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Low concentrations of nicotine differentially desensitize nicotinic acetylcholine receptors that include $\alpha 5$ or $\alpha 6$ subunits and that mediate synaptosomal neurotransmitter release

Sharon R. Grady^a, Charles R. Wageman^a, Natalie E. Patzlaff^{a,1}, and Michael J. Marks^a

^aInstitute for Behavioral Genetics, University of Colorado, Boulder, CO 80309

Abstract

Desensitization is a complex property of nicotinic acetylcholine receptors (nAChR). Several subtypes of nAChR have high sensitivity to nicotine and mediate effects of nicotine at concentrations found in blood of tobacco smokers. Desensitization of some of these receptor subtypes has been studied in model systems, however, other subtypes have been difficult to express heterologously in native forms. In addition, model systems may not have the same accessory molecules and post-translational modifications found in native populations. We have used wild-type and subunit null mutant mice to study desensitization properties of the high sensitivity $\alpha 4\beta 2$ -nAChRs including those that have $\alpha 5$ subunits at both GABAergic and dopaminergic nerve terminals. In addition, we have studied the desensitization of one subtype of $\alpha 6\beta 2$ -nAChRs at dopaminergic terminals using $\alpha 4$ subunit null mutant mice. Exposure to low nicotine concentrations, leads to rapid, but partial desensitization of activity mediated by these receptors. $\alpha 4\beta 2$ -nAChRs including $\alpha 5$ subunits show faster rates of recovery from desensitization than $\alpha 4\beta 2$ -nAChRs without $\alpha 5$. Inclusion of the $\alpha 5$ subunit significantly shifts the concentration response for desensitization to higher values, indicating that receptors with $\alpha 5$ subunits are less desensitized by a 10-min exposure to low concentrations of nicotine. Receptors with $\alpha 6$ subunits appear to desensitize to a lesser degree than those with $\alpha 4$ subunits, indicating that $\alpha 6\beta 2$ -nAChRs are somewhat resistant to desensitization by nicotine. These results highlight the importance of studying various receptor subtypes in native systems and how they may differentially respond to nicotine and to nicotinic drugs.

Keywords

nicotine; desensitization; chrna5 subunit; chrna6 subunit; dopamine; GABA

1. Introduction

Nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels known to desensitize with prolonged exposure to agonists (Changeux et al., 1984). A cyclical scheme to explain the process of desensitization of nAChRs at the neuromuscular junction was

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Corresponding author: Sharon R. Grady, phone: 303-492-9677, fax: 303-492-8063, sharon.grady@colorado.edu; postal address: University of Colorado, Institute for Behavioral Genetics, 447UCB, Boulder, CO 80309 .

¹Present address: Department of Pharmacology, University of Wisconsin, Madison, WI 53706

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proposed in 1957 by Katz and Thesleff ((Katz and Thesleff, 1957). Since that time it has been recognized that all nAChRs are allosteric proteins that desensitize with varying kinetics (Changeux et al., 1984; Quick and Lester, 2002; Taly et al., 2009). Multiple states of desensitization and/or inactivation exist and many factors, including subunit composition, association with other proteins, lipids, ions, and/or accessory molecules (possibly unique to each cell type), as well as post-translational modifications, are likely to affect desensitization and inactivation (Ibanez-Tallon et al., 2002; Cheng et al., 2009; Neff et al., 2009; Taly et al., 2009).

The $\beta 2^*$ -subtype is the most abundant nAChR in the CNS [* indicates presence of other subunits(Lukas et al., 1999)]. The propensity of the $\beta 2^*$ -nAChR to desensitize with long-term exposure to even low concentrations of agonists, such as nicotine, is a complex property of these receptors (Lippiello et al., 1987; Fenster et al., 1999; Quick and Lester, 2002; Gentry et al., 2003; Guo and Lester, 2007; Taly et al., 2009; Papke et al., 2011). Desensitization by an agonist depends on the concentration and length of exposure, with long exposures resulting in deeper states of desensitization that recover more slowly (Quick and Lester, 2002).

The most common $\beta 2^*$ -nAChR is the $\alpha 4\beta 2$ -nAChR which exists in both a high sensitivity (HS) form with the subunit stoichiometry $(\alpha 4\beta 2)_2\beta 2$ and a low sensitivity (LS) form $(\alpha 4\beta 2)_2\alpha 4$ (Zwart and Vijverberg, 1998; Zhou et al., 2003; Marks et al., 2007; Gotti et al., 2008). The diversity of $\alpha 4\beta 2^*$ -nAChR is further complicated by the $\alpha 5$ subunit, expressed in certain neurons that can assemble as $(\alpha 4\beta 2)_2\alpha 5$ -nAChR, another HS form (Tapia et al., 2007; Bailey et al., 2010). The $\alpha 5$ subunit is an accessory subunit and not part of the ligand binding domain (Kuryatov et al., 2008), although recently a ligand binding site at the $\alpha 4\alpha 4$ interface has been described (Harpsoe et al., 2011; Mazzaferro et al., 2011), raising the possibility that $\alpha 4\alpha 5$ or $\alpha 5\beta 2$ may be tertiary binding sites. The role of the $\alpha 5$ subunit in altering function of $\alpha 4\beta 2^*$ -nAChRs has been studied in expression systems. In one study in oocytes, addition of the $\alpha 5$ subunit resulted in larger currents and more desensitization (Ramirez-Latorre et al., 1996). In another study using tandem constructs to better control populations of receptors, the $(\alpha 4\beta 2)_2\alpha 5$ -nAChR showed equally high sensitivity as $(\alpha 4\beta 2)_2\beta 2$, but with considerably increased Ca^{++} permeability (Tapia et al., 2007). The HS nature of the $(\alpha 4\beta 2)_2\alpha 5$ -nAChR has also been demonstrated in mouse brain by $^{86}\text{Rb}^+$ efflux experiments using synaptosomal preparations from striatum and thalamus (Brown et al., 2007) and in a transgenic mouse in cortico-thalamic terminals (Heath et al., 2010), as well as by $[^3\text{H}]$ -GABA release in cortex (McClure-Begley et al., 2009) and $[^3\text{H}]$ -DA release in striatum (Grady et al., 2010a). In layer VI cortical neurons, the $\alpha 5$ accessory subunit forms HS receptors with $\alpha 4$ and $\beta 2$ subunits, and the presence of the $\alpha 5$ subunit protects against desensitization (Bailey et al., 2010).

Another important but less widely expressed $\beta 2^*$ -nAChR contains the $\alpha 6$ subunit as a ligand binding subunit, and forms nAChRs of predominately the $(\alpha 6\beta 2)_2\beta 3$ and $(\alpha 6\beta 2)(\alpha 4\beta 2)\beta 3$ subtypes which are expressed in DAergic terminals [for review see (Quik et al., 2011)]. Both of these subtypes are HS forms, with the latter having the highest sensitivity for activation by nicotine of any reported subtype (Salminen et al., 2007).

All HS subtypes respond to low concentrations of nicotine [EC_{50} values of $\sim 1 \mu\text{M}$ or lower and nM DC_{50} values (half-maximal effective concentration for desensitization)]. These subtypes will mediate effects of nicotine *in vivo* [with concentrations measured in venous blood of smokers reported to be in the range of 10-50 ng/ml equivalent to 60-310 nM (Jarvik et al., 2000; Brody, 2006)].

Furthermore, genome wide association studies have implicated the $\alpha 5$ and $\alpha 6$ subunits in aspects of smoking dependence [for review see (Greenbaum and Lerer, 2009)]. Thus, information on how these subunits influence desensitization properties of the $\beta 2^*$ -nAChR is of major importance in understanding the actions of nicotine as well as the actions of various therapeutic drugs (Picciotto et al., 2008; Andreasen et al., 2009; Buccafusco et al., 2009; Mansvelder et al., 2009; Taly et al., 2009).

This study was undertaken to assess the roles of $\alpha 5$ and $\alpha 6$ subunits on desensitization properties of $\beta 2^*$ -nAChRs from native tissues which have intact the accessory molecules and post-translational modifications unique to neurons. Using $\alpha 5$ subunit null mutant mice ($\alpha 5$ KO) as well as wild-type (WT) mice, desensitization of $(\alpha 4\beta 2)_2\beta 2$ and $(\alpha 4\beta 2)_2\alpha 5$ was studied on GABA terminals. Similar methods with $\alpha 5$ KO and $\alpha 6$ KO mice and the selective antagonist α -conotoxin MII (α -CtxMII) were used to compare these same subtypes on DAergic terminals. In addition, using $\alpha 4$ KO mice, the desensitization of $\alpha 6\beta 2^*$ -nAChR on dopaminergic terminals was assessed.

2. Methods

2.1. Mice

Mice were bred and housed at the Institute for Behavioral Genetics, University of Colorado (Boulder, CO). All subunit null mutant mice were bred onto the C57BL/6 background for minimum of 10 generations. A 12 hour light/dark cycle was maintained (lights on 7AM to 7PM), at 22°C and mice had free access to food (Teklad Rodent Diet, Harlan, Madison WI) and water. Genotypes were assessed by PCR as previously described (Salminen et al, 2004, 2007). The $\alpha 4$ null mutant mice ($\alpha 4$ KO) were originally obtained from Dr. John Drago (University of Melbourne, Victoria, Australia), the $\alpha 5$ KO mice from Dr. Arthur Beaudet (Baylor College, Houston TX) and the $\alpha 6$ KO mice from Dr. Uwe Maskos (Pasteur Institute, Paris, France). The guidelines for care and use of mice provided by the National Institutes of Health were followed and all protocols were approved by the Animal Care and Utilization Committee of the University of Colorado, Boulder. In addition, all efforts were made to minimize the number of animals used.

2.2. Materials

[3 H]- γ -aminobutyric acid (GABA) (25-40 Ci/mmol), [3 H]-dopamine (DA) (20-40 Ci/mmol) and Optiphase SuperMix scintillation cocktail were purchased from Perkin-Elmer Life and Analytical Sciences (Boston, MA). α -Conotoxin MII (α -CtxMII) was obtained from Dr. J. Michael McIntosh (University of Utah, Salt Lake City, UT). The following were products of Sigma-Aldrich (St. Louis, MO): bovine serum albumin (BSA), (-)-nicotine tartrate, 1-[2-[[[(diphenylmethylene)imino]oxy]ethyl]-1,2,5,6-tetrahydro-3-pyridinecarboxylic acid hydrochloride (NO-711), atropine sulphate, pargyline, diisopropylfluorophosphate (DFP), aminooxyacetic acid and nomifensine. N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES) and sodium salt were products of BDH Chemicals distributed by VWR (Denver, CO). All other chemicals were reagent grade.

2.3. Crude synaptosomal neurotransmitter release

For [3 H]-GABA release, the methods of (McClure-Begley et al., 2009) were followed. Briefly, mice were killed by cervical dislocation, the brain removed and dissected on ice. The regions of interest were homogenized in ice-cold isotonic sucrose buffered with HEPES (5 mM, pH 7.4) using 0.5 ml for thalamus and 1 ml for cortex in a glass-teflon homogenizer. After dilution (final volume 2 ml for thalamus; 3 ml for cortex), the crude synaptosomal preparation was divided into aliquots sufficient for 8 filters (1 ml thalamus; 0.25 ml cortex) and centrifuged at 4°C, 12,000g for 20 min. Pellets were resuspended in 0.8 ml of GABA

uptake buffer (128 mM NaCl, 2.4 mM KCl, 3.2 mM CaCl₂, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 10 mM glucose, 1.25 mM aminooxyacetic acid, 25 mM HEPES, pH 7.5). After incubation for 10 min at 37°C, [³H]-GABA (final concentration ~0.1 μM) and DFP (final concentration 10 μM) were added and the incubation continued for 10 min longer. At this point aliquots (80 μl) were pipetted onto individual filters for superfusion at 22°C at 0.7 ml/min with GABA perfusion buffer (uptake buffer without aminooxyacetic and with atropine (1 μM), NO-711 (100 nM) and 0.1% BSA added). For Ca⁺⁺-free buffer, an equi-osmotic amount of Na⁺ was substituted. Protocols illustrated in Figure 1 and described below were followed. The perfusate of interest was collected into 96-well plates as 10-s fractions using a Gilson F204 fraction collector (Middleton WI). After addition of 0.15 ml of Optiphase SuperMix scintillation cocktail, radioactivity was determined in a 1450 MicroBeta Trilux counter (Perkin Elmer Life Sciences - Wallac Oy, Turku, Finland).

For [³H]-dopamine release, the methods of (Salminen et al., 2007) were used. The above procedure was used with the following modifications. Striatal tissue was dissected and homogenized in 0.5 ml ice-cold isotonic sucrose buffered with HEPES (5 mM, pH 7.4); diluted to 2 ml and 0.5 ml aliquots centrifuged. For DA uptake buffer, aminooxyacetic acid was omitted and ascorbic acid (1 mM) and pargyline (10 μM) were added. After the initial 10 min incubation at 37°C, [³H]-DA (~0.1 μM) and DFP (final concentration=10 μM) were added and the incubation continued for 5 min. DA superfusion buffer consisted of DA uptake buffer with atropine (1 μM), nomifensine (1 μM) and 0.1% BSA added. When α-CtxMII (50 nM) was used, it was included in the superfusion buffer for 5 min prior to the stimulation.

2.4. Experimental protocols

Using crude synaptosomal preparations and [³H]-neurotransmitter release as our functional assay, we measured desensitization by nicotine at several concentrations and recovery from desensitization for various nAChR subtypes. In order to avoid any influx of Ca⁺⁺ that may lead to various intra-synaptosomal changes during the desensitization phase (Rusakov, 2006), we have conducted this portion of the experiment in Ca⁺⁺-free buffer. This design necessitates a switch to buffer containing Ca⁺⁺ for the functional measurements (see Figure 1 for a diagram of perfusion protocols). The use of Ca⁺⁺-free buffer for superfusion results in a measurably lower baseline than the baseline with superfusion buffer containing Ca⁺⁺ (see Figure 2 for representative superfusion data). Upon switching to Ca⁺⁺-containing buffer, the baseline is elevated immediately and in approximately 20-30 seconds establishes a new level baseline, approximately twice the previous baseline. In the first 20-30s, there is a small rise higher than the new baseline (a “shift peak”). This shift peak is not affected by nicotine in the Ca⁺⁺-free buffer. For example, in n = 6 experiments, the mean shift peak was 2.39 ± 0.22 units for the no nicotine condition and 2.54 ± 0.27 for 300 nM nicotine, both in the Ca⁺⁺-free buffer for 10 min [the conditions shown in Figure 2 and where units are [(cpm released – baseline cpm)/(baseline cpm)]. Parallel samples were always used to determine this shift peak and it was subtracted from the release stimulated for 20s by either 10 μM ACh or 20 mM K⁺, when these were perfused simultaneously with the buffer switch (also shown in Figure 2). We have used 20 mM K⁺-induced release as a control for the effect of nicotine vs the effect of [Ca⁺⁺] change (see Results). K⁺-induced release was not affected by the prior exposure of the samples to nicotine in the Ca⁺⁺-free buffer.

Using the α4KO, α5KO and α6KO mice as well as the selective α6β2* antagonist, α-CtxMII, to alter the receptor subtypes being measured, we have compared α4β2- and α4α5β2-nAChRs on both DAergic and GABAergic synaptosomes as well as α6β2β3-nAChRs on DAergic synaptosomes as follows:

1. WT mice for GABA release from cortex mediated by α4β2 plus α4α5β2-nAChR

2. $\alpha 5$ KO mice for GABA release from cortex mediated by $\alpha 4\beta 2$ -nAChR only
3. WT mice for GABA release from thalamus mediated by $\alpha 4\beta 2$ -nAChR only
4. $\alpha 6$ KO mice for DA release from striatum mediated by $\alpha 4\beta 2$ plus $\alpha 4\alpha 5\beta 2$ -nAChR
5. $\alpha 5$ KO mice with α -CtxMII block for DA release from striatum mediated by $\alpha 4\beta 2$ -nAChR only
6. $\alpha 4$ KO mice for DA release from striatum mediated by $\alpha 6\beta 2\beta 3$ -nAChR only

Regions assayed were chosen based on previous data indicating significant function mediated by receptors with $\alpha 5$ subunits (McClure-Begley et al., 2009). GABA release is ~50% lower in CX in the $\alpha 5$ KO mouse than in WT ((McClure-Begley et al., 2009). DA release from striatum mediated by $\alpha 4\beta 2^*$ is ~70% lower in the $\alpha 5$ KO mouse than in WT (Grady et al, 2010a). In addition, thalamus was assayed from WT mice for GABA release as a region without significant $\alpha 4\beta 2\alpha 5$ -nAChR on GABA terminals ((McClure-Begley et al., 2009).

2.5. Data analysis

Analysis by non-linear curve-fitting was, in all cases, to all the individual data points using SigmaPlot and the following equations:

Onset— $f = V * (e^{-kt}) + V_r$ where V is the desensitizable amount of release, V_r is the remaining, undesensitized portion of release, t is the time of exposure to nicotine (300 nM), and k is the rate constant for onset of the effect of nicotine in min^{-1} .

Recovery—Equation: $f = V * (1 - e^{-kt}) + V_r$ where V is the desensitized but recoverable amount of release, V_r is the remaining, undesensitized portion of release, t is the time of recovery in min, k is the rate constant for recovery in min^{-1} . The half-time for recovery (τ) = $0.693/k$ min. Constraints applied: for GABA: $V + V_r = 100$, and for DA: $V + V_r = \text{value at 30 min (87 or 86)}$.

DC₅₀—Equation: $f = V / (1 + ([\text{nic}] / \text{DC}_{50})) + V_r$ where V is the maximum desensitized but recoverable amount of release, V_r is the remaining undesensitized portion of release, $[\text{nic}]$ is the concentration of nicotine used for desensitization (10 min, nM) and DC_{50} is the concentration of nicotine (nM) necessary for half-maximal effect.

For all curve fits, after all three variables were determined, V and V_r were fixed and k or DC_{50} recalculated for better accuracy. The half-time (τ) = $0.693/k$ min.

Statistics—One-way ANOVA with the Duncan post-hoc test was used to determine significant differences between variables determined by curve-fits.

3. Results

3.1 Measurement of desensitization

Neurotransmitter release assays measure an event that occurs several steps beyond the actual ion flux through the nAChR. Consequently, the potential for measuring desensitization of other steps in the chain of events leading to neurotransmitter release exists. However, in order to measure the effect of the $\alpha 5$ and $\alpha 6$ subunits, this approach may be our only way of isolating populations of nAChR. Therefore, we explored the use of Ca^{++} -free buffer during the desensitizing exposure to see if we could prevent many of the downstream changes that might occur with ion flux via small amounts of channel opening with exposure to low concentrations of nicotine. Following the procedures illustrated in Figure 1, data of the type

presented in Figure 2 were obtained. The baseline release, as shown previously, is lower in the absence of Ca^{++} indicating that some of the baseline is active vesicle-mediated release. When buffer is switched to Ca^{++} -containing buffer, a new baseline is established with a small increase over the new baseline for about 30s (shift peak). This small increase was found to be independent of exposure to nicotine during the Ca^{++} -free superfusion. In addition, release stimulated by exposure to high K^+ (20 mM for 20s), was also independent of nicotine exposure. Furthermore, the shift peak and the K^+ -stimulated release are independent of nicotine exposure for both [^3H]-GABA and [^3H]-DA release as well as for WT and all null mutant mouse brain regions assayed. Therefore, these assays are likely able to distinguish desensitization properties of the different nAChR populations found on the selected nerve terminals.

3.2. Onset of desensitization

For measurements of onset of inhibition by 300 nM nicotine, protocol 1 illustrated in Figure 1, was followed. For this protocol, all samples were superfused with Ca^{++} -free buffer for 10 min while time of exposure to 300nM nicotine varied between 0 and 10 min.

[^3H]-GABA release from crude synaptosomal preparations of cortex was stimulated by 10 μM ACh (a concentration that activates only HS forms of nAChRs) to measure the onset of desensitization from GABA terminals. A significant fraction (~50%) of [^3H]-GABA release from cortex of WT mice is mediated by $(\alpha 4\beta 2)_2\alpha 5$ -nAChR (McClure-Begley et al., 2009). In order to isolate the $(\alpha 4\beta 2)_2$ -nAChR subtype, we assayed [^3H]-GABA release from crude synaptosomal preparations of cortex from $\alpha 5\text{KO}$ mice. We compared results with WT mice in which this assay measures a combination of the HS forms, $(\alpha 4\beta 2)_2\beta 2$ - and $(\alpha 4\beta 2)_2\alpha 5$ -nAChR to data from $\alpha 5\text{KO}$ mice which measures $(\alpha 4\beta 2)_2$ -nAChR selectively. The results of these [^3H]-GABA release experiments are shown in Figure 3a and Table 1. Onset of desensitization was quite rapid, and leveled off at a maximum 48-56% of control response (samples treated with Ca^{++} -free buffer but without nicotine). For $\alpha 5\text{KO}$ mice, half-time (τ) was 0.39 ± 0.04 min, and for WT mice, $\tau \leq 0.17 \pm 0.06$ min (note that the onset of desensitization could actually be faster as the first time points measurable with this assay are longer than the calculated half-time). Figure 3a also shows a control response for the stimulation of [^3H]-GABA release by K^+ (20 mM) indicating that nicotine treatment for 10 min has no effect on this response and, therefore, the treatment is not affecting the release process in general, but only that mediated by nAChRs.

Similarly, we used the [^3H]-DA release assay with crude synaptosomal preparations from striatum to assess desensitization of various nAChR subtypes. We have previously shown that significant (~70%) DA release activity in this preparation is mediated by the $(\alpha 4\beta 2)_2\alpha 5$ -nAChR (Salminen et al, 2004; Grady et al, 2010a). Onset of desensitization was measured from crude synaptosomal preparations of striatum from $\alpha 6\text{KO}$ mice (in which combination of $(\alpha 4\beta 2)_2\beta 2$ - and $(\alpha 4\beta 2)_2\alpha 5$ -nAChRs are retained) and from $\alpha 5\text{KO}$ mice using α -CtxMII to block all $\alpha 6^*$ -nAChRs (to measure only $(\alpha 4\beta 2)_2\beta 2$ -nAChR). This comparison is shown in Figure 3b and Table 1. The results found with this assay were similar to those for the [^3H]-GABA release in cortex. For $(\alpha 4\beta 2)_2\beta 2$ -nAChR alone, $\tau = 0.32 \pm 0.03$ and for the combination of $(\alpha 4\beta 2)_2\beta 2$ - and $(\alpha 4\beta 2)_2\alpha 5$ -nAChRs, $\tau \leq 0.18 \pm 0.05$, with a maximum of 48-60% of control (note that the onset of desensitization could actually be faster as the first time points measurable with this assay are longer than the calculated half-time). Again the K^+ -stimulated release is not affected by treatment with nicotine.

We used the [^3H]-DA release assay with crude synaptosomal preparations from $\alpha 4$ null mutant mice to measure the onset of desensitization of $\alpha 6^*$ -nAChR. This design isolates the $(\alpha 6\beta 2)_2\beta 3$ -nAChR. Unfortunately, we have no way of isolating the more complex, and likely important subtype, $(\alpha 4\beta 2)(\alpha 6\beta 2)\beta 3$ -nAChR, as there is no sufficiently selective

inhibitor of $\alpha 4\beta 2^*$ (non $\alpha 6$)-nAChRs, available. The results of these experiments are presented in Figure 3c and Table 1. These receptors rapidly desensitized ($\tau \leq 0.052 \pm 0.008$) (Note that the onset of desensitization could actually be faster as the first time points measurable with this assay are longer than the calculated half-time), but to a lower extent (25%) than the $\alpha 4\beta 2^*$ subtypes (48-60%). As for previous experiments, K⁺-stimulated release was not affected by treatment with nicotine.

Because of the rapid rates of desensitization, these measurements of half-times are underestimates as noted above and were not analyzed by ANOVA. Significant differences were found for percent desensitization by one-way ANOVA ($F(4,10)=14.51$, $P<0.001$ with only the $\alpha 4KO$ by DA release ($(\alpha 6\beta 2)_2\beta 3$ -nAChR) differing from all other groups and showing a lower extent of desensitization.

3.3. Recovery from desensitization

Using protocol 2 shown in Figure 1, we measured the recovery from desensitization induced by 10 min of exposure to 300 nM nicotine in Ca⁺⁺-free buffer. Using the [³H]-GABA release assay and crude synaptosomal preparation from cortex of WT or $\alpha 5KO$ mice, recovery was assessed by delaying the 20s test stimulation of 10 μ M ACh for various lengths of time following cessation of the exposure to nicotine in Ca⁺⁺ free medium. Ca⁺⁺ was present during the recovery phase. Results of these experiments are presented in Figure 4a and Table 2. Recovery was essentially complete by 30 min and was faster in WT than in $\alpha 5KO$ mice, indicating that $(\alpha 4\beta 2)_2\alpha 5$ -nAChRs recover more quickly than $(\alpha 4\beta 2)_2\beta 2$ -nAChRs.

Recovery was also assessed for these two subtypes using the [³H]-DA release assay and crude striatum synaptosomal preparations from $\alpha 6KO$ mice (combination of $(\alpha 4\beta 2)_2\alpha 5$ - and $(\alpha 4\beta 2)_2\beta 2$ -nAChRs) and in $\alpha 5KO$ mice with α -CtxMII to block all $\alpha 6^*$ -nAChRs ($(\alpha 4\beta 2)_2\beta 2$ -nAChR only). These data are shown in Figure 4b and Table 2. Recovery was not quite complete but had leveled off by 30 min. As for the [³H]-GABA release assay, presence of the $\alpha 5$ subunit increased the rate of recovery.

The rate of recovery of the $(\alpha 6\beta 2)_2\beta 3$ -nAChRs was measured using the [³H]-DA release assay with crude synaptosomal preparations from striatum of $\alpha 4KO$ mice. These data are shown in Figure 4c and Table 2. Complete recovery was seen with a rate similar to the $(\alpha 4\beta 2)_2\beta 2$ -nAChR subtype.

Analysis by one-way ANOVA for half-time of recovery for all 5 groups (Table 2) showed significant differences ($F(4,10)=12.44$, $P=0.001$). By Duncan's post-hoc test ($P<0.05$), there were three subsets, group 1: WT by GABA release and $\alpha 6KO$ by DA release (both assays for combination of $(\alpha 4\beta 2)_2\beta 2$ - and $(\alpha 4\beta 2)_2\alpha 5$ -nAChRs), group 2: the $\alpha 4KO$ by DA release [$(\alpha 6\beta 2)_2\beta 3$ -nAChR] and $\alpha 5KO$ with α -CtxMII by DA release [$(\alpha 4\beta 2)_2\beta 2$ -nAChR only] and group 3: the $\alpha 4KO$ by DA release [$(\alpha 6\beta 2)_2\beta 3$ -nAChR only] and $\alpha 5KO$ by GABA release [$(\alpha 4\beta 2)_2\beta 2$ -nAChR only]. By this analysis, $(\alpha 4\beta 2)_2\alpha 5$ recovered significantly faster than the other subtypes. The $(\alpha 4\beta 2)_2\beta 2$ in DA terminals subtype recovered faster than in GABA neurons, while the $(\alpha 6\beta 2)_2\beta 3$ subtype did not significantly differ from either measure of $(\alpha 4\beta 2)_2\beta 2$ in recovery half-time.

Significant differences were also found for % desensitization by one-way ANOVA ($F(4,10)=10.28$, $P=0.001$ with group 1: the $\alpha 4KO$ by DA release ($(\alpha 6\beta 2)_2\beta 3$ -nAChR), group 2: WT by GABA release and $\alpha 6KO$ by DA release (both assays for combination of $(\alpha 4\beta 2)_2\beta 2$ - and $(\alpha 4\beta 2)_2\alpha 5$ -nAChRs) and $\alpha 5KO$ with α -CtxMII by DA release [$(\alpha 4\beta 2)_2\beta 2$ -nAChR only] and group 3: the $\alpha 5KO$ with α -CtxMII by DA release and $\alpha 5KO$ by GABA release [both assays for $(\alpha 4\beta 2)_2\beta 2$ -nAChR only] plus the $\alpha 6KO$ by DA release [$(\alpha 4\beta 2)_2\beta 2$ -

nAChR only]. Therefore, by this analysis, $(\alpha 6\beta 2)_2\beta 3$ showed significantly less desensitization than the other subtypes after the 10 min exposure to nicotine replicating the data for onset.

3.4. Concentration dependence of desensitization

Desensitization by varying concentrations of nicotine exposure for 10 min in Ca^{++} -free buffer was determined for various subtypes of nAChR using protocol 3 from Figure 1. The concentration dependence of this desensitization (as DC_{50}) was assessed, using the $[^3\text{H}]$ -GABA release assay and crude cortex synaptosomal preparations from WT and $\alpha 5$ null mutant mice. Data are presented in Figure 5a and DC_{50} values in Table 3 where $\text{DC}_{50} = 71 \pm 17$ nM for the WT [$(\alpha 4\beta 2)_2\beta 2$ -plus $(\alpha 4\beta 2)_2\alpha 5$ -nAChRs] and in the $\alpha 5\text{KO}$, $\text{DC}_{50} = 9 \pm 4$ nM [$(\alpha 4\beta 2)_2\beta 2$ -nAChR alone]. Somewhat surprisingly, the presence of the $\alpha 5$ subunit in the WT significantly shifted the concentration response curve to the right.

Because of the unexpected shift found with the presence of the $\alpha 5$ subunit, we assayed another region of WT mice, thalamus, that previous data indicated would not have $\alpha 5$ subunits assembled with $\alpha 4\beta 2$ -nAChRs on GABA terminals (McClure-Begley et al, 2009). We expected this region would have a concentration response curve similar to the $\alpha 5$ null mutant cortex. These results are also shown in Figure 5a and Table 3. This data is consistent with our hypothesis; the DC_{50} value seen for thalamus = 11 ± 4 nM was similar to the value determined from $\alpha 5\text{KO}$ cortex (9 ± 4 nM).

This procedure was repeated using the $[^3\text{H}]$ -DA release assay with crude striatum synaptosomes from $\alpha 6\text{KO}$ mice and from $\alpha 5\text{KO}$ mice with addition of α -CtxMII to block all $\alpha 6$ -mediated responses. Data are shown in Figure 5b and Table 3. Again, the presence of the $\alpha 5$ subunit in the $\alpha 4\beta 2$ -nAChR (WT) shifted the curve to the right; (DC_{50} value of 8 ± 2 in the $\alpha 5\text{KO}$ and 117 ± 12 nM with the $\alpha 5$ subunit present in the WT).

Next, we measured the DC_{50} value for nicotine-induced desensitization of $(\alpha 6\beta 2)_2\beta 3$ -nAChR using the $[^3\text{H}]$ -DA assay and crude striatum synaptosomal preparations from $\alpha 4\text{KO}$ mice. These data are presented in Figure 5c and Table 3. Results indicate this subtype desensitizes to a maximum of about 38% with an intermediate DC_{50} value (22 ± 11 nM).

Analysis by one-way ANOVA for DC_{50} values for all 6 groups (Table 3) showed significant differences in DC_{50} values ($F(5,12)=61.26$, $P<0.001$). By Duncan's post-hoc test ($P<0.05$), there were three subsets, group 1: $\alpha 5\text{KO}$ in cortex and WT in thalamus by GABA release and $\alpha 5\text{KO}$ with α -CtxMII by DA release (all assays for $(\alpha 4\beta 2)_2\beta 2$ -nAChR only) plus the $\alpha 4\text{KO}$ by DA release ($\alpha 6\beta 2)_2\beta 3$ -nAChR), group 2: WT cortex by GABA release ($\alpha 4\beta 2)_2\beta 2$ - and $(\alpha 4\beta 2)_2\alpha 5$ -nAChRs) and group 3: $\alpha 6\text{KO}$ by DA release ($\alpha 4\beta 2)_2\beta 2$ - and $(\alpha 4\beta 2)_2\alpha 5$ -nAChRs). By this analysis all assays for $(\alpha 4\beta 2)_2\beta 2$ gave lower DC_{50} values than assays including $(\alpha 4\beta 2)_2\alpha 5$. The two assays for $(\alpha 4\beta 2)_2\beta 2$ plus $(\alpha 4\beta 2)_2\alpha 5$ -nAChRs were significantly different with the DA release assay having a higher DC_{50} value than the GABA release assay, possibly because a higher percentage of the measured function on DA neurons being mediated by $(\alpha 4\beta 2)_2\alpha 5$ -nAChRs. The DC_{50} value for the $(\alpha 6\beta 2)_2\beta 3$ subtype did not differ significantly from those measured for the $(\alpha 4\beta 2)_2\beta 2$ subtype but was lower than assays including $(\alpha 4\beta 2)_2\alpha 5$.

Significant differences were also found for maximum extent of desensitization in 10 min by one-way ANOVA ($F(5,12)=11.41$, $P=0.001$). By Duncan's post-hoc test ($P<0.05$), there were four subsets with overlap of groups among them. No group differed from all other groups; however, the $(\alpha 6\beta 2)_2\beta 3$ subtype showed significantly less desensitization than $(\alpha 4\beta 2)_2\alpha 5$ on DA terminals.

4. Discussion

4.1. $\alpha 4\beta 2$ - and $\alpha 4\alpha 5\beta 2$ -nAChR

Experiments presented here utilized relatively short term (10 min) exposures to a nicotine concentration (300 nM) likely to be found in plasma of tobacco smokers (Matta et al., 2007) to study the desensitization of nAChRs mediating neurotransmitter release from mouse brain synaptosomes. The exposure to nicotine was conducted in the absence of external Ca^{++} to eliminate or minimize any Ca^{++} -induced changes elicited by nicotine and any subsequent down-stream events. Under these conditions, $\alpha 4\beta 2$ -nAChRs are probably in an intermediate state of desensitization, and able to recover relatively rapidly (Fenster et al., 1999; Gentry et al., 2003). We chose these conditions as a simple model to investigate the effect of the addition of the $\alpha 5$ subunit to $\alpha 4\beta 2$ -nAChR in native nAChR populations. Our results indicate that, under these conditions, none of the nAChRs-mediated neurotransmitter release studied desensitized completely when exposed to nicotine for 10 min. However, the extent of functional desensitization in WT and $\alpha 5$ KO mice was similar indicating that inclusion of the $\alpha 5$ subunit did not affect steady-state transmitter release following a 10 min exposure to 300 nM nicotine. The data indicate that responses mediated by $\alpha 4\beta 2$ -nAChR which include the $\alpha 5$ subunit recover from desensitization more quickly than $\alpha 4\beta 2$ -nAChRs that do not include $\alpha 5$. This effect of the $\alpha 5$ subunit was seen for two neurotransmitter release assays ($[^3\text{H}]$ -GABA release from cortex and $[^3\text{H}]$ -DA release from striatum), indicating that the desensitization properties of $\alpha 4\beta 2^*$ -nAChRs do not differ greatly in these two types of nerve terminals. It should be noted that responses of $\alpha 4\beta 2\alpha 5$ -nAChR were measured in WT mice that also include significant populations of $\alpha 4\beta 2$ -nAChR such that the effects of the $\alpha 5$ subunit are likely underestimated with these experiments as we are not able to isolate a pure population of $(\alpha 4\beta 2)_2\alpha 5$ -nAChRs.

In addition, DC_{50} values for nicotine were considerably higher in WT mice that express both $\alpha 4\beta 2\alpha 5$ - and $\alpha 4\beta 2$ -nAChR than in $\alpha 5$ KO mice that express only $\alpha 4\beta 2$ -nAChR. The lower DC_{50} values in the absence of $\alpha 5$ are consistent with K_i values of 8-15 nM measured for nicotine binding (Marks et al., 1986). A larger difference in DC_{50} values between WT and $\alpha 5$ KO mice was seen using $[^3\text{H}]$ -DA release from striatum (8 nM vs 117 nM) than for $[^3\text{H}]$ -GABA release from cortex (9 nM vs 71 nM), possibly because a greater percentage of the activity in DA terminals is mediated by receptors with the $\alpha 5$ subunit. This DC_{50} effect is not an artifact of use of the $\alpha 5$ KO mice, as a lower DC_{50} value was also seen in WT mouse thalamus (11 nM) where GABA terminal $\alpha 4\beta 2$ -nAChR function is devoid of any effect of $\alpha 5$ subunit deletion (McClure-Begley et al., 2009). Thus, this regional difference in DC_{50} values for WT mice appears to be the result of the absence of the $\alpha 5$ subunit on GABA terminals in thalamus and its presence in cortex.

The effect of the $\alpha 5$ subunit expressed with $\alpha 4$ and $\beta 2$ subunits on desensitization has been reported for studies using transfected cell lines and heterologously expressed receptors. In a study using HEKtsA201 cells expressing human $\alpha 4\beta 2$ -nAChRs, addition of the $\alpha 5$ subunit increased the rate and amount of acute desensitization when exposed to 20 μM ACh compared to a line with mostly $(\alpha 4\beta 2)_2\alpha 4$, a low sensitivity (LS) form of $\alpha 4\beta 2^*$ -nAChR (Kuryatov et al., 2008). With long-term (6 hr) desensitization by lower concentrations of nicotine, the DC_{50} values were 6.1 ± 3.6 nM for $(\alpha 4\beta 2)_2\beta 2$ and 8.9 ± 1.5 nM for $(\alpha 4\beta 2)_2\alpha 5$ (Kuryatov et al., 2008), an insignificant shift by addition of the $\alpha 5$ subunit. However in a study using concatamers to control subtypes and expression in *Xenopus* oocytes, a DC_{50} value of 87.2 ± 2.7 nM was reported for $(\alpha 4\beta 2)_2\alpha 5$ using a 1.5 hr exposure to 300 nM nicotine that resulted in ~70% desensitization (Kuryatov et al., 2011). Both this DC_{50} value and the extent of desensitization are comparable to data reported here for $(\alpha 4\beta 2)_2\alpha 5$ -nAChR. It is not yet known whether a longer exposure to nicotine would decrease this DC_{50} value for concatamers or those we have measured for $(\alpha 4\beta 2)_2\alpha 5$ -nAChR.

4.2. $\alpha 6\beta 2\beta 3$ -nAChR

The $\alpha 6$ subunit is a ligand binding subunit when paired with $\beta 2$, and forms nAChRs of predominately the $(\alpha 6\beta 2)_2\beta 3$ - and $(\alpha 6\beta 2)(\alpha 4\beta 2)\beta 3$ -nAChR subtypes in dopaminergic neurons [for review see (Quik et al., 2011)]. Both of these subtypes are HS forms, with the latter having the highest sensitivity to nicotine of any reported subtype (Salminen et al., 2007). These receptors have been difficult to express in oocytes or cell lines [for review see (Letchworth and Whiteaker, 2011)] and, therefore, little is known about their desensitization properties. Recent advances with expression of concatamers have facilitated study of the $\alpha 6\beta 2^*$ -nAChR subtypes and desensitization has been shown to occur, but perhaps to a lesser extent than for $\alpha 4\beta 2$ subtypes (Capelli et al., 2011; Kuryatov et al., 2011). Our results with the $(\alpha 6\beta 2)_2\beta 3$ -nAChR in striatum preparations from $\alpha 4$ KO mice presented here indicate that this receptor subtype desensitizes rapidly with exposure to 300 nM nicotine, but to a quite limited degree (only ~25%). The rate of recovery from desensitization is similar to that of $(\alpha 4\beta 2)_2\beta 3$ -nAChR and slower than that of $(\alpha 4\beta 2)_2\alpha 5$ -nAChR. The DC_{50} value (22 ± 11 nM) is similar to the K_i value for nicotine inhibition of α -CtxMII binding (22.6 ± 9.3 nM) (Salminen et al., 2005) or for nicotine inhibition of [125 I]-epibatidine binding in $\alpha 4$ KO tissue (44.9 ± 8.8 nM) (Grady et al., 2010b) both measures of the affinity of nicotine for the $\alpha 6\beta 2$ site. The extent of desensitization is significantly less than that seen under similar conditions for $\alpha 4(\text{non } \alpha 6)\beta 2^*$ -nAChRs as measured here.

4.3. Incomplete desensitization and shifts of DC_{50} values

With more classical methods, measurements of EC_{50} values for activation and K_i values for binding by a number of agonists indicates the ratio of these values for $\alpha 6\beta 2^*$ -nAChR is ~5-10, considerably lower than the ratio for $\alpha 4\beta 2^*$ -nAChR of ~100 (Salminen et al., 2005; Grady et al., 2010b). This observation implies that the ground state and desensitized state of $\alpha 6\beta 2^*$ -nAChR may be almost equally stable (Quick and Lester, 2002). The prediction from this observation is that there may not be as much (or as long lasting) desensitization of $\alpha 6\beta 2^*$ -nAChR (with ratio of ~5-10) as there is of $\alpha 4\beta 2^*$ -nAChR where the ratio of EC_{50} value to binding K_i is ~100. The small amount of desensitization measured for $\alpha 6\beta 2\beta 3$ -nAChR is consistent with this prediction.

The possibility of spare receptors cannot be ignored when using neurotransmitter release assays (Feuerstein and Limberger, 1999). If activation of only a percentage of receptors on each unit (synaptosome in this case) results in full measured activity (release) the apparent EC_{50} (or DC_{50}) value will be shifted from values obtained in the absence of spare receptors. Spare receptor theory predicts measured EC_{50} value $< K_d$ (or $DC_{50} > K_d$) when spare receptors are present, as well as deviations in slope of concentration response curves (Agneter et al., 1997). However, EC_{50} values also differ from K_d in allosteric models, because of different affinities for different conformational states (Changeux et al., 1984). In the absence of the $\alpha 5$ subunit, good correspondence of DC_{50} value with binding K_i was obtained for $\alpha 4\beta 2$ -nAChR, perhaps indicating a lack of spare receptors. This correspondence was found for the $\alpha 6\beta 2\beta 3$ -nAChR measurement, also. To explain the large shift we measured for $\alpha 4\alpha 5\beta 2^*$ -nAChR compared to $\alpha 4\beta 2$ -nAChR by spare receptor theory, a large change in spare receptors would need to be postulated.

4.4. Relevance to smoking levels of nicotine

These results suggest, at least with short term (10 min) exposure to nicotine, that functional responses mediated by nAChR including the $\alpha 5$ subunit would be more resistant to desensitization (owing to their relatively high DC_{50}) than nAChR that do not include $\alpha 5$. Because the $\alpha 5$ subunit has limited distribution in the brain (Wada et al., 1990; Marks et al., 1992), tobacco smoking could then change relative activity of nicotinic receptors regionally, and/or by cell-type when assembled with $\alpha 4$ and $\beta 2$ subunits. Since plasma levels of nicotine

range from ~1nM for second-hand smoke exposure (Brody et al., 2011), and ~6 nM after overnight abstinence (Jarvik et al., 2000; Brody et al., 2006) to ~150-250 nM shortly after smoking (Jarvik et al., 2000), the shift in DC₅₀ from ~10 nM for $\alpha 4\beta 2$ -nAChR to ~100 nM for $\alpha 4\beta 2\alpha 5$ -nAChR could have a significant effect on desensitization of these receptors following tobacco smoking. In addition, after exposure to 300 nM nicotine, the $\alpha 6\beta 2\beta 3$ -nAChR desensitized to a maximum of only ~25%, suggesting that these receptors may retain significant activity in the presence of smoking levels of nicotine.

Serum levels of nicotine in smokers remain at mid-nM levels for considerably longer than the 10 min exposure time used for the experiments reported here. Prolonged exposures possibly lead to deeper desensitization states that may not recover easily and, in addition, may affect DC₅₀ values. Study of long exposures to nicotine will require different experiments than those reported here. The level of nicotine likely modifies the amount of active receptor available, and our results suggest that different subtypes of HS nAChR, especially those with $\alpha 5$ or $\alpha 6$ subunits, may not fully desensitize thereby retaining some activity in smokers.

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Highlights

1. Desensitization of presynaptic nAChRs was assessed by neurotransmitter release.
2. $\alpha 4\alpha 5\beta 2$ -nAChR recovers from desensitization more quickly than $\alpha 4\beta 2$ -nAChR.
3. The desensitization concentration for $\alpha 4\alpha 5\beta 2$ -nAChR is higher than for $\alpha 4\beta 2$ -nAChR.
4. The extent of desensitization of $\alpha 6\beta 3\beta 2$ -nAChR is less than $\alpha 4\beta 2$ - or $\alpha 4\alpha 5\beta 2$ -nAChR.

Figure 1.
Schematic presentation of experimental protocols. Timelines are shown for buffer superfusion for each of three types of experiments.

Figure 2.

Representative superfusion scans illustrating the effect of the buffer switch protocol. Data shown is for [^3H]-DA release from $\alpha 6\text{KO}$ striatum. Units of release are net release as a percentage of baseline. The $+\text{Ca}^{++}$ baseline is calculated as a single exponential decay from cpm collected in fractions 15-23. Open symbols represent only a buffer switch ($\square$ with 300 nM nicotine in Ca^{++} -free buffer, $\triangle$ with no nicotine in Ca^{++} -free buffer), a shift peak is seen which is not affected by the presence of nicotine in the Ca^{++} -free buffer. The effect of nicotine treatment on stimulation by 10 μM ACh is shown by closed symbols ($\blacksquare$ with nicotine exposure, and $\blacktriangle$ no nicotine exposure). As seen, nicotine treatment substantially decreases release promoted by ACh.

Figure 3.

Onset of desensitization to 300 nM nicotine. Panel a shows data for [3 H]-GABA release from cortex for WT and $\alpha 5$ KO mice stimulated by 10 μ M ACh for 20s (WT, n=5 experiments; $\alpha 5$ KO, n=6) or by 20 mM K^+ for 20s (WT, n=4; $\alpha 5$ KO n=3). Panel b presents data for [3 H]-DA release from striatum for $\alpha 6$ KO mice and for $\alpha 5$ KO mice in the presence of α -CtxMII (50 nM) using the same stimulations as for Panel a (all, n=3). Panel c shows data for [3 H]-DA release from striatum for $\alpha 6$ KO mice using the same stimulations as for Panel a (n=8 for ACh; n=3 for K^+).

Figure 4.

Recovery from desensitization by exposure to 300 nM nicotine for 10 min. Panel a shows data for [3 H] GABA release from cortex of WT mice (n=5) and α 5KO mice (n=5). Panel b shows data for [3 H]-DA release from striatum of α 6KO mice (n=6) and α 5KO mice (n=5) in the presence of α -CtxMII (50 nM). Panel c presents data for [3 H]-DA release from striatum of α 4KO mice (n=13).

Figure 5.

Concentration response for desensitization by nicotine. Panel 5a shows data for [^3H] GABA release from CX (n=10) and thalamus (n=6) of WT mice, as well as cortex of $\alpha 5\text{KO}$ mice (n=7). Panel 5b shows data for [^3H] -DA release from striatum of $\alpha 6\text{KO}$ mice (n=4) and $\alpha 5\text{KO}$ mice (n=5) in the presence of α -CtxMII (50 nM). Panel 5c presents data for [^3H] -DA release from striatum of $\alpha 4\text{KO}$ mice (n=5).

Table 1

Onset parameters for 300 nM nicotine desensitization

Subtype assayed	Genotype/ Antagonist	Region	Assay	Half-time (τ) of onset (min) ^a	% desensitization by 300 nM nic
(α 4 β 2) β 2	α 5KO	Cortex	GABA	0.39 $\pm$ 0.04	56 $\pm$ 4
(α 4 β 2) β 2	α 5KO/ α CtxMII	Striatum	DA	0.32 $\pm$ 0.03	60 $\pm$ 4
(α 4 β 2) β 2 + (α 4 β 2) α 5	WT	Cortex	GABA	0.17 $\pm$ 0.06	48 $\pm$ 6
(α 4 β 2) β 2 + (α 4 β 2) α 5	α 6KO	Striatum	DA	0.18 $\pm$ 0.05	48 $\pm$ 11
(α 6 β 2) β 3	α 4KO	Striatum	DA	0.052 $\pm$ 0.008	25 $\pm$ 1

^a Half-times of onset should be considered estimates.

Table 2

Recovery parameters for 300 nM nicotine desensitization

Subtype assayed	Genotype/ Antagonist	Region	Assay	Half-time (τ) of recovery (min)	Starting % desensitization
($\alpha 4\beta 2$) $_2\beta 2$	$\alpha 5$ KO	Cortex	GABA	8.8 $\pm$ 0.9	76 $\pm$ 10
($\alpha 4\beta 2$) $_2\beta 2$	$\alpha 5$ KO/ α CtxMII	Striatum	DA	5.8 $\pm$ 0.5	59 $\pm$ 5
($\alpha 4\beta 2$) $_2\beta 2$ + ($\alpha 4\beta 2$) $_2\alpha 5$	WT	Cortex	GABA	2.4 $\pm$ 0.4	54 $\pm$ 6
($\alpha 4\beta 2$) $_2\beta 2$ + ($\alpha 4\beta 2$) $_2\alpha 5$	$\alpha 6$ KO	Striatum	DA	1.1 $\pm$ 0.2	64 $\pm$ 8
($\alpha 6\beta 2$) $_2\beta 3$	$\alpha 4$ KO	Striatum	DA	6.8 $\pm$ 3.3	27 $\pm$ 16

Table 3DC₅₀ values for 10 min nicotine desensitization

Subtype assayed	Genotype/ Antagonist	Region	Assay	nicotine DC ₅₀ (nM)	Max % desensitization
(α 4 β 2) β 2	α 5KO	Cortex	GABA	9 $\pm$ 4	53 $\pm$ 9
(α 4 β 2) β 2	WT	Thalamus	GABA	11 $\pm$ 4	65 $\pm$ 9
(α 4 β 2) β 2	α 5KO + α CtxMII	Striatum	DA	8 $\pm$ 2	44 $\pm$ 4
(α 4 β 2) β 2 + (α 4 β 2) α 5	WT	Cortex	GABA	71 $\pm$ 17	63 $\pm$ 8
(α 4 β 2) β 2 + (α 4 β 2) α 5	α 6KO	Striatum	DA	117 $\pm$ 12	77 $\pm$ 4
(α 6 β 2) β 3	α 4KO	Striatum	DA	22 $\pm$ 11	38 $\pm$ 9