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Stimulation of dopamine release by nicotinic acetylcholine receptor ligands in rat brain slices correlates with the profile of high, but not low, sensitivity α4β2 subunit combination

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ABSTRACT

α4β2 neuronal nicotinic receptors (nAChRs) can exist in high and low sensitivity states possibly due to distinct stoichiometries during subunit assembly: $(\alpha4)_2(\beta2)_3$ pentamer (high sensitivity, HS) and $(\alpha4)_3(\beta2)_2$ pentamer (low sensitivity, LS). To determine if there is a linkage between HS or LS states and receptor-mediated responses in brain, we profiled several clinically studied α4β2* nAChR agonists for the displacement of radioligand binding to α4β2 [^{3}H]-cytisine sites in rat brain membranes, effects on stimulation of [^{3}H]-dopamine release from slices of rat prefrontal cortex and striatum, and activation of HS and LS human α4β2 nAChRs expressed in *Xenopus laevis* oocytes. Binding affinities (pK_i) and potency (pEC_{50}) values for [^{3}H]-dopamine release closely correlated with a rank order: varenicline > (−)-nicotine > AZD3480 > dianicline ≅ ABT-089. Further, a good correlation was observed between [^{3}H]-dopamine release and HS α4β2 pEC_{50} values, but not between [^{3}H]-dopamine release and LS α4β2. The relative efficacies of the agonists ranged from full to partial agonists. Varenicline behaved as a partial agonist in stimulating [^{3}H]-dopamine release and activating both HS and LS α4β2 nAChRs expressed in oocytes. Conversely, ABT-089, AZD3480 and dianicline exhibited little efficacy at LS α4β2 (<5%), were more effective at HS α4β2 nAChRs, and in stimulating cortical and striatal [^{3}H]-dopamine release ≥30%. In the presence of α-conotoxin MII to block α6β2* nAChRs, the α4β2* α-conotoxin-insensitive [^{3}H]-dopamine release stimulated by these ligands correlates well with their interactions at HS, but not LS. In summary, this study provides support for HS α4β2* nAChR involvement in neurotransmitter release.

1. Introduction

Neuronal nicotinic acetylcholine receptors (nAChRs) belong to the family of pentameric acetylcholine-gated cation channels and are assembled from twelve subunits, α2–α10 and β2–β4. These subunits combine to form homomeric or heteromeric nAChRs that mediate a wide range of physiological and pharmacological effects in the nervous system. All are thought to be pentameric, comprised of a mixture of alpha-subunits (α2–α6) and beta-subunits (β2–β4) with exceptions of homomers of α7–α9 subunits, or heteromers of α9–α10 subunits [1]. A recent report provided evidence for the existence of a functional α7β2 nAChR in rodent basal forebrain [2]. The α4β2-containing (α4β2*) nAChRs account for 90% of the high affinity nicotine binding sites and are widely distributed throughout the brain including in the cortex, hippocampus, substantia nigra, and ventral tegmental area (reviewed in [3]). The last two regions are particularly important since they are rich in dopaminergic neurons whose function is regulated by various nicotinic subunits including α4β2* subtype and thought to participate in modulating the reinforcing effects of nicotine and other addictive substances (reviewed in [4]) and in CNS disorders such as Parkinson's disease.

Initially, heteromeric α4β2 subunits were thought to exist as a single subunit entity exhibiting high affinity to nicotine [5]. However, a variety of combinations and stoichiometries are possible as evidenced by injecting varying ratios of α4 and α2 cRNA or cDNA [6–9].

Combination of excess α4 with respect to β2 subunit is thought to generate predominantly $(\alpha4)_3(\beta2)_2$ pentamer with low sensitivity (LS) to ACh ($EC_{50} \sim 100\ \mu M$), whereas the reverse ratio

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is thought to favor the $(\alpha4)_2(\beta2)_3$ expression with high sensitivity (HS) to ACh ($EC_{50} \sim 1\ \mu M$). This concept is supported by studies using linked α4β2 subunits assembled with β2 subunits as $(\alpha4\beta2)_2\beta2$ pentamers and with α4 subunits as $(\alpha4\beta2)_2\alpha4$ pentamers to address the stochiometry of functional receptor assemblies [10]. Other compounds exhibiting differential profiles at HS and LS profiles include a variety of agonists (nicotine, epibatidine, cytisine, 3-Br-cytisine, 3-[2(S)-azetidinylmethoxy]-pyridine (A-85380), 5-ethoxy-metanicotine (TC-2559), A-163554 and A-168939) and some antagonists, particularly chlorisondamine [9,11]. The LS form is thought to be more permeable to Ca^{2+} than the HS form whereas the HS form is more sensitive to upregulation by chronic nicotine and reduced ambient temperature [12]. Other combinations with various degrees of sensitivities and selectivities for nAChR ligands can be formed with the substitution of other subunits such as α2, α3, α6 or β4 [3]. Like α4β2, HS and LS forms of α3β2 also have been demonstrated, suggesting this may be a general feature of β2-containing nAChR [9]. The α5 and β3 subunits do not participate in the formation of acetylcholine binding sites, but may have accessory or regulatory roles in the receptor affecting the properties of the expressed pentamer. For instance, $(\alpha4\beta2)_2$ α5 nAChRs were reported to be more permeable to Ca^{2+} than either $(\alpha4\beta2)_2\beta2$ and $(\alpha4\beta2)_2\alpha4$ pentamers [13]. One brain region that has been demonstrated to be especially rich in the variety of nAChRs present is the striatum with up to six nAChR combinations proposed [14]. Through the use of null mutant mice, immunoprecipitation, and inhibition with α-conotoxin MII, it has been ascertained that there likely exist α4β2, α4α5β2, α6β2, α4α6β2β3, and α6β2β3 combinations as well as the homomeric α7 in the striatum.

However, HS and LS forms of β2-containing nAChR are defined mainly by kinetic (EC_{50}) parameters. Whether both forms are expressed in the CNS and, if so, whether they have different functional roles is not yet fully elucidated. In mouse thalamic synaptosomes, responses to HS α4β2 nAChR selective agonists such as A-163554 and A-168939 suggest that at least the HS form is expressed functionally in that brain region [9]. Whether LS α4β2 nAChR also is expressed and whether the pattern may vary among brain regions remain open questions.

In this study, we hypothesized that there may be differential contributions of HS, $(\alpha4)_2(\beta2)_3$, and LS, $(\alpha4)_3(\beta2)_2$, nAChRs in neurotransmitter release. We addressed the contribution of HS and LS α4β2 to nAChR-mediated [^{3}H]-dopamine release from slices of rat prefrontal cortex and striatum by using a set of five agonists, and compared the pharmacological profiles of transmitter release with those of HS and LS α4β2 nAChRs expressed in Xenopus oocytes and α4β2* ([^{3}H]-cytisine) binding in rat brain. Additionally, since multiple receptor subtypes are involved in nAChR-mediated [^{3}H]-dopamine release in the striatum, α-conotoxin MII was used to distinguish the role of α4β2* from α6β2* nAChRs. Our results suggest that the HS, but not the LS, form of α4β2 nAChR is primarily involved in nicotine-stimulated dopamine release.

2. Methods

2.1. Materials

Membranes for radioligand binding studies were prepared from frozen rat brains (PelFreez, Rogers, AR, USA). For neurotransmitter release studies, male Sprague–Dawley rats were used from Charles River Laboratories (Portage, MI, USA, 250–400 g). Oocytes were obtained from adult female *Xenopus laevis* frogs (Blades Biological Ltd., Cowden, Edenbridge, Kent, UK). Animals were cared for in accordance with the Institutional Animal Care Committee guidelines that meet the guidelines of the National Institutes of Health. Acetylcholine and nicotine were obtained from Sigma (St. Louis, MO, USA or Oslo, Norway). ABT-089 (2-methyl-3-([(2S)-pyrrolidin-2-yl]methoxy)pyridine), varenicline (7,8,9,10-tetrahydro-6,10-methano-6*H*-pyrazino (2,3-h)(3) benzazepine), AZD3480 (S-E-[4-(5-isopropoxy-pyridin-3-yl)-1-methyl-but-3-enyl]-methyl-amine), and dianicline ((5aS,8S,10aR)-5a,6,9,10-tetrahydro,7H,11H-8,10a-methanopyrido[2′,3′:5,6]pyrano[2,3-d]azepine) were synthesized in house (Abbott Park, IL, USA). α-Conotoxin MII was obtained from Tocris Bioscience (Ellisville, MO, USA or Bristol, UK). [^{3}H]-cytisine and [^{3}H]-dopamine were obtained from PerkinElmer Life Sciences (Boston, MA, USA). All other chemicals and reagents were obtained from Sigma (St. Louis, MO, USA) or Fisher Scientific (Essex, UK).

2.2. [^{3}H]-cytisine binding to rat brain

[^{3}H]-Cytisine binding to α4β2 nAChRs was determined under equilibrium conditions using membrane enriched fractions from rat brain (minus cerebellum) as previously described [15]. Brains were homogenized in 15 volumes of 0.32 M sucrose and centrifuged at $1000 \times g$ at 4 °C for 10 min. Supernatants were centrifuged at $20{,}000 \times g$ for 20 min. The resulting P_2 pellets were homogenized with a Polytron (10 s at setting 7) in 15 volumes ice-cold H_2O and centrifuged at $8000 \times g$ at 4 °C for 20 min. The supernatant and loose buffy coat were centrifuged at $40{,}000 \times g$. The pellet was washed in 15 volumes ice-cold H_2O and recentrifuged before storage at −80 °C. Pellets were thawed at 4 °C, washed and resuspended with a Polytron at a setting of 7 in 30 volumes of BSS-Tris buffer (120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, and 50 mM Tris–Cl, pH 7.4, 4 °C) to yield 100–200 μg of protein. For concentration-inhibition assays, seven log-dilution concentrations of test compounds in duplicate, homogenate containing 100–200 μg of protein, and 0.7 nM [^{3}H]-cytisine were incubated in a final volume of 500 μl for 75 min at 4 °C. Non-specific binding was determined in the presence of 10 μM (−)-nicotine. Bound radioactivity was collected by vacuum filtration onto Millipore MultiScreen® harvest plates FB presoaked with 0.3% PEI using a PerkinElmer cell harvester and the filters were rapidly rinsed with 2 ml of ice-cold BSS-Tris buffer. PerkinElmer MicroScint-20® scintillation cocktail (40 μl) was added to each well and bound radioactivity was determined using a PerkinElmer TopCount® instrument.

2.3. Electrophysiological characterization in X. laevis oocytes

High sensitivity (HS) and low sensitivity (LS) human α4β2 nAChR were expressed in *X. laevis* oocytes by methods described previously [16,17]. In brief, three to four lobes from ovaries of female adult *X. laevis* frogs were removed and defolliculated after treatment with collagenase type 1A (2 mg/ml; Sigma) prepared in low-Ca^{2+} Barth's solution (90 mM NaCl, 1.0 mM KCl, 0.66 mM $NaNO_3$, 2.4 mM $NaHCO_3$, 10 mM HEPES, 2.5 mM sodium pyruvate, 0.82 mM $MgCl_2$, and 0.5% (v/v) penicillin–streptomycin solution, pH 7.55 (Sigma)) for 1.5–2 h at ~18 °C under constant agitation to obtain isolated oocytes. The oocytes were co-injected with human α4 and β2 nAChR cRNA (0.2–30 ng of each), kept at 18 °C in a humidified incubator in low-Ca^{2+} Barth's solution and used 2–7 days after injection. Responses were measured by two-electrode voltage clamp using parallel oocyte electrophysiology test station (Abbott, Abbott Park, IL) [18]. During recordings, the oocytes were bathed in Ba^{2+}-OR2 solution (90 mM NaCl, 2.5 mM KCl, 2.5 mM $BaCl_2$, 1.0 mM $MgCl_2$, 5.0 mM HEPES, and 0.0005 mM atropine, pH 7.4) to prevent activation of Ca^{2+}-dependent currents and held at −60 mV at room temperature (~20 °C). No acetylcholinesterase inhibitor was included in the bath buffer. To obtain either predominantly HS or LS combinations, ratios of 1:1 (HS) or 100:1 (LS) α4 and β2 cRNAs were injected. Ion current amplitudes

were measured and normalized to the amplitude of control responses induced by acetylcholine (ACh): 100 μM for HS (EC_{50} = 2 μM) and 1 mM for LS (EC_{50} = 84 μM).

2.4. [^{3}H]-dopamine release from rat striatum and prefrontal cortex slices

Methods for measurement of [^{3}H]-DA release was modified from those described elsewhere [19]. Rat striata and/or prefrontal cortices were dissected from male Sprague–Dawley rats. Tissue from two rat brains yielded enough material for each 96-well plate. The tissue was chopped 3× at 250 mm at 60° angles, then washed 3× with buffer (130 mM NaCl; 1.25 mM NaH_2PO_4; 25 mM $NaHCO_3$; 2.8 mM KCl; 1.3 mM $MgS0_4$; 2.5 mM $CaCl_2$; 0.1 mM sodium ascorbate; 10 mM dextrose; 10 μM pargyline; pH 7.4, 37 °C, oxygenated with 95/5% O_2/CO_2). Slices were incubated in 4 ml buffer with 100 nM [^{3}H]-DA and 1 mM sodium ascorbate for 25 min, 37 °C, with solution surface blowing with 95/5% O_2/CO_2. The slices were washed with buffer 4×, resuspended in 10 ml/96-well plate, and distributed 100 μl per well in triplicate or quadruplicate to 96-well filter plates (Millipore Multiscreen® MABVN1210). The tissue was gently washed with 100 μl buffer by low-vacuum filtration. Then 100 μl buffer containing 0.5 μM nomefensine was added and the plates were preincubated 10 min at 37 °C. Nomefensine was included for the rest of the assay. When α-conotoxin MII was used, it was included in the appropriate wells and the preincubation was extended to 20 min. The plates were vacuumed and the effluent discarded. 75 μl buffer or antagonist was added, followed by 10 min incubation at 37 °C. The medium was separated from the tissue with low-vacuum filtration and the effluent collected (Basal) in PerkinElmer PicoPlates®. 75 μl (buffer, drug, or drug + antagonist) were added and incubated for 10 min at 37 °C, followed by vacuum filtration where the effluent was collected (Stimulated). 70 μl of Solvable™ were added to each well and incubated 45+ min to dissolve the tissue. The fluid was collected into PicoPlates by centrifugation at 2000 rpm (tissue) and the wells were washed with 25 μl water and 150 μl Microscint 40™ (PerkinElmer) into the same plate. 150 μl Microscint 40™ were added to basal and stimulated plates. Radioactivity was determined using a PerkinElmer TopCount® instrument.

2.5. Data analyses

For radioligand binding, the IC_{50} values were determined by nonlinear regression in Microsoft Excel or Assay Explorer (Symyx, San Ramon, CA). K_i values were calculated from the IC_{50}s using the Cheng–Prusoff equation, where $K_i = IC_{50}/(1 + [\text{ligand}/K_d])$; K_d = 0.14 nM. Dopamine release data was expressed as a fractional % of total radioactivity collected for each well. Basal release was subtracted from stimulated release for each sample. These values were normalized to maximal release evoked by nicotine. Each concentration of control or test compound was applied in replicates of 3–4. Concentration–response parameters were determined by nonlinear regression analysis using Prism 4.0 (GraphPad, San Diego, CA). Averaged replicates of evoked release of [^{3}H]-DA for nicotinic agonists in individual experiments were normalized to the maximal response to nicotine which ranged from 4.5 to 7.8% of total [^{3}H]-DA in striatal slices and from 2.9 to 4.8% in cortical slices. Pooled normalized data were analyzed in Prism to determine EC_{50} and E_{max} as a % of nicotine response. Standard errors were determined by Prism. Global (shared) fits were performed with *F*-test in Prism for the EC_{50} and E_{max} for each data set. In two-electrode voltage clamp studies, concentration–response curves, current amplitudes were measured and normalized to the amplitude of control responses induced by acetylcholine (ACh): 100 μM for HS and 1 mM for LS. Pooled data were analyzed as above.

3. Results

Binding affinities of nAChR ligands at α4β2* nAChRs were assessed by displacement of [^{3}H]-cytisine binding to rat brain membranes (Table 1). Varenicline had a K_i value of 0.08 nM, which was 10-fold higher than (−)-nicotine (K_i = 0.94 nM). The other three compounds also had relatively high affinity with K_i values of 2.7 nM for AZD3480, 11.4 nM for dianicline, and 14.1 nM for ABT-089.

Changes in the stoichiometry of the nAChR pentameric structure can be influenced by the ratio of human subunit cRNAs injected into Xenopus oocytes. Two different sensitivity states can be produced. Under the conditions of this study, when the α4:β2 ratio injected was 1:1, a predominantly high sensitivity (HS) state resulted, presumably an $(\alpha4)_2(\beta2)_3$ pentamer, as confirmed by ACh concentration responses yielding an EC_{50} values of 1.6 μM (pEC_{50} 5.79 ± 0.10, n = 4). Conversely, when the α4:β2 ratio injected was 100:1, the result was a predominantly low sensitivity (LS) state, an $(\alpha4)_3(\beta2)_2$ pentamer. The EC_{50} for ACh for LS form was 84 μM (pEC_{50} 4.07 ± 0.05, n = 6). The five nicotinic agonists used in this study evoked a wide range of responses at the HS and LS forms of the α4β2 nAChR (Table 1 and Fig. 1). Nicotine evoked HS currents with an EC_{50} of 0.45 μM and an efficacy of 32% relative to ACh. The LS response to nicotine had an EC_{50} of 12.5 μM and an efficacy of 68%. Likewise, varenicline produced a greater response at the LS α4β2

Table 1
Comparative in vitro properties of nAChR ligands in binding and functional assays.

Drug	[^{3}H]-cyt.[a]	[^{3}H]-DA release		α4β2 current (oocytes)			
	Rat brain	Rat striatum		HS α4β2 (1:1)		LS α4β2 (100:1)	
	K_i (nM) (pK_i ± S.E.)	EC_{50}[b,c] (nM) (pEC_{50} ± S.E.)	E_{max}[d,e] (S.E.)	EC_{50}[f,c] (μM) (pEC_{50} ± S.E.)	E_{max}[g,e] (S.E.)	EC_{50}[f,h] (μM) (pEC_{50} ± S.E.)	E_{max}[g,e]
Nicotine	0.94 (9.03 ± 0.30)	86 (7.07 ± 0.11)	101.0% (3.8)	0.46 (6.34 ± 0.05)	32.5% (1.1)	12.5 (4.90 ± 0.05)	68.1% (2.2)
Varenicline	0.08 (10.1 ± 0.03)	10 (7.99 ± 0.29)	48.7% (4.1)	0.16 (6.79 ± 0.19)	17.1% (2.3)	2.1 (5.68 ± 0.20)	23.0% (2.2)
AZD3480	2.67 (8.57 ± 0.02)	337 (6.47 ± 0.13)	102.0% (4.2)	1.0 (5.99 ± 0.04)	89.7% (2.0)	1.3 (5.87 ± 0.12)	3.5% (0.3)
ABT-089	14.1 (7.85 ± 0.25)	1330 (5.88 ± 0.24)	64.6% 10.9	2.4 (5.63 ± 0.11)	11.4% (0.7)	N.R	N.R
Dianicline	11.4 (7.94 ± 0.06)	1110 (5.96 ± 0.36)	51.7% (4.7)	1.7 (5.76 ± 0.10)	26.0% (1.6)	12.9 (4.89 ± 0.09)	2.8% (0.2)

[a] Displacement of [^{3}H]-cytisine binding to rat brain homogenates nicotinic agonists. The K_is represent mean values obtained from independent experiments where $n \geq 3$.
[b] EC50 values represent potencies of compounds determined from pooled data from independent experiments where $n \geq 3$, assessed by evoked efflux of [^{3}H]-DA from rat striatal slices.
[c] EC_{50} values as determined by a *F*-test in Prism were significantly different from each other, $P < 0.05$, DFn = 4.
[d] Max values represent maximal response of the ligand relative to the peak response for nicotine obtained from independent experiments where $n \geq 3$.
[e] Global E_{max} values were significantly different from each other, $P < 0.05$.
[f] Values represent mean EC_{50}s of compounds, assessed by POETs using human α4β2 nAChRs expressed in Xenopus oocytes.
[g] Max values represent maximal response of the ligand relative to the control responses induced by 100 μM ACh for HS and 1 mM ACh for LS. N.R., no detectable response. All values for [^{3}H]-DA release and α4β2 currents were determined in GraphPad Prism.
[h] EC_{50} values of LS were not significantly different from each other, $P > 0.05$.

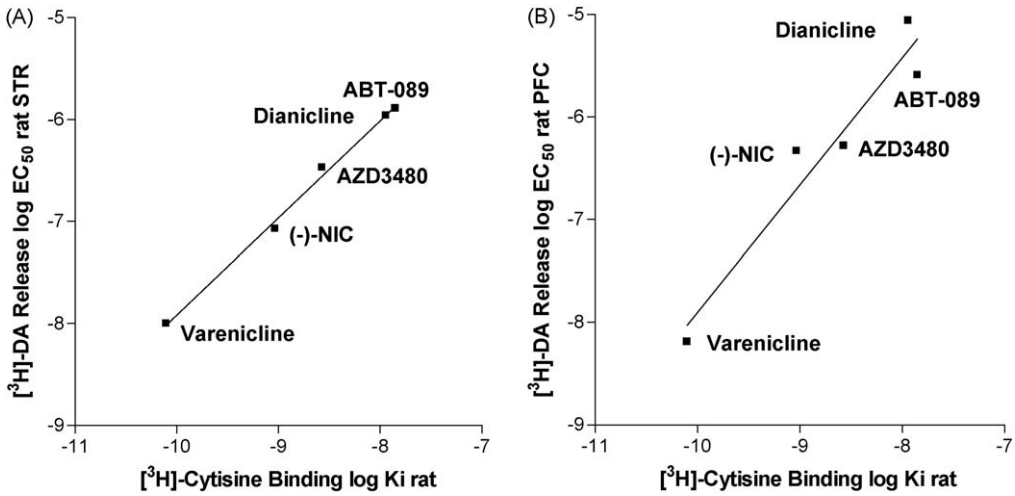

Fig. 1. Concentration–response curves of nAChR ligands at HS (1:1 cRNA) and LS (100:1 cRNA) α4β2 nAChRs expressed in Xenopus oocytes. Activity was measured by two-electrode voltage clamp using POETs. Ion currents were normalized to acetylcholine at 100 μM for HS and 1 mM for LS. Values are in Table 1. Nicotine (A) and varenicline (B) were partial agonists at HS and had greater responses at LS α4β2. AZD3480 (C) was a nearly full agonist at HS, but had a very small effect at LS. Dianicline (D) and ABT-089 (E) had 26% and 11% respective responses at HS and very small or negligible effects at LS.

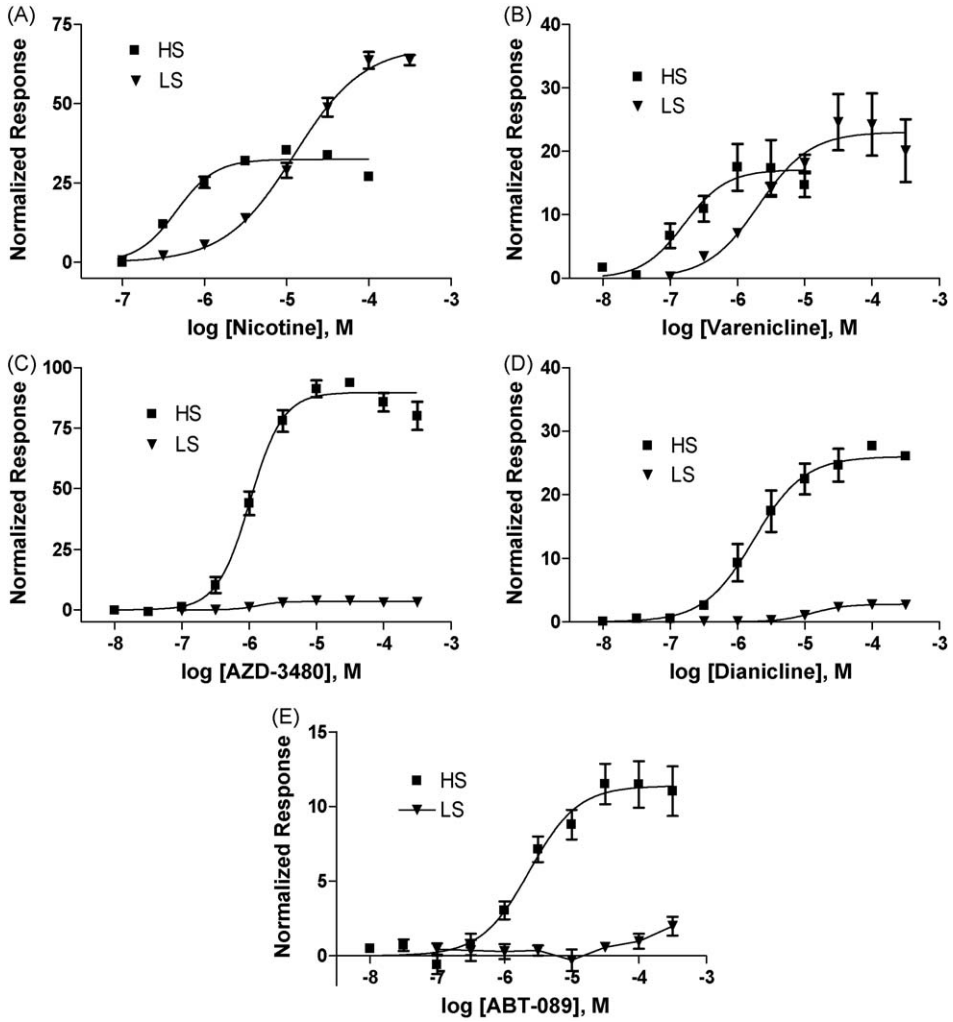

Fig. 2. Concentration–response curves of agonist-evoked [^{3}H]-DA release from rat brain slices. (A) In rat striatum, the rank order of estimated EC_{50} values and % efficacies were: varenicline, 10 nM and 49%; nicotine, 86 nM and 100%; AZD3480, 337 nM and 102%; dianicline, 1.11 μM and 51%; and ABT-089, 1.33 μM and 65%. Data points are the mean ± S.E. from 3 to 9 independent determinations done in triplicate. (B) In rat prefrontal cortex, the rank order was: varenicline, which had a biphasic curve with a high affinity component of 6.4 nM EC_{50} and 22% efficacy and a lower affinity component of 2.6 μM and 48%; nicotine, 463 nM and 100%; AZD3480, 522 nM and 67%; ABT-089, 2.6 μM and 32%; and dianicline, 8.6 μM and 93%. Data points are the mean ± S.E. (n = 4–8 in quadruplicate, except dianicline n = 2).

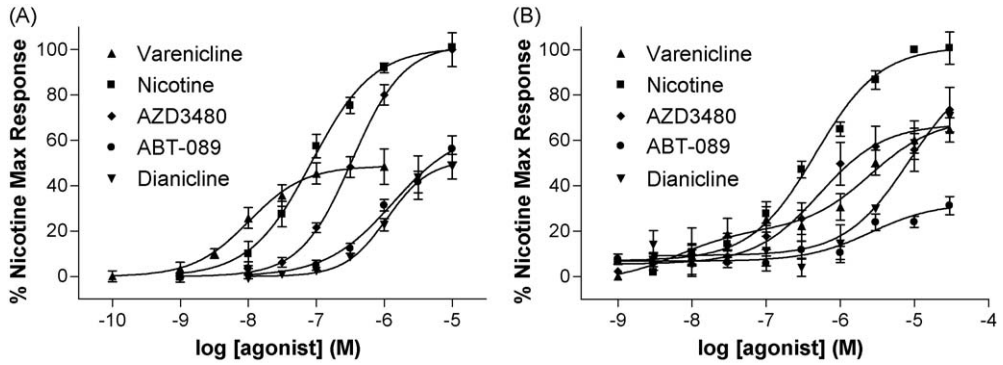

Fig. 3. Comparison of [^{3}H]-cytisine binding to rat brain membrane α4β2* nAChRs to [^{3}H]-DA release from (A) rat striatum slices and (B) rat prefrontal cortex slices. Log K_i and log EC_{50} values presented in Table 1 were plotted and correlations were determined from linear regression lines. For (A), the r^2 value was 1.0 and the slope was 0.95, and for (B), the r^2 was 0.93 and the slope was 1.25.

nAChR with an efficacy of 23% vs. 17% at HS. The EC_{50} values for HS and LS were 0.16 μM and 1.3 μM, respectively. The remainder of the test compounds evoked responses at the predominantly HS α4β2 nAChR, but little or no response at the predominantly LS form. AZD3460 was a nearly full agonist at HS α4β2 nAChR with an efficacy of 90% and an EC_{50} of 1.0 μM. ABT-089 and dianicline were less efficacious and potent at HS α4β2 nAChR with respective efficacy values of 11% and 26% and EC_{50} values of 2.4 and 1.7 μM.

Agonist potencies and efficacies were evaluated in evoked release of [^{3}H]-dopamine from rat brain slices derived from the

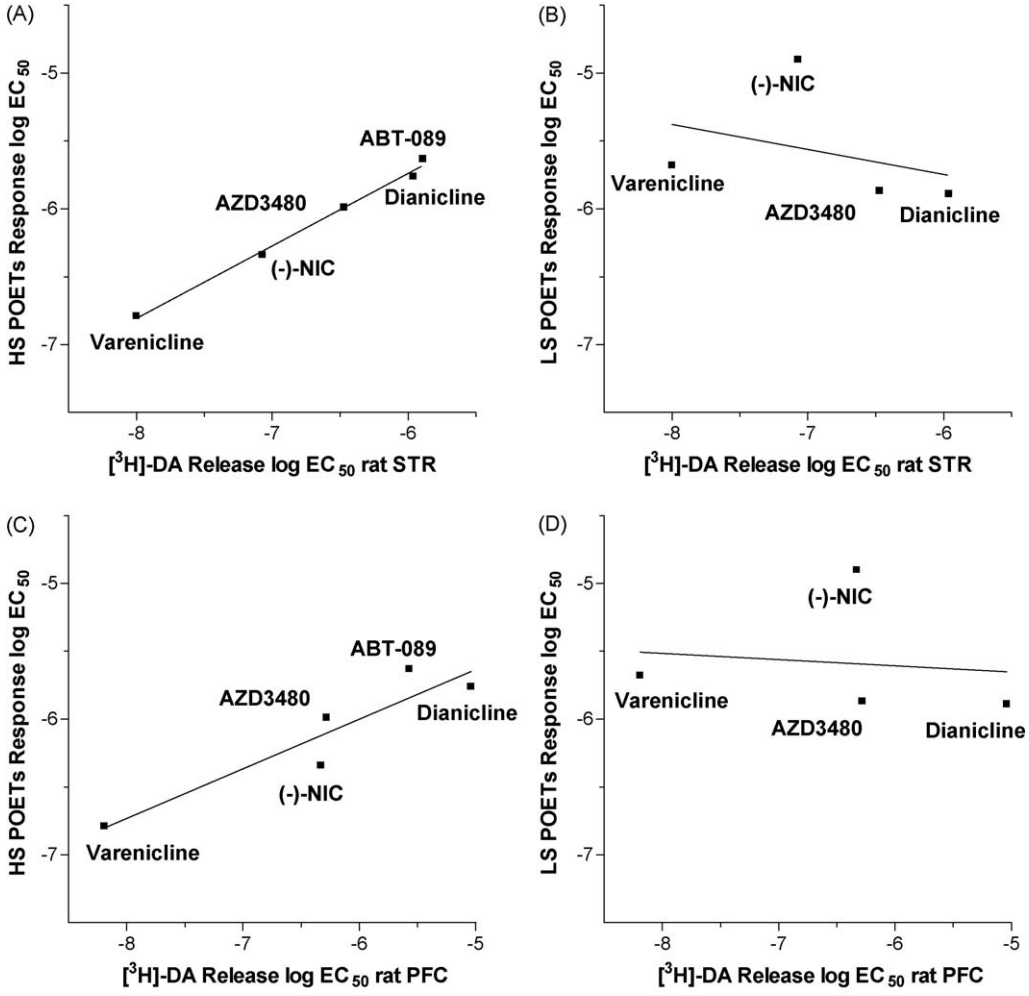

Fig. 4. Comparison of log EC_{50} values of agonist-evoked [^{3}H]-DA release from rat striatum slices (A and B) and rat prefrontal cortex slices (C and D) to log EC_{50} values of agonist responses at HS (A and C) and LS (B and D) α4β2 nAChRs expressed in Xenopus oocytes. The responses of [^{3}H]-DA release from both striatal slices and cortical slices had high degrees of correlation with the response at HS α4β2 nAChRs with r^2 values of 0.99 and 0.87, respectively. The respective slopes were 0.53 and 0.37. There was no correlation between [^{3}H]-DA release and responses at the LS α4β2 nAChR.

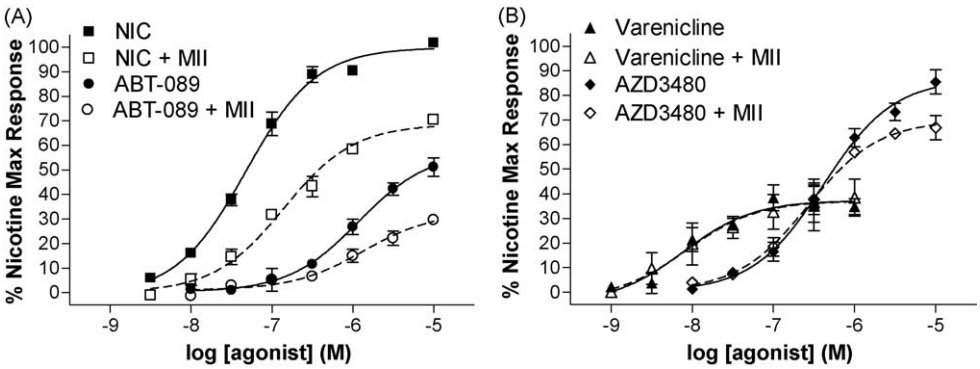

Fig. 5. Concentration–response curves of agonist-evoked [^{3}H]-DA release from rat striatum slices. Agonists were incubated in absence (solid lines and symbols) or presence (dashed lines and open symbols) of 300 nM α-CtxMII. (A) Nicotine (■) had an EC_{50} of 48 nM (pEC_{50}, 7.32 ± 0.06) and 100% efficacy alone and EC_{50} of 140 nM (6.85 ± 0.08) and 69% efficacy with α-CtxMII. ABT-089 (●) had an EC_{50} of 1.4 μM (5.85 ± 0.08) and 62% efficacy alone and EC_{50} of 1.3 μM (5.87 ± 0.18) and 33% efficacy with α-CtxMII. (B) Varenicline (▲) had an EC_{50} of 11 nM (7.95 ± 0.16) and 36% efficacy alone and no change was observed with α-CtxMII. AZD3480 (◆) had an EC_{50} of 404 nM (6.39 ± 0.06) and 87% efficacy alone and EC_{50} of 261 nM (6.58 ± 0.06) and 70% efficacy with α-CtxMII. Data points are the mean ± S.E. from three independent determinations done in triplicate.

striatum and prefrontal cortex. As expected, nicotine-evoked [^{3}H]-DA from striatal slices with an EC_{50} of 86 nM (Fig. 2 and Table 1) with a maximal release of 4–8% of total [^{3}H]-DA. In each experiment, the maximum nicotine-evoked release derived from nonlinear regression was assigned the relative value of 100%. Varenicline was a potent, partial agonist with an EC_{50} of 10 nM and an efficacy of 49% relative to nicotine. Other compounds were less potent than nicotine and exhibited varying degrees of efficacy. AZD3480 was a full agonist with an EC_{50} of 2.67 nM and relative efficacy of 102%. ABT-089 and dianicline were both partial agonists with EC_{50} values of 14.1 nM (efficacy 65%) and 11.4 nM (efficacy 51%), respectively.

While [^{3}H]-DA release from prefrontal cortex slices was substantially lower and more difficult to assess than from striatum, the order of agonist potency was comparable between the two tissues (Fig. 2). The EC_{50} values were right-shifted in prefrontal cortex, possibly due to a different nAChR subtype population in cortex relative to striatum. In the prefrontal cortex, the [^{3}H]-DA release response to varenicline fitted best in Prism to a two-site model with a high affinity component that had an EC_{50} of 6.4 nM (pEC_{50} 8.2 ± 0.8, n = 5) with 22% efficacy and a low affinity component with an EC_{50} of 2.6 μM (pEC_{50} 5.6 ± 0.3, n = 5) with 48% efficacy. Data obtained with other nAChR agonists statistically fitted better to a one-site model in Prism. Nicotine had an EC_{50} of 463 nM (pEC_{50} 6.3 ± 0.1, n = 8) with 100% efficacy (2–4% of total [^{3}H]-DA). AZD3480 was a partial agonist with 67% efficacy and an EC_{50} of 522 nM (pEC_{50} 6.3 ± 0.2, n = 5). ABT-089 also exhibited lower efficacy at 32% and an EC_{50} of 2.6 μM (pEC_{50} 5.6 ± 0.2, n = 4). Conversely, dianicline had higher efficacy (93%) with an EC_{50} of 8.6 μM (pEC_{50} 5.1 ± 0.2, n = 2).

Potencies of compounds to stimulate [^{3}H]-DA release from striatal slices and from cortical slices closely correlated with binding affinities to α4β2* nAChR as measured by [^{3}H]-cytisine binding to rat brain membranes (Fig. 3). For [^{3}H]-cytisine binding vs. striatal [^{3}H]-DA release the coefficient of correlation (r^2) was 0.996 with a slope of 0.95. The relationship was nearly as good for binding vs. cortical release with the r^2 value of 0.93 and a slope 1.25.

The rank order of potencies was similar between HS α4β2 nAChR and [^{3}H]-DA release in both striatal and cortical slices (Fig. 4). The square of the coefficient of correlation was 0.99 for the HS response vs. striatal release, while the value was 0.87 for the HS response vs. cortical release. Both of the slopes were considerably less than 1. However, the rank order of potencies of the agonists at the LS α4β2 nAChR was different than for [^{3}H]-DA release in the striatum and prefrontal cortex and no correlation of potencies was noted.

As noted earlier, α6-containing nAChRs also substantially contribute to striatal dopamine release [14]. In order to further determine the relative contribution of α4β2* nAChRs in [^{3}H]-DA release minus the effect of agonists on α6β2* nAChRs, concentration–response curves were measured in striatal slices in the presence and absence of 300 nM α-conotoxin MII (α-CtxMII), a concentration that had been determined to block α-CtxMII-sensitive [^{3}H]-DA release from slices. α-CtxMII had a differential effect on the response curves evoked by test compounds (Fig. 5). The maximal response to nicotine was inhibited 31 ± 2% by α-CtxMII and the EC_{50} was shifted from 48 nM to 140 nM. The maximal response to ABT-089 was also inhibited by α-CtxMII from 62 ± 3% to 33 ± 4% which represents a 47% decrease, but there was no apparent shift in the EC_{50} values. Likewise, there was no shift in EC_{50} values in the presence of α-CtxMII for either AZD3480 or varenicline, which both apparently have less effect at α6β2* nAChRs. The response of AZD3480 was reduced from 87 ± 3% to 70 ± 3%. Thus, the amount of α-CtxMII-sensitive response evoked by AZD3480 represented less than 20% of the total release of [^{3}H]-DA. Varenicline, on the other hand, had no apparent effect on the α6β2* component of [^{3}H]-DA release from striatal slices.

4. Discussion

Previous studies have demonstrated that α4β2 nAChRs can exist in high sensitivity (HS) and low sensitivity (LS) states dependent on stoichiometry [6–9]. However, the contributions of high and low sensitivity combinations to functional effects mediated by nAChR ligands, as for example dopamine release, remain unclear. Release of [^{3}H]-dopamine from rat brain slices evoked by nicotine and nAChR agonists is mediated by multiple receptor subtypes, including α4β2-containing and α6-containing receptors. Our present study attempted to elucidate the relationship by assessing the pharmacological profile of a set of nAChR ligands by radioligand binding, activation of high and low affinity α4β2 nAChRs in Xenopus oocytes and by [^{3}H]-dopamine release in rat brain slices.

Radioligand binding of [^{3}H]-cytisine to rat brain membranes provided a determination of affinity of a set of compounds for α4β2* nAChRs. The data were in good agreement with previously published data [20–23]. As such, the relative affinities do not reveal whether compounds are agonists or antagonist, or what degree of efficacy can be expected. Although it is not clear what the stoichiometry of native α4β2* nAChRs is, it is possible to force artificially expressed α4β2 nAChRs into two conformations by adjusting the ratio of α4 and β2 cRNAs that are injected into Xenopus oocytes. Such constructs enable the elucidation of the pharmacological roles of the individual subtypes in relative isolation, but may not truly reflect the native receptor. None-

theless, it is instructive to study the electrophysiology of expressed nAChRs in oocytes in order to associate receptor attributes to their roles in native tissue.

As noted earlier, the $(\alpha4)_2(\beta2)_3$ HS receptor is favored by a 1:1 α4:β2 injection, while the formation of the LS $(\alpha4)_3(\beta2)_2$ receptor is favored by a 100:1 ratio of α4:β2 under the experimental conditions of this study. Previous studies in HEK-293 cells transfected with equal amounts of α4 and β2 subunits resulted in the expression of nAChRs, predominantly in the LS $(\alpha4)_3(\beta2)_2$ stoichiometry, and exhibiting EC_{50} values of 74 and 0.7 μM for ACh at LS and HS forms, respectively[8]. Despite differences in expression systems (HEK-293 vs. oocytes), the EC_{50} values for ACh were comparable to those observed in our present study. The electrophysiological responses elicited by the five additional agonists reflect a range of potencies and selectivities (Fig. 1). In accordance with its high binding affinity, varenicline was the most potent at the HS α4β2 with a maximal efficacy of 17% relative to ACh. Nicotine was nearly 3-fold less potent with a maximal efficacy of 32%. Both compounds also evoked LS α4β2 currents with efficacies that were greater that at HS α4β2 nAChRs. In contrast, AZD3480, dianicline, and ABT-089 all evoked responses at the HS α4β2, but little or no responses at the LS α4β2. AZD3480 was a nearly full agonist at the HS α4β2 with only a 3% response at the LS α4β2. Likewise, dianicline had only a 3% response at the LS α4β2 and a 26% response at HS α4β2. ABT-089 has a much smaller response at only 11% efficacy at HS α4β2 and no measurable response at the LS form. It should be noted that a major limitation of the oocyte expression system to study the HS and LS expression attributable to $(\alpha4)_2(\beta2)_3$ and $(\alpha4)_3(\beta2)_2$ subunit stoichimetries, respectively, is that only predominant expression of the dominant form can be achieved. It is likely that less dominant form is also co-expressed, albeit marginally. For example, we show that for AZD3480 the agonist activities at HS and LS α4β2 nAChRs were 1.0 μM (90%) and 1.3 μM (3%), respectively (see Table 1). A potential explanation might be that it is the HS form that is responsible for the activity of the system predominantly expressing LS form.

While there are certain advantages for studying α4β2 nAChRs in an isolated and controlled environment, its relationship to physiological nAChR-mediated responses, such as neurotransmitter release remain to be elucidated. One way to address this was to measure agonist-evoked release of [^{3}H]-DA from two regions of rat brain, the striatum and the prefrontal cortex (Fig. 2). These regions were chosen for their ability to release measurable amounts of dopamine in response to nicotinic agonists and due to their potential involvement in the pathophysiology of conditions such as smoking cessation, Parkinson's disease, and cognitive deficits. Small tissue slices provide enough cytoarchitecture to maintain the circuitry that may be involved in neurotransmitter release is preserved and they also enable the usage of a 96-well format to perform side-by-side comparisons of concentration–response curves. Direct comparison of agonist-evoked responses in the two systems (slices and synaptosomes) yields similar results. For example in this study using slices, AZD3480 showed EC_{50} and B_{max} values of 340 nM and 102% (relative to nicotine) comparing well to the corresponding values of 106 nM and 113% [22] obtained in synaptosomes for dopamine release.

The rank order of potency of compounds in evoking dopamine release was reflective of their affinity for α4β2* nAChRs. In fact, the coefficients of correlation between [^{3}H]-cytisine binding vs. [^{3}H]-DA release from both regions was close to 1, suggesting that a significant component of dopamine release involves α4β2* nAChRs as has been suggested previously [24–26]. An interesting aspect of dopamine release is the relative efficacy of various ligands examined. Varenicline, the most potent of the compounds tested, is a partial agonist, the aspect that is claimed to underpin the rationale for its use in smoking cessation [27]. Interestingly, of the compounds tested in the cortex, only varenicline produced a biphasic curve. It may be that, at higher concentrations, another receptor subtype or another mechanism of release may contribute. Further investigation would be necessary to definitively address this phenomenon. AZD3480 was found to be a full agonist, but somewhat less potent than nicotine in the striatum; whereas in the cortex, AZD3480 is as potent as nicotine, but less efficacious. ABT-089 and dianicline have comparable partial agonist efficacy in the striatum; but in the cortex, dianicline appears to have considerably greater efficacy than ABT-089.

The relationship of the ability of the test compounds to evoke for [^{3}H]-DA release and their functional responses at the HS α4β2 nAChR is rather clear. In terms of potency, dopamine release from both striatal and cortical slices correlates with HS responses with r^2 values close to one. It is notable that the slopes are not close to one. Since the range of EC_{50} values for responses at the HS α4β2 nAChR is very narrow compared to the range of potencies for dopamine release, the result is a flat slope. It may be argued that it is not an appropriate comparison between human α4β2 nAChRs in oocytes and native rat α4β2* nAChRs in binding and [^{3}H]-DA release. However, radioligand affinities for α4β2* nAChRs in both rat brain and human cortex were virtually identical for nicotine and ABT-089 in our hands [unpublished observations] and for varenicline [23]. Additionally, dianicline displayed comparable binding affinities for rat α4β2* nAChRs and human α4β2 nAChRs expressed in HEK-293 cells [21].

Although our analysis demonstrates a good association of α4β2* nAChR involvement in [^{3}H]-DA release, it has been demonstrated by multiple investigators that α6β2-containing nAChRs also participate in [^{3}H]-DA release in the striatum [26,28–30]. This may have important implications for clinical applications such as smoking cessation and for mechanisms underlying reward and addiction. To examine this, effects on [^{3}H]-DA release by nicotine, ABT-089, varenicline, and AZD3480 were studied in the absence and presence of 300 nM α-CtxMII to block α6β2* nAChRs. These compounds exhibited a range of selectivity for α6-mediated dopamine release. The EC_{50} value of nicotine was significantly shifted in the presence of α-CtxMII which inhibited the maximal response by 31%. In contrast, the [^{3}H]-DA release in response to varenicline was insensitive to α-CtxMII blockade which indicates that it is devoid of α6β2* activity. Likewise, AZD3480 had only a minor α6β2* component with α-CtxMII-sensitive [^{3}H]-DA release representing only 20% of the total. In a preliminary communication using mouse synaptosomes [31], the [^{3}H]-DA release response to AZD3480 was shown to be insensitive to α-CtxMII. ABT-089 had a significant α6-mediated effect, as suggested by significant attenuation (∼47%) in the presence of α-CtxMII. A similar profile of ABT-089 stimulation of [^{3}H]-DA release was observed in a recent study [32, this issue] of mouse striatal synaptosomes. In that study, ABT-089 exhibited 57% and 31% efficacy in the absence and presence of α-CtxMII, respectively, suggesting that α6-mediated component is ∼46%. The α-CtxMII-insensitive response for the four compounds, presumably primarily mediated through α4β2-containing nAChRs, maintains a close correlation with the HS (4(2 nAChR response with an r^2 value of 0.96 for the pEC_{50}. These observation support our interpretation that HS α4β2* nAChRs could be involved in neurotransmitter release in the mammalian brain.

In summary, the present study demonstrates an association between the binding affinity of nicotinic agonists for α4β2* nAChRs in rat brain and dopamine release from two regions of the rat brain. The functional pharmacology of these ligands in stimulating dopamine release correlates well with their interactions at high sensitivity $(\alpha4)_2(\beta2)_3$ combination. Further, a component of evoked dopamine release by nicotine, ABT-089

and AZD3480, but not varenicline, involves α6-containing nAChRs. The differential effects of nAChR ligands as evidenced for in vitro studies may have implications for the behavioral and clinical effects of these compounds.

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