ORIGINAL INVESTIGATION

Effectiveness of Nicotinic Agonists as Desensitizers at Presynaptic $\alpha 4\beta 2$ - and $\alpha 4\alpha 5\beta 2$ -Nicotinic Acetylcholine Receptors

Charles R. Wageman BA, Michael J. Marks PhD, Sharon R. Grady PhD

Institute for Behavioral Genetics, University of Colorado, Boulder, CO

Corresponding Author: Sharon R. Grady, PhD, Institute for Behavioral Genetics, 447UCB, University of Colorado, Boulder, CO 80309, USA. Telephone: 303-492-9677; Fax: 303-492-8063; E-mail: sharon.grady@colorado.edu

Received April 2, 2013; accepted August 6, 2013

ABSTRACT

Introduction: Nicotine interacts with nicotinic acetylcholine receptors (nAChRs) and modifies neuronal functions. The net result of nicotine exposure is difficult to assess because multiple nAChR subtypes exist and are expressed on multiple classes of neurons. Nicotine, unlike the natural agonist acetylcholine, remains in tissues for hours, and during this extended exposure nAChRs desensitize. Therefore, agonists can block the natural functions of nAChRs. Higher nicotine concentrations are required to desensitize $\alpha 4\beta 2$ -nAChRs containing the $\alpha 5$ subunit. The aim of these experiments was to determine if this property holds true for compounds other than nicotine.

Methods: [^3H]-dopamine release from crude mouse striatal synaptosomal preparations was used to measure activation and desensitization of the $[(\alpha 4\beta 2)_2\beta 2]$ and $[(\alpha 4\beta 2)_2\alpha 5]$ nAChR subtypes. Affinity was measured by competition with [^{125}I]-epibatidine.

Results: Nine compounds of varying affinity and efficacy were tested. All compounds partially desensitized both subtypes; concentration necessary for desensitization correlated with binding site affinity but not efficacy. All compounds showed a similar, significant shift in concentration necessary for a 50% effect when the $\alpha 5$ subunit was included (averaging 8-fold higher). The extent of desensitization produced by a 10-min exposure did not correlate with affinity or efficacy of compound.

Conclusion: Full or partial nicotinic agonists used as medications may effectively desensitize $\alpha 4\beta 2$ -nAChRs. However, significantly higher concentrations of all compounds tested were required to elicit desensitization of $\alpha 4\alpha 5\beta 2$ -nAChRs than $\alpha 4\beta 2$ -nAChRs. If desensitization is the important property for a smoking cessation drug, basic screening at both subtypes may provide a mechanistic foundation for effectiveness.

INTRODUCTION

Neuronal nicotinic acetylcholine receptors (nAChRs) are the initial sites of action for nicotine. Nicotine intake via smoking tobacco alters the state of brain nAChRs. The direct effects of nicotine and other nicotinic agonists on nAChRs are complex: an initial activation of the receptors is followed by desensitization after prolonged or repeated exposure or after prolonged exposure to subactivating concentrations (Barik & Wonnacott, 2009). Desensitization of nAChRs renders them unresponsive for a time to the natural agonist, acetylcholine, and therefore prevents their normal function (Maskos, 2010). In vivo studies on physiological effects of nicotine have shown that desensitization to a dose of nicotine occurs in animals, rendering a second dose less effective for a time (Fonck et al., 2005; Hulihan-Giblin, Lumpkin, & Kellar, 1990; Miner & Collins, 1988; Sharp & Beyer, 1986). Nicotine, during active smoking, rises to blood levels of ~30 ng/ml (~200 nM) (Jarvik

et al., 2000) and falls with overnight abstinence to ~1 ng/ml (~6 nM), with levels of ~5 ng/ml (~30 nM) 2 hr after smoking (Brody et al., 2006; Jarvik et al., 2000). Free brain nicotine levels assessed by positron emission tomography scans have been estimated to be 25 nM after overnight abstinence, 265 nM after one cigarette, and from 200 to 465 nM during the afternoon in smokers (Rose et al., 2010).

The most abundant nicotinic receptor subtype, the $\alpha 4\beta 2^{*}$ -nAChR (* indicating possible other subunits; Lukas et al., 1999), in its high-sensitivity (HS) form $(\alpha 4\beta 2)_2\beta 2$ can be activated by levels of nicotine achieved in smokers. These receptors are desensitized by chronic exposure to nicotine (Barik & Wonnacott, 2009; De Biasi & Dani, 2011). The daily variations of levels of nicotine are consistent with concentrations that would desensitize the $\alpha 4\beta 2$ -nAChRs during active smoking, but possibly allow partial recovery between cigarettes and after overnight abstinence.

An additional HS form $(\alpha 4\beta 2)_2\alpha 5$ contains the $\alpha 5$ subunit, a subunit that is not part of the agonist binding site and

doi:10.1093/ntr/ntt146

Advance Access publication September 19, 2013

© The Author 2013. Published by Oxford University Press on behalf of the Society for Research on Nicotine and Tobacco. All rights reserved. For permissions, please e-mail: journals.permissions@oup.com.

is, therefore, considered an accessory subunit. While agonist activation of the two subtypes is similar, there is evidence that $(\alpha 4\beta 2)_2\alpha 5$ -nAChR is more resistant to desensitization induced by smoking levels of nicotine than $(\alpha 4\beta 2)_2\beta 2$ -nAChR (Bailey, De Biasi, Fletcher, & Lambe, 2010; Grady, Wageman, Patzlaff, & Marks, 2012; Kuryatov, Berrettini, & Lindstrom, 2011; Poorthuis, Bloem, Verhoog, & Mansvelder, 2013). Consequently, the $(\alpha 4\beta 2)_2\alpha 5$ subtype may retain more activity or recover function more easily between cigarettes than the $(\alpha 4\beta 2)_2\beta 2$ -nAChR subtype. Differential desensitization properties could have important consequences for smoking behavior.

In mouse brain preparations, important functions depend on the $\alpha 4\alpha 5\beta 2$ -nAChR. nAChR-evoked dopamine release from striatal terminals is mediated to a large extent by $\alpha 4\alpha 5\beta 2$ -nAChR (Grady, Salminen, McIntosh, Marks, & Collins, 2010). In addition, regulation of the function of dopaminergic neurons that terminate in the dorsal striatum depends on these presynaptic $\alpha 4\alpha 5\beta 2$ -nAChRs (Exley, McIntosh, Marks, Maskos, & Cragg, 2012). The $\alpha 4\alpha 5\beta 2$ -nAChR at presynaptic locations shifts the desensitization profile induced by nicotine to higher concentrations (Grady et al., 2012). A critical role for $\alpha 4\alpha 5\beta 2$ -nAChR has been shown in murine attentional behavior via function in layer VI pyramidal neurons (Bailey et al., 2010). These layer VI pyramidal neurons of prefrontal cortex $\alpha 4\alpha 5\beta 2$ -nAChRs desensitize less following exposure to 100–300 nM nicotine than interneurons that express only $\alpha 4\beta 2$ -nAChRs (Poorthuis et al., 2013).

Smoking cessation drugs such as varenicline and cytisine interact at the same $\alpha 4\beta 2$ -nAChR binding sites as nicotine but are partial agonists compared to nicotine (Hurst, Rollema, & Bertrand, 2013). It is of interest to determine whether these drugs have a similar profile of short-term desensitization shifted by $\alpha 5$ subunits, as is seen with nicotine. Knowing more about the mechanisms of action of these compounds may guide future studies. This article presents results of a study comparing effects of 10 nicotinic agonists on short-term desensitization.

METHODS

Mice

Mice, bred at the Institute for Behavioral Genetics, University of Colorado, were housed five or fewer to a cage and had free access to food and water. The room was maintained at 22 °C with a 12-h light/dark cycle. All mice had been bred for a minimum of 10 generations onto C57Bl/6 background. The $\alpha 5$ and $\alpha 6$ null mutant mice ($\alpha 5$ KO and $\alpha 6$ KO) were originally provided by Dr. Arthur Beaudet (Baylor College) and Dr. Uwe Maskos (Pasteur Institute), respectively. Mice were bred by mating heterozygotes and genotyped upon weaning. The care and use of mice followed the guidelines of The National Institutes of Health, and all protocols were approved by the Animal Care and Utilization Committee of the University of Colorado, Boulder.

Materials

[¹²⁵I]-Epibatidine and [³H]-dopamine were products of Perkin Elmer Life and Analytical Sciences. α -Conotoxin MII (α -CtxMII) was obtained from Dr. J. Michael McIntosh

(University of Utah). Varenicline tartrate was synthesized and donated by Targacept, Inc. and sazetidine-A was a gift from Dr. Ken Kellar (Georgetown University). Atropine sulfate, pargyline, diisopropylfluorophosphate (DFP), nomifensine, (–)-nicotine tartrate (L-nicotine), carbachol, (+)-nicotine (D-nicotine), (±)-epibatidine, cytisine, nornicotine, anabasine, and bovine serum albumin (BSA) were products of Sigma Aldrich (St. Louis, MO). I-A85380 was purchased from Tocris Biosciences. Ecoscint XR, a product of National Diagnostics, was obtained from Life Sciences, Inc.

[³H]-Dopamine Release

[³H]-dopamine release from crude mouse striatal synaptosome preparations was used to evaluate agonist-induced desensitization of nAChRs. This is a robust assay for function of the $\alpha 4\beta 2$ - and $\alpha 4\alpha 5\beta 2$ -nAChR subtypes we wanted to measure. About 70% of α CtxMII-resistant activity (i.e., non- $\alpha 6\beta 2$ mediated) is attributed to the $\alpha 4\alpha 5\beta 2$ subtype (Grady, Salminen, et al., 2010). Function of the $\alpha 4\beta 2$ -nAChR alone can be measured from the $\alpha 5$ KO mouse in combination with the selective antagonist α CtxMII to block all $\alpha 6\beta 2$ -nAChR subtypes (Grady et al., 2012; Salminen et al., 2004). The function of a mixture of two subtypes, the $\alpha 4\beta 2$ - and $\alpha 4\alpha 5\beta 2$ -nAChR, can be measured using either C57Bl/6 striatal preparations in combination with α CtxMII to block the $\alpha 6\beta 2$ -nAChRs or from $\alpha 6$ KO mice that lack any $\alpha 6\beta 2$ -nAChR subtypes (Champtiaux et al., 2003; Grady et al., 2012).

The methods described in Grady et al. (2012) were followed with modifications. Briefly, crude synaptosomal preparations of freshly dissected striatal tissue from $\alpha 6$ KO, $\alpha 5$ WT, or $\alpha 5$ KO mice were prepared. For experiments where time of exposure to agonist was varied from 10 to 60 min, synaptosomes were exposed to agonist prior to and during the uptake of [³H]-dopamine as well as for the initial 10 min of superfusion. In these experiments, synaptosomes were resuspended in uptake buffer without Ca^{2+} (133 mM NaCl, 2.4 mM KCl, 1.2 mM KH_2PO_4 , 1.2 mM MgSO_4 , 10 mM glucose, 1 mM ascorbic acid, and 1 μ M pargyline) with or without agonist and incubated at room temperature for 50 min. [³H]-dopamine (100 nM) and DFP (10 μ M) were added for the last 15 min of this incubation. For assays to determine concentration response of compounds by 10-min desensitization exposures, uptake incubations were for 15 min at 37 °C in uptake buffer with Ca^{2+} (as above, but with 128 mM NaCl and 3.2 mM CaCl_2). [³H]-dopamine (100 nM) and DFP (10 μ M) were added for the last 5 min.

After uptake, aliquots were transferred to filters and superfused at 0.7 ml/min with Ca^{2+} -free buffer (uptake buffer without Ca^{2+} and with 1 μ M atropine, 1 μ M nomifensine, and 0.1% BSA) with or without agonist for 10 min. For samples from $\alpha 5$ WT or $\alpha 5$ KO mice, α CtxMII (50 nM) was added for the last 3 min of the superfusion. [³H]-dopamine release was stimulated for 20 s by 10 μ M acetylcholine in buffer with Ca^{2+} followed by buffer with Ca^{2+} for the rest of the superfusion. Shifting external Ca^{2+} concentration produces a small shift peak that can be measured from additional parallel aliquots without acetylcholine stimulation. All data were corrected for this shift response (Grady et al., 2012). Fractions (10 s, ~115 μ l) of superfusate were collected from 1 min before and 3 min during and after stimulation into 96-well plates using a Gilson FC204 fraction collector. Radioactivity was determined in a

1450 Microbeta Trilux counter (Perkin Elmer Life Sciences – Wallac Oy) after addition of 150 μ l Ecoscint XR scintillation cocktail to each well. After baseline subtraction, total units of release were determined by normalization to baseline release. Maximal ACh-stimulated release ($R_{\max}$) and amount of drug necessary for 50% inhibition (DC_{50}) were determined by fitting the data to the equation: $f = R_{\max}/[1 + (\text{drug})/DC_{50}] + R_{\text{rem}}$, where R_{rem} is the uninhibited portion of release.

[125 I]-Epibatidine Binding

Previously published methods were followed (Whiteaker, Jimenez, McIntosh, Collins, & Marks, 2000). Briefly, membranes were prepared from cortex of C57BL/6 mouse brain as a source of $\alpha 4\beta 2$ -nAChR binding sites (Marks, Whiteaker, & Collins, 2006). Incubations were performed in 96-well polystyrene plates, in 30 μ l of binding buffer (144 mM NaCl, 1.5 mM KCl, 2 mM CaCl_2 , 1 mM MgSO_4 , 20 mM HEPES pH 7.5). Inhibition binding experiments were performed using 200 pM [125 I]-epibatidine. The amount of membrane protein added was chosen to produce maximum binding of ligand to the tissue (less than 10% of total ligand added, minimizing the effects of ligand depletion). Various concentrations of competing drugs were included in duplicate wells, and L-nicotine (10 μ M) was used to determine blank. IC_{50} values were calculated by fitting data from a minimum of three separate experiments to the equation: $B = B_0/(1 + ([I]/IC_{50}))$ where B = bound [125 I]-epibatidine and B_0 = uninhibited bound [125 I]-epibatidine, $[I]$ = concentration of inhibiting compound. K_i values were calculated from IC_{50} values by the formula $K_i = IC_{50}/(1 + [\text{epi}]/K_d)$.

Data Analysis

Significant differences in DC_{50} and $R_{\max}$ were assessed by t test comparing two groups, the $\alpha 6$ KO ($\alpha 4\alpha 5\beta 2 + \alpha 4\beta 2$) and $\alpha 5$ KO with α CtxMII ($\alpha 4\beta 2$ alone). One-way analysis of variance was used when three groups were compared. Degrees of freedom represent the number of mice assayed.

RESULTS

Previously, we have shown that exposure to 300 nM nicotine desensitizes presynaptic nAChRs of both ($\alpha 4\beta 2$) $_2\beta 2$ and ($\alpha 4\beta 2$) $_2\alpha 5$ subtypes at dopaminergic terminals (Grady et al.,

2012). Onset half-times were less than 30 s. This concentration achieves near maximal, though incomplete, desensitization in this experiment after 10 min. Even though no further increase in the extent of desensitization was seen between 2 and 10 min, this could be a result of nonequilibrium conditions. In order to assess whether longer exposure to 300 nM nicotine could elicit greater steady-state desensitization, we extended the length of exposure to 300 nM L-nicotine to 40 or 60 min. No further increase in amount of desensitization was achieved with longer exposure for either receptor subtype (Table 1).

With high-affinity compounds, using very low concentrations, exposure time necessary to achieve equilibrium may be considerably longer than for 300 nM nicotine. If the assay is not at equilibrium for low concentrations, the DC_{50} calculated may be affected. We have investigated this possibility using 0.1 nM epibatidine, the lowest concentration that showed differential desensitization of the ($\alpha 4\beta 2$) $_2\beta 2$ -nAChR population compared to the population of $\alpha 4\beta 2$ -nAChR containing $\alpha 5$ subunits. No increase in desensitization was seen with longer (60 min compared to 10 min) exposure to 0.1 nM epibatidine (Table 1). Thus, a 10-min exposure appears to be sufficient to reach equilibrium. Subsequent experiments were, therefore, carried out using the 10-min exposure time.

While differences between these subtypes (genotypes) are not apparent by maximum desensitization with 300 nM nicotine even after extended exposure, a significant difference is seen in concentration response relationships for L-nicotine (Grady et al., 2012). We have repeated the concentration response for desensitization by L-nicotine using $\alpha 5$ KO tissue in the presence of α CtxMII (measuring only the ($\alpha 4\beta 2$) $_2\beta 2$ -nAChR) and compared this to $\alpha 5$ WT also in the presence of α CtxMII measuring combined populations of ($\alpha 4\beta 2$) $_2\beta 2$ -nAChR and ($\alpha 4\beta 2$) $_2\alpha 5$ -nAChR to be certain there are no unexpected effects of α CtxMII. These data are presented in Figure 1 along with comparison data for $\alpha 6$ KO, the model previously used for measuring both subtypes (Grady et al., 2012). No $\alpha 5$ WT + α CtxMII datapoints differed from $\alpha 6$ KO datapoints; however, datapoints for $\alpha 5$ KO were significantly shifted to the left as shown previously (Grady et al., 2012). For subsequent compound analysis, the $\alpha 6$ KO model was used.

Desensitization of nAChR-mediated [3 H]-dopamine release by nine compounds was measured and compared to L-nicotine. The compounds included four full agonists (($\pm$)-epibatidine, I-A85380, L-nicotine, and carbachol) and six compounds with varying degrees of partial agonism at HS $\alpha 4\beta 2^*$ -nAChRs

Table 1. Effect of Time of Exposure on Desensitization

Minutes of exposure	Percent desensitization after 300 nM L-nicotine ($\alpha 4\beta 2$) $_2\beta 2$	Percent desensitization after 300 nM L-nicotine ($\alpha 4\beta 2$) $_2\beta 2$ plus ($\alpha 4\beta 2$) $_2\alpha 5$
10	66.7 $\pm$ 4.4 (n = 3)	60.9 $\pm$ 6.5 (n = 3)
40	61.2 $\pm$ 9.4 (n = 3)	69.1 $\pm$ 7.6 (n = 5)
60	67.1 $\pm$ 6.4 (n = 5)	60.2 $\pm$ 4.2 (n = 4)
	Percent desensitization after 0.1 nM epibatidine ($\alpha 4\beta 2$) $_2\beta 2$	Percent desensitization after 0.1 nM epibatidine ($\alpha 4\beta 2$) $_2\beta 2$ plus ($\alpha 4\beta 2$) $_2\alpha 5$
10	33.1 $\pm$ 7.1 (n = 6)	8.2 $\pm$ 3.4 (n = 6)
60	34.7 $\pm$ 7.6 (n = 6)	8.8 $\pm$ 4.7 (n = 6)

Note. No significant differences were found with time of exposure by one-way analysis of variance for L-nicotine or by t test for epibatidine.

Effectiveness of nicotinic agonists as desensitizers

compared to acetylcholine (sazetidine-A, varenicline, cytisine, nornicotine, anabasine, and D-nicotine) as previously determined by rubidium efflux assays (Marks et al., 1999) (and current study). These efficacies as fraction of effect of ACh are summarized in Table 2. Concentration effect curves were constructed for all test compounds in order to determine maximum extent of desensitization of ACh-stimulated [3 H]-dopamine release achieved by each compound in 10 min of exposure as determined by a leveling off of the inhibition curve. Two curves were generated for each compound, one using $\alpha 6$ KO striatal preparations, which measures combined populations of

($\alpha 4\beta 2$) $_2\beta 2$ - and ($\alpha 4\beta 2$) $_2\alpha 5$ -nAChRs and another using $\alpha 5$ KO striatal preparations with α CtxMII to block $\alpha 6\beta 2^*$ -nAChRs thereby isolating a population of only ($\alpha 4\beta 2$) $_2\beta 2$ -nAChRs. Representative curves are shown in Figure 2 for varenicline, epibatidine, cytisine, and sazetidine-A. Each compound elicited concentration-dependent reduction in activity. When $\alpha 5$ subunits were present, maximum desensitization values (reported in Table 2) varied from virtually complete desensitization of the [3 H]-dopamine release response (90% or more) by ($\pm$)-epibatidine, sazetidine-A, carbachol, and D-nicotine to about 50% inhibition by cytisine and anabasine. Without $\alpha 5$

Figure 1. Concentration response curves for L-nicotine-induced desensitization. Two models for assaying combined populations of $\alpha 4\alpha 5\beta 2$ - plus $\alpha 4\beta 2$ -nAChR were compared: the $\alpha 6$ KO (filled triangle), and the $\alpha 5$ WT in the presence of α CtxMII (filled square). These data did not differ. Data for the $\alpha 4\beta 2$ -nAChR population alone from $\alpha 5$ KO in the presence of α CtxMII (open square) differ from both models with $\alpha 5$ subunits present.

Table 2. Parameters for Agonists of $\alpha 4\beta 2^*$ -nAChR

Compound	Efficacy compared to ACh ^a	Maximum percent desensitization $\alpha 4\beta 2$	Maximum percent desensitization $\alpha 4\alpha 5\beta 2 + \alpha 4\beta 2$	DC ₅₀ (nM) $\alpha 4\beta 2$	DC ₅₀ (nM) $\alpha 4\alpha 5\beta 2 + \alpha 4\beta 2$	Binding K _i (nM) $\alpha 4\beta 2$ sites	DC ₅₀ /K _i $\alpha 4\beta 2$	DC ₅₀ /K _i $\alpha 4\alpha 5\beta 2 + \alpha 4\beta 2$
I-A85380	0.81	83 ± 6	82 ± 5	0.06 ± 0.01	0.23 ± 0.06 ^b	0.014 ± 0.001	4.3	16
Anabasine	0.23	60 ± 8	40 ± 3	73 ± 99	433 ± 123	92 ± 2	0.8	5
Carbachol	1.26	85 ± 16	99 ± 3	700 ± 500	2800 ± 400 ^b	157 ± 9	4.5	18
Cytisine	0.11	67 ± 6	52 ± 4	0.5 ± 0.2	7 ± 2 ^b	0.34 ± 0.04	1.5	21
Epibatidine	1.94	76 ± 5	90 ± 7	0.09 ± 0.03	0.4 ± 0.1 ^b	0.010 ± 0.001	9.0	40
D-Nicotine	0.32	82 ± 16	94 ± 8	150 ± 100	1700 ± 600 ^b	158 ± 10	0.9	11
L-Nicotine ^c	0.75	44 ± 4	77 ± 4 ^b	8 ± 2	117 ± 12 ^b	3.5 ± 0.4	2.3	33
L-Nicotine ^d	0.75	54 ± 6	73 ± 4 ^b	9 ± 4	68 ± 15 ^b	1.8 ± 0.1	5.0	38
Nornicotine	0.34	55 ± 10	79 ± 4	60 ± 40	3500 ± 800 ^b	61 ± 6	1.0	57
Sazetidine-A	0.49	77 ± 7	90 ± 5	0.12 ± 0.05	0.63 ± 0.10 ^b	0.06 ± 0.01	2.0	11
Varenicline	0.20	56 ± 8	64 ± 5	0.80 ± 0.42	4.4 ± 1.5 ^b	0.20 ± 0.02	4.0	22

^aFrom Marks et al. (1999) and current study.

^bDifferent from $\alpha 4\beta 2$ alone.

^cFrom Whiteaker et al. (2000), Grady et al. (2012), and Marks et al. (1999).

^dFrom data with $\alpha 5$ WT (see Figure 1).

Figure 2. Representative concentration response curves for inhibition of nAChR-mediated dopamine release. Aliquots of crude synaptosomes from striatal tissue of either $\alpha 6$ KO mice to measure $\alpha 4\alpha 5\beta 2$ - plus $\alpha 4\beta 2$ -nAChR (filled triangle) or $\alpha 5$ KO mice to measure only $\alpha 4\beta 2$ -nAChR with $\alpha 6\beta 2$ -nAChR activity blocked by α CtxMII (open square) were exposed to various concentrations of agonist for 10 min in buffer without Ca^{2+} followed by ACh (10 μM) for 20 s. Panels A–D, respectively, with 10-min exposure to varenicline, epibatidine, cytisine, and sazetidine-A. Parameters of curve-fit lines are given in Table 1.

subunits, maximum desensitization varied from 85% with carbachol to 49% with L-nicotine. Only L-nicotine showed a significant difference in extent of desensitization comparing presence and absence of the $\alpha 5$ subunit. Overall, for the 10 compounds, mean maximum desensitization at $(\alpha 4\beta 2)_2\beta 2$ -nAChRs (using $\alpha 5$ KO tissue plus α CtxMII) was $68.5 \pm 4.5\%$ control, while at $(\alpha 4\beta 2)_2\beta 2$ - plus $(\alpha 4\beta 2)_2\alpha 5$ -nAChRs (using $\alpha 6$ KO tissue), maximum desensitization tended to be higher ($76.7 \pm 6.1\%$ control); however, overall this difference was not significant.

DC_{50} values (the concentration required to desensitize to 50% of maximum value) were also calculated for each compound. As for maximum desensitization, these parameters were determined for conditions where only $(\alpha 4\beta 2)_2\beta 2$ -nAChRs were measured (using $\alpha 5$ KO mice along with α CtxMII to block all $\alpha 6\beta 2$ -nAChRs) and the combined populations of $(\alpha 4\beta 2)_2\beta 2$ - plus $(\alpha 4\beta 2)_2\alpha 5$ -nAChR mediated the $[^3\text{H}]$ -dopamine release (using $\alpha 6$ KO mice). These DC_{50} values are given in Table 2. For all compounds, DC_{50} values were higher when $\alpha 5$ subunits were present and this difference was significant for all compounds except anabasine. DC_{50} values ranged from 0.06 nM for

I-A85380 to 700 nM for carbachol at $(\alpha 4\beta 2)_2\beta 2$ -nAChRs and from 0.23 nM for I-A85380 to 3500 nM for normicotine for the combined populations of $(\alpha 4\beta 2)_2\beta 2$ - plus $(\alpha 4\beta 2)_2\alpha 5$ -nAChR.

The competitive agonists used for the desensitization studies were assayed for ability to inhibit $[^{125}\text{I}]$ -epibatidine binding. Membranes from mouse (C57Bl/6) cerebral cortex, a region where virtually all high affinity $[^{125}\text{I}]$ -epibatidine binding is to $\alpha 4\beta 2$ -nAChR sites (Marks et al., 2006), were used. Previous experiments have indicated that K_d for $[^{125}\text{I}]$ -epibatidine binding does not change with presence of the $\alpha 5$ subunit (Brown, Collins, Lindstrom, & Whiteaker, 2007). All compounds tested completely inhibited $[^{125}\text{I}]$ -epibatidine binding with varying potency; K_i values calculated from data of three separate experiments are given in Table 2. Affinity (K_i) varied from 0.01 nM for $(\pm)$ -epibatidine to 158 nM for D-nicotine.

A correlation of K_i values, as a measure of affinity for $\alpha 4\beta 2$ -nAChR binding sites, and DC_{50} values, as a measure of potency for desensitization, is shown in Figure 3. An excellent correlation of binding affinity (K_i) to desensitization potency (DC_{50}) was seen for $(\alpha 4\beta 2)_2\beta 2$ -nAChRs alone ($r^2 = .97$) and for $(\alpha 4\beta 2)_2\beta 2$ - plus $(\alpha 4\beta 2)_2\alpha 5$ -nAChRs ($r^2 = .97$). As previously

Figure 3. Correlation of binding K_i values with inhibition DC_{50} values. Binding K_i values were determined by inhibition of [125 I]-epibatidine binding to $\alpha 4\beta 2$ -nAChR sites. DC_{50} values are concentrations required to produce 50% maximal desensitization of nAChR-mediated dopamine release as shown in Figure 1. For $\alpha 4\beta 2$ - plus $\alpha 4\alpha 5\beta 2$ -nAChR (filled triangle) and only $\alpha 4\beta 2$ -nAChR (open square) parameters from regression lines are respectively: $r^2 = .97$ and $.97$, slope = 0.96 and 0.87 .

measured for L-nicotine (Grady et al., 2012), the desensitization potency for all the compounds measured is shifted to higher concentrations by the presence of the $\alpha 5$ subunit. The mean ratio of DC_{50} to K_i (see Table 2) for $(\alpha 4\beta 2)_2\beta 2$ -nAChR alone measured with 10 compounds was 3.2 ± 0.8 and for the combination of $(\alpha 4\beta 2)_2\beta 2$ - plus $(\alpha 4\beta 2)_2\alpha 5$ -nAChR was 26.6 ± 6.5 (see Table 2), a significant 8.3-fold shift ($p < .01$).

In a subsequent analysis, maximum desensitization was compared to binding K_i (plot not shown). These parameters were not correlated for either $(\alpha 4\beta 2)_2\beta 2$ -nAChRs or for $(\alpha 4\beta 2)_2\beta 2$ - plus $(\alpha 4\beta 2)_2\alpha 5$ -nAChRs ($r^2 = .06$ and $.01$, respectively). Maximum desensitization appears to be relatively invariant. The only agonist that showed a significant difference for the nAChR subtypes was L-nicotine, a difference that was reported previously (Grady et al., 2012).

The DC_{50} and maximum desensitization values were also compared to agonist efficacy. Efficacy was measured, as previously reported, by comparison to acetylcholine in a Rb^+ efflux assay for activation of HS $\alpha 4\beta 2$ -nAChR (Grady, Drenan, et al., 2010; Marks et al., 1999) (and current study) and is compiled in Table 2. Neither the DC_{50} nor the maximum desensitization values correlated well with efficacy for either subtype population ($r^2 = .03$ and $.07$ for DC_{50} compared to efficacy, and $r^2 = .18$ and $.37$ for maximum desensitization compared to efficacy, respectively, for $(\alpha 4\beta 2)_2\beta 2$ -nAChRs and for $(\alpha 4\beta 2)_2\beta 2$ - plus $(\alpha 4\beta 2)_2\alpha 5$ -nAChRs; plots not shown).

DISCUSSION

Normal function of nAChRs depends on the balance of activation and desensitization. The natural agonist, ACh, activates and desensitizes but is degraded quickly and receptors recover from desensitization quickly. Compounds that are metabolized

and cleared slowly elicit prolonged desensitization. This persistent desensitization appears to be a likely mode of action for nicotine as well as nAChR agonists that are being developed as drugs for smoking cessation and treatment for other disorders (Buccafusco, Beach, & Terry, 2009; Picciotto, Addy, Mineur, & Brunzell, 2008; Pidoplichko, DeBiasi, Williams, & Dani, 1997).

Previously reported data indicate that the $(\alpha 4\beta 2)_2\alpha 5$ -nAChR is more resistant to desensitization induced by low concentrations of nicotine than the $(\alpha 4\beta 2)_2\beta 2$ -nAChR subtype (Grady et al., 2012; Kuryatov et al., 2011; Poorthuis et al., 2013). For nicotine at 300 nM, both subtypes may only partially desensitize and the $(\alpha 4\beta 2)_2\alpha 5$ -nAChR subtype may recover substantially between bouts of smoking. The $(\alpha 4\beta 2)_2\alpha 5$ -nAChR is expressed in limited amounts and in only certain brain regions (Brown et al., 2007; Mao, Perry, Yasuda, Wolfe, & Kellar, 2008). Both $(\alpha 4\beta 2)_2\alpha 5$ - and $(\alpha 4\beta 2)_2\beta 2$ -nAChR appeared to retain considerable activity even after a 60-min exposure to L-nicotine. This seems contrary to *in vivo* experiments indicating that a single injection of nicotine will inhibit effects of a subsequent nicotine injection preventing hormone release response (Hulihan-Giblin et al., 1990; Sharp & Beyer, 1986) or seizure induction (Fonck et al., 2005; Miner & Collins, 1988). However, it is not known whether these systemic effects of nicotine-mediated desensitization are results of nAChRs located somatically or presynaptically. There may be some presynaptic population of nAChR that is more resistant to desensitization than somatodendritic nAChR populations, perhaps because of associated molecules or post-translational modifications (Pennock, Dicken, & Hentges, 2012; Vardya, Drasbek, Gibson, & Jensen, 2010; Wetherington & Lambert, 2002). If so, nAChR activity may not be uniformly depressed across the brain by smoking levels of nicotine. It is possible that this incomplete desensitization reflects some partial fast recovery phase during the 20-s ACh exposure of our

assay method and/or that a partial desensitization is sufficient to lower the threshold for hormone release or seizures promoted by nicotine. We were interested in determining whether these properties are unique to L-nicotine or generally applicable to other nicotinic agonists. These studies investigated the interplay of desensitization, affinity, and efficacy, and how they might inform the important search for useful smoking cessation treatments or therapies for other disorders.

We chose nine compounds including full and partial agonists with a range of affinity for $\alpha 4\beta 2$ -nAChRs to compare to L-nicotine. We included compounds currently used as smoking cessation agents (varenicline and cytisine), as well as sazetidine-A, a compound being considered as a possible candidate drug (Husmann et al., 2012; Levin et al., 2010). These were assessed for affinity (by competition with [125 I]-epibatidine) and desensitization as measured by maximum percent desensitization in 10 min as well as DC_{50} . Data were compared to determine if desensitization parameters correlated with affinity for binding sites or previously measured agonist efficacy. For both receptor populations, DC_{50} correlated well to affinity as measured by binding K_i . DC_{50} did not correlate to efficacy of compound for activating the HS form of $\alpha 4\beta 2$ -nAChRs as measured by Rb^+ efflux, shown in Table 2 (Marks et al., 1999) (and current study). The maximum percent desensitization in 10 min did not correlate with either affinity of the compound for the binding site or the efficacy as an agonist and appeared to be relatively constant, but not complete, at the 10-min exposure time.

As for L-nicotine, all nine other compounds required higher concentrations for equivalent desensitization of $\alpha 4\alpha 5\beta 2$ -nAChR subtype compared to $\alpha 4\beta 2$ without the $\alpha 5$ subunit. This relative resistance to desensitization of the $\alpha 4\alpha 5\beta 2$ -nAChR presynaptic receptors on dopaminergic terminals may contribute to the continued reward properties of nicotine in smokers. Concentrations of nicotine in blood of smokers after overnight abstinence from smoking are in the range of DC_{50} for nicotine at $(\alpha 4\beta 2)_2\beta 2$ -nAChRs (Table 2). However, this level of nicotine would be well below the DC_{50} at $(\alpha 4\beta 2)_2\alpha 5$ -nAChRs. Our measurements of DC_{50} are for a mixed population of subtypes (with and without $\alpha 5$). For this mixed population, biphasic curves might be expected. However, the DC_{50} difference of ~8-fold is likely not sufficiently great to be resolved. In addition, the size of the two populations is unclear. By the measure of functional loss in the $\alpha 5$ KO, 65%–70% of the HS activity is via $\alpha 4\alpha 5\beta 2$ -nAChR (Grady, Salminen et al., 2010); however, this could be an underestimate of what is present in a wild-type mouse. With one population being small (perhaps considerably smaller than 30%), biphasic curves may be even less apparent. Because of this mixed population, it is possible that we have somewhat underestimated the effect of the $\alpha 5$ subunit in shifting this parameter. As a consequence, neurons that express significant levels of $\alpha 5$ subunits paired with $\alpha 4$ and $\beta 2$ subunits may retain considerable nAChR function even during smoking. After short intervals of abstinence, such as between bouts of smoking $(\alpha 4\beta 2)_2\alpha 5$ -nAChRs may recover much of their activity. Therefore, acetylcholine will be able to trigger function of $(\alpha 4\beta 2)_2\alpha 5$ -nAChRs; whereas, the $(\alpha 4\beta 2)_2\beta 2$ subtype may be less functional leading to regional differences based on where $\alpha 5$ is expressed as a component of the $\alpha 4\beta 2$ -nAChR subtype. This differential activity could result in region-specific adaptations to nicotine and/or other drugs. Actions that differ between $(\alpha 4\beta 2)_2\beta 2$ and $(\alpha 4\beta 2)_2\alpha 5$

may contribute to the association of the CHRNA5 D398N variant with smoking.

Varenicline has a much longer *in vivo* half-life than nicotine and remains at a stable concentration for longer, with effective concentrations of 7–10 ng/ml blood levels (30–50 nM) (Faessel et al., 2006). This concentration of varenicline is just sufficient to maximally desensitize both $(\alpha 4\beta 2)_2\beta 2$ - and $(\alpha 4\beta 2)_2\alpha 5$ -nAChRs (see Figure 2A). A behaviorally effective brain concentration of sazetidine-A in rats was estimated to be ~30 nM (Husmann et al., 2012), a concentration that would also confer maximal desensitization of both $(\alpha 4\beta 2)_2\beta 2$ - and $(\alpha 4\beta 2)_2\alpha 5$ -nAChRs (see Figure 2D). Because the concentrations of varenicline and sazetidine-A would be relatively stable, the balance of functional versus desensitized receptors may not fluctuate with these compounds as much as with L-nicotine derived from smoking.

SUMMARY AND CONCLUSIONS

Smoking may differentially modify function of nAChRs in different types of neurons and in different locations depending on the expression of the $\alpha 5$ subunit, site of the identified CHRNA5 smoking risk variant highlighting the importance of this subunit. Differential effects on desensitization of $(\alpha 4\beta 2)_2\beta 2$ - and $(\alpha 4\beta 2)_2\alpha 5$ -nAChRs were found for all compounds tested including a range of full and partial agonists with varying affinity. The effectiveness of smoking cessation drugs in blocking reward of nicotine may require concentrations that effectively block considerable activity of both subtypes. Blocking nAChRs by a drug present in more stable concentrations than nicotine in a smoker may prolong desensitization and, additionally, inhibit restoration of a nonsmoker state. There are indications that even if nAChR levels return to normal upon withdrawal of nicotine, other adaptations may occur in the CA1 region of hippocampus establishing cue-induced craving rather than returning to a “predrug” state (Penton, Quick, & Lester, 2011). Other drug- and cue-evoked persistent synaptic adaptations, common to all addictive drugs, have also been described for the mesocorticolimbic dopamine system (Lüscher & Malenka, 2011). Studies with selective allosteric modulators of $\alpha 3\alpha 5\beta 4$ - or $\alpha 4\alpha 5\beta 2$ -nAChR (Berrettini & Doyle, 2012; Kuryatov et al., 2011) might be useful in determining whether such compounds would be helpful smoking cessation aids or, alternatively, whether additional downstream targets should be explored.

FUNDING

This work was supported by the National Institutes of Health (U19DA019375, RO1DA003194, and P30DA015663 to MJM).

DECLARATION OF INTERESTS

None declared.

ACKNOWLEDGMENTS

We thank William Van Morter, Erin Meyers, and Esteban Loetz for animal husbandry and genotyping.

REFERENCES

- Bailey, C. D., De Biasi, M., Fletcher, P. J., & Lambe, E. K. (2010). The nicotinic acetylcholine receptor $\alpha 5$ subunit plays a key role in attention circuitry and accuracy. *The Journal of Neuroscience*, 30, 9241–9252. doi:10.1523/JNEUROSCI.2258-10.2010
- Barik, J., & Wonnacott, S. (2009). Molecular and cellular mechanisms of action of nicotine in the CNS. *Handbook of Experimental Pharmacology*, 192, 173–207. doi:10.1007/978-3-540-69248-5_7
- Berrettini, W. H., & Doyle, G. A. (2012). The CHRNA5-A3-B4 gene cluster in nicotine addiction. *Molecular Psychiatry*, 17, 856–866. doi:10.1038/mp.2011.122
- Brody, A. L., Mandelkern, M. A., London, E. D., Olmstead, R. E., Farahi, J., Scheibal, D., ..., Mukhin, A. G. (2006). Cigarette smoking saturates brain $\alpha 4 \beta 2$ nicotinic acetylcholine receptors. *Archives of General Psychiatry*, 63, 907–915. doi:10.1001/archpsyc.63.8.907
- Brown, R. W., Collins, A. C., Lindstrom, J. M., & Whiteaker, P. (2007). Nicotinic $\alpha 5$ subunit deletion locally reduces high-affinity agonist activation without altering nicotinic receptor numbers. *Journal of Neurochemistry*, 103, 204–215. doi:10.1111/j.1471-4159.2007.04700.x
- Buccafusco, J. J., Beach, J. W., & Terry, A. V., Jr. (2009). Desensitization of nicotinic acetylcholine receptors as a strategy for drug development. *The Journal of Pharmacology and Experimental Therapeutics*, 328, 364–370. doi:10.1124/jpet.108.145292
- Champtiaux, N., Gotti, C., Cordero-Erausquin, M., David, D. J., Przybylski, C., Lena, C., ..., Changeux, J. P. (2003). Subunit composition of functional nicotinic receptors in dopaminergic neurons investigated with knock-out mice. *The Journal of Neuroscience*, 23, 7820–7829.
- De Biasi, M., & Dani, J. A. (2011). Reward, addiction, withdrawal to nicotine. *Annual Review of Neuroscience*, 34, 105–130. doi:10.1146/annurev-neuro-061010-113734
- Exley, R., McIntosh, J. M., Marks, M. J., Maskos, U., & Cragg, S. J. (2012). Striatal $\alpha 5$ nicotinic receptor subunit regulates dopamine transmission in dorsal striatum. *The Journal of Neuroscience*, 32, 2352–2356. doi:10.1523/JNEUROSCI.4985-11.2012
- Faessel, H. M., Gibbs, M. A., Clark, D. J., Rohrbacher, K., Stolar, M., & Burstein, A. H. (2006). Multiple-dose pharmacokinetics of the selective nicotinic receptor partial agonist, varenicline, in healthy smokers. *Journal of Clinical Pharmacology*, 46, 1439–1448. doi:10.1177/0091270006292624
- Fonck, C., Cohen, B. N., Nashmi, R., Whiteaker, P., Wagenaar, D. A., Rodrigues-Pinguet, N., ..., Lester, H. A. (2005). Novel seizure phenotype and sleep disruptions in knock-in mice with hypersensitive $\alpha 4^*$ nicotinic receptors. *The Journal of Neuroscience*, 25, 11396–11411. doi:10.1523/jneurosci.3597-05.2005
- Grady, S. R., Drenan, R. M., Breining, S. R., Yohannes, D., Wageman, C. R., Fedorov, N. B., ..., Marks, M. J. (2010). Structural differences determine the relative selectivity of nicotinic compounds for native $\alpha 4 \beta 2^*$, $\alpha 6 \beta 2^*$, $\alpha 3 \beta 4^*$ and $\alpha 7$ -nicotine acetylcholine receptors. *Neuropharmacology*, 58, 1054–1066. doi:10.1016/j.neuropharm.2010.01.013
- Grady, S. R., Salminen, O., McIntosh, J. M., Marks, M. J., & Collins, A. C. (2010). Mouse striatal dopamine nerve terminals express $\alpha 4 \alpha 5 \beta 2$ and two stoichiometric forms of $\alpha 4 \beta 2^*$ -nicotinic acetylcholine receptors. *Journal of Molecular Neuroscience*, 40, 91–95. doi:10.1007/s12031-009-9263-y
- Grady, S. R., Wageman, C. R., Patzlaff, N. E., & Marks, M. J. (2012). Low concentrations of nicotine differentially desensitize nicotinic acetylcholine receptors that include $\alpha 5$ or $\alpha 6$ subunits and that mediate synaptosomal neurotransmitter release. *Neuropharmacology*, 62, 1935–1943. doi:10.1016/j.neuropharm.2011.12.026
- Hulihan-Giblin, B. A., Lumpkin, M. D., & Kellar, K. J. (1990). Acute effects of nicotine on prolactin release in the rat: Agonist and antagonist effects of a single injection of nicotine. *The Journal of Pharmacology and Experimental Therapeutics*, 252, 15–20.
- Hurst, R., Rollema, H., & Bertrand, D. (2013). Nicotinic acetylcholine receptors: From basic science to therapeutics. *Pharmacology & Therapeutics*, 137, 22–54. doi:10.1016/j.pharmthera.2012.08.012
- Husmann, G. P., Turner, J. R., Lomazzo, E., Venkatesh, R., Cousins, V., Xiao, Y., ..., Kellar, K. J. (2012). Chronic sazetidine-A at behaviorally active doses does not increase nicotinic cholinergic receptors in rodent brain. *The Journal of Pharmacology and Experimental Therapeutics*, 343, 441–450. doi:10.1124/jpet.112.198085
- Jarvik, M. E., Madsen, D. C., Olmstead, R. E., Iwamoto-Schaap, P. N., Elins, J. L., & Benowitz, N. L. (2000). Nicotine blood levels and subjective craving for cigarettes. *Pharmacology, Biochemistry, and Behavior*, 66, 553–558.
- Kuryatov, A., Berrettini, W., & Lindstrom, L. (2011). Acetylcholine receptor (AChR) $\alpha 5$ subunit variant associated with risk for nicotine dependence and lung cancer reduces ($\alpha 4 \beta 2$)($\alpha 5$) AChR function. *Molecular Pharmacology*, 79, 119–125. doi:10.1124/mol.110.066357
- Levin, E. D., Rezvani, A. H., Xiao, Y., Slade, S., Cauley, M., Wells, C., ..., Kellar, K. J. (2010). Sazetidine-A, a selective $\alpha 4 \beta 2$ nicotinic receptor desensitizing agent and partial agonist, reduces nicotine self-administration in rats. *The Journal of Pharmacology and Experimental Therapeutics*, 332, 933–939. doi:10.1124/jpet.109.162073
- Lukas, R. J., Changeux, J. P., Le Novère, N., Albuquerque, E. X., Balfour, D. J., Berg, D. K., ..., Wonnacott, S. (1999). International Union of Pharmacology. XX. Current status of the nomenclature for nicotinic acetylcholine receptors and their subunits. *Pharmacological Reviews*, 51, 397–401.
- Lüscher, C., & Malenka, R. C. (2011). Drug-evoked synaptic plasticity in addiction: From molecular changes to circuit remodeling. *Neuron*, 69, 650–663. doi:10.1016/j.neuron.2011.01.017
- Mao, D., Perry, D. C., Yasuda, R. P., Wolfe, B. B., & Kellar, K. J. (2008). The $\alpha 4 \beta 2 \alpha 5$ nicotinic cholinergic receptor in rat brain is resistant to up-regulation by nicotine in vivo. *Journal of Neurochemistry*, 104, 446–456. doi:10.1111/j.1471-4159.2007.05011.x
- Marks, M. J., Whiteaker, P., Calcaterra, J., Stitzel, J. A., Bullock, A. E., Grady, S. R., ..., Collins, A. C. (1999). Two pharmacologically distinct components of nicotinic receptor-mediated rubidium efflux in mouse brain require the $\beta 2$ subunit. *The Journal of Pharmacology and Experimental Therapeutics*, 289, 1090–1103.
- Marks, M. J., Whiteaker, P., & Collins, A. C. (2006). Deletion of the $\alpha 7$, $\beta 2$, or $\beta 4$ nicotinic receptor subunit genes identifies highly expressed subtypes with relatively low affinity for [3 H]epibatidine. *Molecular Pharmacology*, 70, 947–959. doi:10.1124/mol.106.025338
- Maskos, U. (2010). Role of endogenous acetylcholine in the control of the dopaminergic system via nicotinic receptors. *Journal of Neurochemistry*, 114, 641–646. doi:10.1111/j.1471-4159.2010.06798.x
- Miner, L. L., & Collins, A. C. (1988). Effect of nicotine pretreatment on nicotine-induced seizures. *Pharmacology, Biochemistry, and Behavior*, 29, 375–380.
- Pennock, R. L., Dicken, M. S., & Hentges, S. T. (2012). Multiple inhibitory G-protein-coupled receptors resist acute

- desensitization in the presynaptic but not postsynaptic compartments of neurons. *The Journal of Neuroscience*, 32, 10192–10200. doi:10.1523/jneurosci.1227-12.2012
- Penton, R. E., Quick, M. W., & Lester, R. A. (2011). Short- and long-lasting consequences of in vivo nicotine treatment on hippocampal excitability. *The Journal of Neuroscience*, 31, 2584–2594. doi:10.1523/JNEUROSCI.4362-10.2011
- Picciotto, M. R., Addy, N. A., Mineur, Y. S., & Brunzell, D. H. (2008). It is not “either/or”: activation and desensitization of nicotinic acetylcholine receptors both contribute to behaviors related to nicotine addiction and mood. *Progress in Neurobiology*, 84, 329–342. doi:10.1016/j.pneurobio.2007.12.005
- Pidoplichko, V. I., DeBiasi, M., Williams, J. T., & Dani, J. A. (1997). Nicotine activates and desensitizes midbrain dopamine neurons. *Nature*, 390, 401–404. doi:10.1038/37120
- Poorthuis, R. B., Bloem, B., Verhoog, M. B., & Mansvelder, H. D. (2013). Layer-specific interference with cholinergic signaling in the prefrontal cortex by smoking concentrations of nicotine. *The Journal of Neuroscience*, 33, 4843–4853. doi:10.1523/jneurosci.5012-12.2013
- Rose, J. E., Mukhin, A. G., Lokitz, S. J., Turkington, T. G., Herskovic, J., Behm, F. M., ..., Garg, P. K. (2010). Kinetics of brain nicotine accumulation in dependent and nondependent smokers assessed with PET and cigarettes containing 11C-nicotine. *Proceedings of the National Academy of Sciences of the United States of America*, 107, 5190–5195. doi:10.1073/pnas.0909184107
- Salminen, O., Murphy, K. L., McIntosh, J. M., Drago, J., Marks, M. J., Collins, A. C., & Grady, S. R. (2004). Subunit composition and pharmacology of two classes of striatal presynaptic nicotinic acetylcholine receptors mediating dopamine release in mice. *Molecular Pharmacology*, 65, 1526–1535. doi:10.1124/mol.65.6.1526
- Sharp, B. M., & Beyer, H. S. (1986). Rapid desensitization of the acute stimulatory effects of nicotine on rat plasma adrenocorticotropin and prolactin. *The Journal of Pharmacology and Experimental Therapeutics*, 238, 486–491.
- Vardya, I., Drasbek, K. R., Gibson, K. M., & Jensen, K. (2010). Plasticity of postsynaptic, but not presynaptic, GABAB receptors in SSADH deficient mice. *Experimental Neurology*, 225, 114–122. doi:10.1016/j.expneurol.2010.05.022
- Wetherington, J. P., & Lambert, N. A. (2002). Differential desensitization of responses mediated by presynaptic and postsynaptic A1 adenosine receptors. *The Journal of Neuroscience*, 22, 1248–1255.
- Whiteaker, P., Jimenez, M., McIntosh, J. M., Collins, A. C., & Marks, M. J. (2000). Identification of a novel nicotinic binding site in mouse brain using [(125)I]-epibatidine. *British Journal of Pharmacology*, 131, 729–739. doi:10.1038/sj.bjp.0703616