research tool for understanding nicotinic cholinergic neurobiology, do not allow investigators to fully address the diversity and selectivity challenges in the nAChR field. In particular, KO animals typically only allow for answering questions of necessity—not sufficiency. We and others have studied several strains of knock-in or transgenic mice that are complementary to nAChR KO animals and to pharmacological studies on native receptors: mice expressing hypersensitive nAChR variants that functionally amplify and isolate the actions of specific nAChR subtypes. In this review, we discuss this approach in detail, including the development of new hypersensitive mouse models, key observations from several of these models, and their unique utility in advancing drug discovery efforts.

II. Critical Issues in the Production of Useful Nicotinic Acetylcholine Receptor Gain-of-Function Mice

Unlike production of a KO mouse, where removal or inactivation of the gene of interest can be done in many different ways, creation of a mouse with a single point mutation in a nAChR subunit poses two critical challenges: 1) selection of an appropriate mutation and 2) selection of an appropriate genetic targeting strategy. For the former, several amino acid residues in nAChR subunits are known to alter receptor sensitivity when mutated, but the preferred region of the receptor to mutate is the M2 transmembrane domain (Fig. 1) (Lester et al., 2003). The M2 region is an α -helix that contributes to the formation of the ion conducting pore of the pentameric ion channel (Miyazawa et al., 2003). Several highly conserved hydrophobic residues line the channel pore (Fig. 1), and when five contributing subunits are assembled, these hydrophobic residues are thought to create a channel gate that opens upon ligand binding (Miyazawa et al., 2003). Revah et al. (1991) first described mutations in a conserved leucine residue in the chick $\alpha 7$ homomeric receptor that significantly increased receptor sensitivity to agonist. Labarca et al. (1995) later demonstrated a similar effect in the muscle-type nAChR and further demonstrated that the number of nAChR subunits within a pentameric receptor with a sensitizing mutation is roughly proportional to the log of the gain in overall receptor sensitivity. This observation continues to prove useful in interpretation of data derived from nAChR knock-in/transgenic hypersensitive mice. Several studies on M2 domain mutations have since been conducted on many neuronal nAChR subunits, including $\alpha 3$ (Boorman et al., 2000), $\alpha 4$ (Fonck et al., 2005), $\alpha 5$ (Groot-Kormelink et al., 2001; Li et al., 2011), $\beta 3$ (Boorman et al., 2000), and

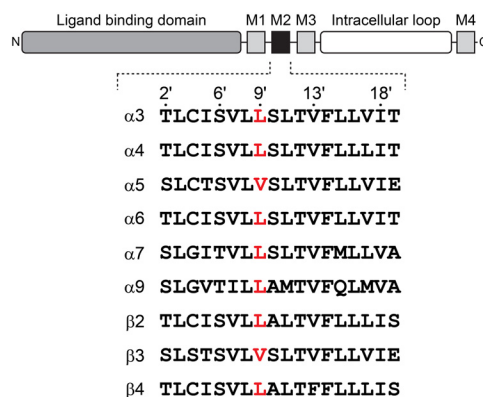


FIG. 1. nAChR M2 domain. nAChRs are composed of an N-terminal ligand binding domain, three adjacent transmembrane segments (M1–M3), a large cytoplasmic intracellular loop, and a fourth transmembrane segment (M4). Mouse M2 transmembrane protein sequences were aligned for the following nAChR subunits between the 2' and 18' positions: $\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 6$, $\alpha 7$, $\beta 2$, $\beta 3$, and $\beta 4$. The 9' Leu residue is highly conserved across nAChRs and is often mutated to Ser or Thr when creating nAChR gain-of-function mutant mice.

$\beta 4$ (Boorman et al., 2000). Although there are conflicting reports on $\alpha 5$ (Groot-Kormelink et al., 2001; Li et al., 2011), results from nearly all these studies are consistent: polar substitutions at the 9' or 13' position in the M2 domain (Fig. 1) produce a marked (10- to 100-fold) leftward shift in the concentration-response curve, and thus create a useful feature to better understand these proteins in vitro and in vivo [in the useful "prime" nomenclature, the 1' position denotes the most N-terminal, cytoplasmic residue in the M2 region (Charnet et al., 1990)]. These mutations also produce biophysical effects that may be unwanted, leading to difficulty in interpretation of experimental results. For example, 9' serine or threonine mutations may alter receptor desensitization (Revah et al., 1991), create alternative conducting states (Bertrand et al., 1992), abolish or reduce voltage rectification (Revah et al., 1991; Bertrand et al., 1992; Xiu et al., 2009), and increase receptor open time (Labarca et al., 1995). Although it is possible to understand a particular subunit of interest quite well in vitro, it is not often predictable how a specific mutation will perform in vivo.

Once a "hypersensitive" mutation is chosen for incorporation into the genome of a mouse, the second challenge is choosing the appropriate genetic targeting strategy. The knock-in approach uses homologous recombination in embryonic stem (ES) cells to replace one WT allele of the nAChR gene of interest with an allele containing the hypersensitive point mutation (Doyle et al., 2012). This approach has the advantage of being highly precise, only modifying the endogenous nAChR gene and usually leaving behind only one 34-base pair loxP sequence (Doyle et al., 2012). The knock-in approach is more time-consuming, however, usually requiring 1 to 3 years to produce a usable strain. Creation of a transgenic mouse is the alternative approach to a knock-in. In this method, exogenous DNA that directs expression of the hypersensitive nAChR subunit of interest is randomly inserted into the genome (Gong et al., 2003; Doyle et al., 2012). Because the ectopic DNA is capable of incorporating into undesired loci in the genome, possibly disrupting expression of other important genes, several founder strains must be studied at the outset, and misexpression of the nAChR gene of interest in ectopic brain areas must be mitigated. The use of bacterial artificial chromosomes (BACs) containing the nAChR gene of interest along with important regulatory sequences can reduce expression artifacts (Gong et al., 2003). Although the transgenic approach suffers from imprecision, the time to product is much shorter (~3 months), and creation of a targeting vector is simpler compared with the knock-in approach (Gong et al., 2003; Doyle et al., 2012). Regardless of the molecular biological route taken to produce the desired mouse strain, the resulting hypersensitive mutation should produce a substantial leftward shift in the concentration-response curve for the particular receptor of interest (Fig. 2). In the future, newer methods, such as conditional gene knock-in/KO approaches, inducible expression

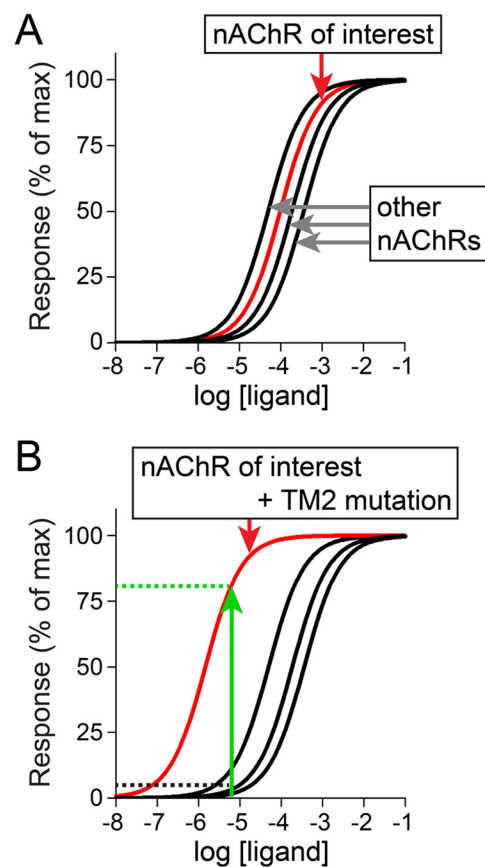


FIG. 2. Effect of a transmembrane (TM) 2 mutation on nAChR sensitivity. A, the particular nAChR subtype of interest will often, broadly speaking, have a sensitivity to ligand similar to that of other nAChR subtypes. In the absence of ligands that specifically activate the nAChR subtype of interest, an alternative approach to isolating the biological action of the receptor is to increase the sensitivity of the receptor by 10- to 100-fold. B, in the presence of a sufficient fraction of nAChR subunits of interest harboring a TM2 sensitizing mutation, receptor sensitivity is dramatically increased and the action of the receptor can be pharmacologically isolated relative to other nAChRs.

systems (e.g., tetracycline on/off systems), and/or targeted expression of nAChR genes with viral vectors (Molles et al., 2006) could be used to create new mouse models expressing hypersensitive nAChRs.

III. Critical Molecular, Neurochemical, and Behavioral Observations from Mice Expressing Hypersensitive Nicotinic Acetylcholine Receptor Subunits

1. $\alpha 7$ Nicotinic Acetylcholine Receptor Subunits. To date, mouse $\alpha 7$, $\alpha 4$, $\alpha 6$, and $\alpha 9$ nAChR genes have been targeted using the hypersensitive nAChR approach (Table 1). Orr-Urtreger et al. (2000) first reported the production of a mouse expressing hypersensitive, $\alpha 7$ L250T nAChRs. Mice homozygous for the L250T substitution mutation died perinatally, possibly as a result of excitotoxic cell death resulting from excess Ca^{2+} entry through mutant channels. Because $\alpha 7$ -dependent currents in L250T mice were prominent at low concentrations of nicotine, this report suggested that recording

TABLE 1
Summary of key results obtained from studies on mice with hypersensitive nAChR subunit mutations

nAChR Subunit and TM2 Mutation	Key Results	Reference
$\alpha 4$ L9'S	Increased anxiety, poor motor learning, and locomotor hyperactivity in heterozygote mice	Labarca et al., 2001
L9'A	Sensitization to the seizure-causing action of nicotine and other nAChR agents Sensitization to the antinociceptive action of nicotine Selective activation of $\alpha 4^*$ nAChRs is sufficient for nicotine reward, tolerance, and sensitization Seizure and sleep phenotypes associated with hypersensitive $\alpha 4$ subunits $\alpha 4^*$ nAChRs regulate respiratory rhythm Demonstrated strong expression of $\alpha 4^*$ nAChRs in ventrolateral medial habenula Selective activation of $\alpha 4^*$ nAChRs is necessary and sufficient for varenicline's ability to reduce alcohol consumption	Fonck et al., 2003 Damaj et al., 2007 Tapper et al., 2004 Fonck et al., 2005 Shao et al., 2008 Fonck et al., 2009 Hendrickson et al., 2010
$\alpha 6$ L9'S	Selective $\alpha 6^*$ nAChR activation causes spontaneous and nicotine-elicited locomotor hyperactivity Altered frequency-dependent DA release in $\alpha 6$ L9'S mice; $\alpha 4$ subunits are required for all significant behavioral effects of the $\alpha 6$ L9'S mutation Voluntary exercise is inversely correlated with the degree of $\alpha 6^*$ nAChR hypersensitivity	Drenan et al., 2008 Drenan et al., 2010 Cohen et al., 2012
$\alpha 7$ L250T	Perinatal death of $\alpha 7$ L250T homozygotes but survival of L250T heterozygotes Hypersensitivity to nicotine-induced seizures Somatodendritic expression of $\alpha 7$ nAChRs on midbrain DA neurons	Orr-Urtreger et al., 2000 Broide et al., 2002 Wooltorton et al., 2003
$\alpha 9$ L9'T	Mutant mice are protected from hearing loss in response to intense noise	Taranda et al., 2009

from neurons in brain slices derived from mice with hypersensitive nAChRs is a viable method for assaying functional expression of a channel of interest without the need for complex pharmacological inhibition of other nAChRs. In contrast to homozygous L250T mice, animals heterozygous for the L250T mutation were viable and fertile (Orr-Urtreger et al., 2000). These mice exhibit augmented nicotine-elicited currents in hippocampal neurons, and are hypersensitive to nicotine-induced seizures (Broide et al., 2002). It is noteworthy that Dani and colleagues demonstrated somatodendritic expression of functional $\alpha 7$ nAChRs in VTA and substantia nigra pars compacta (SNc) DA neurons (Wooltorton et al., 2003), implicating native $\alpha 7$ nAChRs in nicotine action at midbrain DA neuron cell bodies (Fig. 3A). In the future, these mice could prove useful in determining the role of heightened $\alpha 7^*$ nAChR activity in nicotine reward behavior.

2. $\alpha 4$ Nicotinic Acetylcholine Receptor Subunits. After the pioneering proof-of-concept studies on $\alpha 7$ L250T mice, knock-in mice expressing $\alpha 4$ L9'S mutant receptors were reported (Labarca et al., 2001). Like homozygous $\alpha 7$ L250T mice, $\alpha 4$ L9'S homozygous mice died perinatally (Labarca et al., 2001), probably from excitotoxic cell death of midbrain DA neurons (Labarca et al., 2001; Orb et al., 2004). $\alpha 4$ L9'S receptor sensitivity in homozygous animals may have been elevated such that normal levels of extracellular choline were able to constitutively activate $\alpha 4$ L9'S receptors (Labarca et al., 2001; Orb et al., 2004), leading to cell death. Heterozygous $\alpha 4$ L9'S mice retaining the neomycin (neo) selection cassette in the $\alpha 4$ gene were viable and fertile, and they also showed deficits in DA neuron survival, specifically in SNc (Labarca et al., 2001; Orb et al., 2004).

These mice had reduced expression of the mutant subunit, suggesting that a mixture of $\alpha 4$ L9'S and WT alleles was more compatible with survival (Labarca et al., 2001; Orb et al., 2004). Similar to homozygous versus heterozygous $\alpha 7$ L250T mice (Orr-Urtreger et al., 2000), a mixture of mutant and WT $\alpha 4$ alleles may have produced $\alpha 4^*$ nAChRs with only modest changes in channel properties (Labarca et al., 2001). This was a key observation leading to the design of BAC transgenic $\alpha 6$ L9'S mice (Drenan et al., 2008a). Heterozygous $\alpha 4$ L9'S mice have a number of behavioral abnormalities, including elevated anxiety, poor motor learning, locomotor hyperactivity, and hypersensitivity to nicotine-induced reduction in ambulation (Labarca et al., 2001). Midbrain DA neurons survive better in $\alpha 4$ L9'S neo-intact heterozygous animals (Labarca et al., 2001), but ~35% of these neurons die, and the remaining neurons exhibit elevated sensitivity to nicotine (Labarca et al., 2001; Orb et al., 2004). In $\alpha 4$ L9'S heterozygote mice injected with a helper-dependent, cre enzyme-producing adenovirus into SNc, $\alpha 4$ L9'S receptor expression was increased because of cre-mediated removal of the neo selection cassette (Schwarz et al., 2006). Increased $\alpha 4$ L9'S receptor expression was associated with 1) increased DA neuron death due to cholinergic excitotoxicity and 2) a reduction in amphetamine-stimulated locomotion (Schwarz et al., 2006). This approach may be a useful way to inducibly destroy midbrain DA neurons similar to the DA neuron loss seen in Parkinson's disease (Schwarz et al., 2006). $\alpha 4$ L9'S mice also highlight the importance of $\alpha 4^*$ nAChRs in analgesic pathways and mechanisms, because $\alpha 4$ L9'S heterozygotes were 6-fold more sensitive to the antinociceptive effect of nicotine in the hot-plate assay (Damaj et al., 2007).

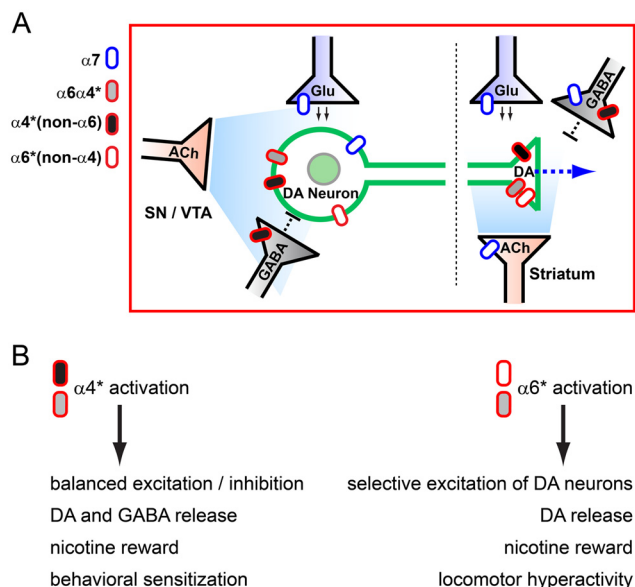


FIG. 3. DA transmission regulated by the nicotinic cholinergic system: insights from hypersensitive nAChR mice. A, DA neurons express both $\alpha 4^*$ (non- $\alpha 6$), $\alpha 6^*$ (non- $\alpha 4$), and $\alpha 4\alpha 6^*$ nAChRs on their soma and dendrites, and a fraction of DA neurons also express $\alpha 7$ nAChRs. $\alpha 7$ nAChRs are also known to be expressed on glutamatergic terminals that synapse onto DA neurons. Midbrain GABAergic neurons express $\alpha 4^*$ (non- $\alpha 6$) nAChRs. Cholinergic input to the ventral midbrain is capable of activating all of these nAChRs. In striatum, DA terminals also express $\alpha 4^*$ (non- $\alpha 6$), $\alpha 6^*$ (non- $\alpha 4$), and $\alpha 4\alpha 6^*$ nAChRs, which respond to local ACh to modulate DA release. Striatal $\alpha 4^*$ (non- $\alpha 6$) nAChRs are known to participate in cholinergic regulation of GABA release. In striatum, $\alpha 7$ nAChRs are expressed in GABAergic and cholinergic cells, but are largely excluded from DA terminals. B, in $\alpha 4$ L9'A mice, selective activation of $\alpha 4^*$ nAChRs largely recapitulates the action of nicotine, producing both activation of DA neurons via the action of somatodendritic $\alpha 4^*$ nAChRs and attenuation of DAergic activity through stimulation of inhibitory GABAergic interneurons. Selective $\alpha 4^*$ activation with nicotine produces nicotine reward as well as behavioral sensitization and tolerance. In $\alpha 6$ L9'S mice, selective activation of $\alpha 6^*$ nAChRs can specifically activate DA neurons and cause elevated DA release and locomotor hyperactivity.

Because $\alpha 4$ L9'S mice express nAChRs with M2 domain mutations that probably increase $\alpha 4^*$ nAChR sensitivity in vivo, they are a useful model of human autosomal-dominant nocturnal frontal-lobe epilepsy (ADNFLE) (Scheffer et al., 1994; Scheffer et al., 1995). ADNFLE is a rare disorder caused by M2 domain mutations in either human $\alpha 4$ or $\beta 2$ nAChR subunits (Sutor and Zolles, 2001). ADNFLE begins in childhood or adolescence and is characterized by seizures occurring during sleep and that probably originate in frontal cortex (Scheffer et al., 1994; Scheffer et al., 1995). Heterozygous $\alpha 4$ L9'S mice exhibit increased sensitivity to nicotine-, epibatidine-, tacrine-, and galanthamine-induced seizures, as well as a pronounced tail dorsiflexion (Straub tail) phenotype at low-to-moderate (0.1–1.0 mg/kg) nicotine doses (Fonck et al., 2003). After these studies, investigators created various knock-in mouse models by expressing the mouse equivalent to human ADNFLE mutations in $\alpha 4$ (Klaassen et al., 2006; Teper et al., 2007) or $\beta 2$ (Xu et al., 2010) nAChR genes. These models further support the notion that ADNFLE is mediated by mutant nAChRs, possibly as a result of augmented nAChR sensitivity (Rodrigues-

Pinguet et al., 2005), changes in $\alpha 4\beta 2^*$ nAChR stoichiometry (Son et al., 2009), or altered GABAergic network activity (Steinlein, 2010).

To overcome the problems associated with neonatal lethality of $\alpha 4$ L9'S homozygote mice, a new $\alpha 4$ nAChR knock-in strain was created with L9'A mutant subunits (Tapper et al., 2004). The $\alpha 4$ L9'A mutation produces a marked (~50-fold) left-shift in the concentration-response relation, but the mutant nAChRs are not activated by circulating levels of choline as $\alpha 4$ L9'S receptors are. As a result, the $\alpha 4$ L9'A mice do not experience excitotoxic depolarization. The $\alpha 4$ L9'A heterozygous and homozygous mice are viable and fertile, and they have led to several important insights into $\alpha 4\beta 2^*$ nAChR function. In 2004, a study on $\alpha 4^*$ nAChRs in nicotine action used the $\alpha 4$ L9'A model to show that $\alpha 4^*$ nAChRs are sufficient for nicotine reward (Tapper et al., 2004). Selective activation of $\alpha 4^*$ nAChRs (which may include $\alpha 4$ (non- $\alpha 6$) $\beta 2$ and $\alpha 4\alpha 6\beta 2^*$ nAChRs; Fig. 3A) with low doses of nicotine led to a conditioned place preference (CPP) for the nicotine-paired chamber in CPP assays. Repeated activation of $\alpha 4^*$ nAChRs in vivo also resulted in behavioral sensitization and/or tolerance to the locomotor-suppressive and hypothermia-producing effects of nicotine in $\alpha 4$ L9'A mice (Tapper et al., 2004, 2007). When combined with parallel experiments on $\alpha 4$ KO mice (Tapper et al., 2007), these studies suggest that $\alpha 4^*$ nAChRs are necessary and sufficient for critical aspects of nicotine's addictive properties. These results were later corroborated by elegant studies using lentiviral re-expression of $\alpha 4$ subunits in VTA of $\alpha 4$ KO mice (Pons et al., 2008; Exley et al., 2011). Studies in $\alpha 4$ L9'A mice also point to a role for $\alpha 4^*$ nAChRs in ethanol consumption and to an opportunity to target these receptors in ethanol addiction (Hendrickson et al., 2010, 2011). Parallel experiments in $\alpha 4$ L9'A mice and $\alpha 4$ KO mice show that $\alpha 4^*$ nAChRs are necessary and sufficient for the ability of varenicline, a $\alpha 4\beta 2$ nAChR partial agonist, to inhibit ethanol consumption (Hendrickson et al., 2010).

Like their $\alpha 4$ L9'S predecessors, $\alpha 4$ L9'A mice are hypersensitive to nicotine-induced seizures (Fonck et al., 2005). $\alpha 4$ L9'A mice also exhibit disturbances in their sleep-wake cycles (Fonck et al., 2005) and respiratory rhythms (Shao et al., 2008). The latter is caused by continuous activation of $\alpha 4^*$ nAChRs in medullary pre-Bötzinger complex inspiratory neurons by ACh, leading to nicotinic cholinergic modulation of hypoglossal nerve (XIIIn) activity (Shao et al., 2008). $\alpha 4$ L9'A mice also demonstrate an imbalance in normal dopamine/acetylcholine cross-talk in the striatum (Zhao-Shea et al., 2010). Challenging $\alpha 4$ L9'A mice with quinpirole, a D_2 DA receptor agonist, caused akinesia, rigidity, tremor, and cataplexy (Zhao-Shea et al., 2010). Patch-clamp recordings from striatal cholinergic interneurons in $\alpha 4$ L9'A brain slices suggested that an interaction between D_2 DA receptors and $\alpha 4^*$ nAChRs, which is functionally

augmented in $\alpha 4$ L9'A mice, may lead to this locomotor abnormality (Zhao-Shea et al., 2010). Together, these studies demonstrate the usefulness of $\alpha 4$ hypersensitive mice in understanding the neurobiological and behavioral aspects of nicotine dependence, ethanol consumption, circadian biology, Parkinson's disease, and epilepsy. As approaches such as 1) creation of mice with multiple nAChR gene mutations and 2) ectopic expression of nAChRs in select brain areas continue to mature, we expect $\alpha 4$ -hypersensitive mice to continue to be useful for studying complex behaviors.

In addition to their utility in studying complex behaviors, $\alpha 4$ L9'A knock-in mice are also useful in mapping brain circuits that either contain $\alpha 4^*$ nAChRs, or are activated by $\alpha 4(+)$ neurons. Taking advantage of the ability to selectively activate $\alpha 4^*$ nAChRs, (Fonck et al. (2009)) injected $\alpha 4$ L9'A mice with low doses of nicotine and searched for activated neurons using c-Fos immunohistochemistry. Using this procedure, they discovered a specific group of neurons in the ventrolateral aspect of the MHB that strongly express $\alpha 4^*$ nAChRs (Fonck et al., 2009). Given the recent appreciation for the MHB and IPN in nicotine consumption (Fowler et al., 2011; Frahm et al., 2011) and cholinergic transmission (Ren et al., 2011), these experiments in $\alpha 4$ L9'A mice suggest a role for $\alpha 4^*$ nAChRs in regulating neurotransmission in the MHB during nicotine exposure. Zhao-Shea et al. (2011) recently used $\alpha 4$ L9'A mice to map $\alpha 4^*$ nAChR expression within the VTA, reporting that $\alpha 4^*$ nAChRs coassemble with $\alpha 6$ subunits preferentially in posterior VTA. As a follow-up, Liu et al. (2012) demonstrated that a population of $\alpha 4^*$ nAChRs in VTA DA neurons may be resistant to desensitization by nicotine. These nAChRs probably contain both $\alpha 4$ and $\alpha 6$ nAChR subunits (Fig. 3A), and their activation by nicotine may drive VTA DA neuron firing for several minutes during smoking (Liu et al., 2012).

3. *$\alpha 6$ Nicotinic Acetylcholine Receptor Subunits.* Since the discovery that $\alpha 6$ nAChR subunits are selectively expressed in relatively few brain areas (Le Novère et al., 1996; Léna et al., 1999), including midbrain DA neurons (Azam et al., 2002), $\alpha 6^*$ nAChRs have been the subject of intense basic and translational research. More than a decade after the first comprehensive study (Kuryatov et al., 2000), the ability to reconstitute $\alpha 6^*$ nAChRs in vitro for biophysical studies or drug screening has been improving (Walsh et al., 2008; Kuryatov and Lindstrom, 2011). Xiao et al. (2011) greatly improved expression of functional $\alpha 6^*$ nAChRs by 1) coexpressing green fluorescent protein-labeled $\alpha 6$ subunits (to better identify transfected cells) (Drenan et al., 2008b) and 2) substituting mutant $\beta 2$ subunits with enhanced endoplasmic reticulum export and plasma membrane expression (Srinivasan et al., 2011).

To learn about the role of $\alpha 6^*$ nAChRs in vivo, and to circumvent the problems associated with $\alpha 6^*$ in vitro expression, we constructed and studied BAC transgenic mice expressing hypersensitive $\alpha 6$ L9'S subunits. $\alpha 6$

L9'S mice have a number of spontaneous and induced behavioral alterations, including home cage hyperactivity, nicotine-stimulated hyperactivity, and reduced wheel rotations (Drenan et al., 2008a, 2010; Grady et al., 2010; Cohen et al., 2012). Patch-clamp recordings from $\alpha 6$ L9'S midbrain slices containing SNc and VTA demonstrated that SNc DA neurons—but not nearby SNr GABA neurons—can be selectively activated by low doses of nicotine (Drenan et al., 2008a) or ACh (R. Drenan, unpublished observations). Electrochemical recordings of DA release from $\alpha 6$ L9'S striatal slices also demonstrated altered frequency-dependent DA release in these mice, because a 1-pulse stimulus induced less DA release and a four-pulse burst stimulus induced greater release compared with control (Drenan et al., 2010).

$\alpha 6$ L9'S mouse behavioral phenotypes, as well as neurochemical and physiological results, were sensitive to genetic manipulations that affected the sensitivity of the $\alpha 6^*$ nAChR pool. For example, a line of $\alpha 6$ L9'S mice with fewer copies of the mutant $\alpha 6$ transgene demonstrated normal locomotor activity and agonist-induced DA release from striatal synaptosomes (Cohen et al., 2012). In addition, crossing the $\alpha 4$ KO strain with $\alpha 6$ L9'S mice removed $\alpha 4$ subunits from $\alpha 6^*$ nAChRs, resulting in 1) a reduced sensitivity of the $\alpha 6^*$ nAChR pool, 2) normalized frequency-dependent DA release from striatal slices, and 3) attenuation of most of the behavioral alterations seen in $\alpha 6$ L9'S mice (Drenan et al., 2010).

These studies indicate that $\alpha 6$ L9'S mice are or may be very useful in studying behaviors involving DA transmission (drug addiction, Parkinson's disease, schizophrenia, attention-deficit/hyperactivity disorder, etc.), and specifically those DA-dependent behaviors that may be influenced by activation of brain cholinergic systems. Collectively, studies with $\alpha 6$ L9'S mice point to the need to maintain a precise level of endogenous nicotinic cholinergic input to DA neurons, because moderate perturbations in the strength of nAChR activation by ACh can induce substantial alterations in DA-dependent behaviors (Cohen et al., 2012). In addition to endogenous ACh, studies with $\alpha 6$ L9'S mice have begun to show the role of $\alpha 6^*$ nAChRs in the response to nicotine (Drenan et al., 2008a, 2010; Grady et al., 2010; Cohen et al., 2012). Recent work implicates $\alpha 6^*$ nAChRs in nicotine reward behavior (Jackson et al., 2009; Brunzell et al., 2010; Gotti et al., 2010; Exley et al., 2011), and $\alpha 6$ L9'S mice will be instrumental in determining whether $\alpha 6^*$ nAChR activation is sufficient to establish and/or maintain nicotine reward.

4. *$\alpha 9$ and $\alpha 10$ Nicotinic Acetylcholine Receptor Subunits.* As discussed in section I, $\alpha 9\alpha 10$ nAChRs are selectively expressed in the cochlea, where they mediate SK2 K^+ channel activation and subsequent inhibition of auditory hair cells (Elgoyhen and Katz, 2012). Taranda et al. (2009) produced $\alpha 9$ nAChR subunit L9'T gain-of-

function mice. In hair cells of $\alpha 9$ L9'T mice, ACh-gated nAChR responses are hypersensitive to agonist and desensitize less compared with responses in WT hair cells (Taranda et al., 2009). Synaptic currents are prolonged in hair cells of $\alpha 9$ L9'T mice, and these mice show reduced hearing loss in response to intense noise relative to WT littermates (Taranda et al., 2009). This study, along with parallel studies in $\alpha 9$ KO mice, show that cholinergic synaptic feedback in the cochlea is important in providing protection from acoustic trauma (Taranda et al., 2009). These mice should continue to be useful in understanding the contribution of the auditory system to complex behaviors, including mating and maternal behavior.

IV. Conceptual Advances in Understanding Dopamine Reinforcement Circuitry

1. $\alpha 7^*$ *Nicotinic Acetylcholine Receptors*. Mice expressing hypersensitive nAChRs have contributed substantially to our understanding of the DA system. Since the seminal work by Mansvelder and McGehee (2000), $\alpha 7$ nAChRs have been strongly implicated in DA reinforcement. Although their work and that of others (Schilström et al., 1998) have implicated $\alpha 7$ nAChRs primarily on glutamatergic presynaptic terminals that synapse onto DA neurons, other results suggested the involvement of $\alpha 7$ nAChRs directly within DA neurons (Pidoplichko et al., 1997). Work with $\alpha 7$ L250T mice directly demonstrated a role for these receptors in DA neuron somata (Wooltorton et al., 2003) (Fig. 3A). These results were corroborated later by Yang et al. (2009), who used acutely dissociated DA neuron preparations to demonstrate expression of functional, $\alpha 7$ nAChR currents in a substantial fraction of DA neurons. In addition, electrophysiology and pharmacology methods have been used to localize $\alpha 7$ nAChR function to several cell types in striatum (Azam et al., 2003; Xiao et al., 2009) (Fig. 3A). Additional work is required, however, to further define the role of $\alpha 7^*$ nAChRs in striatum.

2. $\alpha 4^*$ versus $\alpha 6^*$ *Nicotinic Acetylcholine Receptors*. The role of $\beta 2^*$ nAChRs in DA transmission has also been elucidated in part via mice expressing hypersensitive $\alpha 4$ and $\alpha 6$ nAChR subunits. In particular, $\alpha 4$ L9'A and $\alpha 6$ L9'S mice have revealed subtle but important differences for $\alpha 4$ versus $\alpha 6$ subunits in the DA reward system. $\alpha 4$ L9'A mice lack dramatic spontaneous behavioral phenotypes (Tapper et al., 2004, 2007; Fonck et al., 2005). Most results from $\alpha 4$ L9'A mice injected with $\alpha 4$ -specific doses of nicotine (which activate $\alpha 4(\text{non-}\alpha 6)\beta 2^*$ and $\alpha 4\alpha 6\beta 2^*$ nAChRs), such as nicotine CPP, behavioral sensitization, locomotor suppression and nicotine-induced seizures, largely recapitulate the effects of nicotine in WT mice (Tapper et al., 2004, 2007). These data suggest that $\alpha 4^*$ nAChRs function within DA system circuits such that selective activation of these receptors maintains a roughly WT-like balance in

excitatory, inhibitory, or modulatory transmission (Fig. 3B). This may be due largely to the expression of $\alpha 4\beta 2^*$ nAChRs within both DA neurons and the cells that tonically inhibit them, midbrain GABAergic interneurons (Nashmi et al., 2007) (Fig. 3A). Experiments with $\alpha 4$ KO (McClure-Begley et al., 2009) mice also implicate $\alpha 4^*$ nAChRs in striatal GABA release, which may affect DA transmission via cholinergic signaling (Witten et al., 2010) (Fig. 3A).

In contrast, $\alpha 6$ L9'S mice exhibit various locomotor hyperactivity phenotypes, including spontaneous home cage hyperactivity (Drenan et al., 2008a) (Fig. 3B). In addition, in contrast to results with $\alpha 4$ L9'A mice, repeated daily nicotine administration to $\alpha 6$ L9'S mice does not lead to behavioral tolerance or sensitization (Tapper et al., 2004, 2007; Drenan et al., 2008a) (Fig. 3B). $\alpha 6^*$ nAChRs are strongly implicated in nicotine reward (Jackson et al., 2009; Brunzell et al., 2010; Gotti et al., 2010; Exley et al., 2011), and experiments in $\alpha 6$ L9'S mice testing the role of these receptors are a major priority. These results, when coupled with the ability of nicotinic agonists to selectively activate $\alpha 6$ L9'S DA neurons without stimulating local, inhibitory GABAergic neurons (Drenan et al., 2008a) (Fig. 3A), suggest differing natural roles for $\alpha 4$ versus $\alpha 6$ subunits in the DA system. $\alpha 4\beta 2^*$ nAChRs may be general-purpose, high-sensitivity ACh receptors important for modulating circuit activity in response to cholinergic tone. On the basis of their extremely high sensitivity to ACh (Salminen et al., 2007) and peculiar expression pattern in the brain (Le Novère et al., 1996; Léna et al., 1999), $\alpha 6$ nAChR subunits may confer a more specialized property to nAChR pentamers. More experiments are needed to address this hypothesis.

V. Implications for Development of Therapeutics

nAChR preclinical drug development involves in vitro studies on heterologously expressed ion channels, followed by animal studies to probe both safety and efficacy of the candidate drug. Although the safety of a candidate drug is often reliably tested in animals, efficacy cannot readily be evaluated in the absence of an appropriate assay, where one can be confident that the target receptor is being activated. $\alpha 4$ L9'A and $\alpha 6$ L9'S mice, because of their ability to report selective $\alpha 4^*$ or $\alpha 6^*$ nAChR activation, offer a solution to some of the problems associated with in vivo efficacy testing. In particular, assays that use a behavioral or physiological readout that is specific for the nAChR in question provide instant information regarding a drug's bioavailability. Among several behavioral readouts that result from selective $\alpha 4^*$ nAChR activation in $\alpha 4$ L9'A mice, nicotine-induced hypothermia has proven useful (Tapper et al., 2004). In this assay, mice are implanted with subcutaneous temperature probes that transmit data to a nearby receiver that is connected to a computer (Tapper

et al., 2004, 2007). Mice are administered nicotine or a drug candidate, and body temperature is recorded. Whereas WT mice typically become hypothermic at nicotine doses ranging from 0.5 to 10.0 mg/kg, hypothermia in $\alpha 4$ L9'A mice can be induced selectively through $\alpha 4^*$ nAChRs at doses of 0.01 to 0.03 mg/kg (Tapper et al., 2004, 2007). We used this assay recently in a secondary screen of a small library of compounds for their ability to activate or block $\alpha 4^*$ nAChRs in vivo (Grady et al., 2010).

In $\alpha 6$ L9'S mice, $\alpha 6^*$ nAChRs can be studied in a similar manner using locomotor behavioral assays (Drenan et al., 2008a). In these mice, nicotine selectively activates $\alpha 6^*$ nAChRs and briefly (5–10 min) induces locomotor activity at doses of 0.02 to 0.15 mg/kg (Drenan et al., 2008a). We found that compounds with $\alpha 6$ -selectivity in vitro were able to strongly activate locomotion in vivo (Drenan et al., 2008a). In addition, we used this assay to show that $\alpha 4$ -selective drugs can weakly induce locomotor activity in $\alpha 6$ L9'S mice, implicating $\alpha 4\alpha 6^*$ nAChR pentamers in this locomotor response (Drenan et al., 2008a). These results were later corroborated by studies in $\alpha 6$ L9'S mice that were crossed to the $\alpha 4$ KO (Drenan et al., 2010). Such results are useful in providing a detailed description of the important nAChR subtypes giving rise to behavioral effects in these mice.

Studies in $\alpha 4$ and $\alpha 6$ hypersensitive mice, such as those noted above, are generally useful in identifying compounds that activate or antagonize these receptor subtypes and could improve nAChR drug development for several diseases/disorders that implicate nicotinic cholinergic control of dopamine neurotransmission, including nicotine dependence, alcohol dependence, and Parkinson's disease. Varenicline, a cytosine-derived $\alpha 4\beta 2^*$ and $\alpha 6\beta 2^*$ nAChR partial agonist (Grady et al., 2010), is used as a smoking cessation aid and has been demonstrated to reduce ethanol consumption in rats (Steensland et al., 2007) and mice (Hendrickson et al., 2010, 2011). Varenicline exerts these effects primarily through VTA neurons and the DA system (Hendrickson et al., 2010, 2011) but has undesirable side effects such as GI disturbances (Hays et al., 2008). These results suggest that future nAChR-directed drug therapies for smoking or alcohol-use cessation could include compounds that more selectively target DA system nAChRs, perhaps by selectively activating $\alpha 4\alpha 6\beta 2^*$ nAChRs that are more selectively expressed in DA neurons (Drenan et al., 2010).

Whereas smoking cessation and reduction of alcohol-use may be effectively treated with nAChR partial agonists, full nAChR agonists may be useful in treating Parkinson's disease. PD involves a reduction in forebrain DA levels, and $\alpha 6$ -specific agonists may be very useful in stimulating DA release from residual, surviving DA terminals (Quik and McIntosh, 2006). $\alpha 6$ -activating drugs could be combined with levodopa therapy to potentially allow lower doses of levodopa to effectively control PD symptoms. This approach could delay or fully

prevent the development of levodopa-induced dyskinesias (Quik et al., 2008).

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Wrote or contributed to the writing of the manuscript: Drenan and Lester.

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