Molecular Pharmacology Fast Forward. Published on March 26, 2008 as doi:10.1124/mol.108.045104
This article has not been copyedited and formatted. The final version may differ from this version.

MOL Manuscript # 45104

Sazetidine-A Is a Potent and Selective Agonist at Native and Recombinant α4β2 Nicotinic Acetylcholine Receptors

Ruud Zwart, Anna L. Carbone, Mirko Moroni, Isabel Bermudez, Adrian J. Mogg, Elizabeth A. Folly, Lisa M. Broad, Andrew C. Williams, Deyi Zhang, Chunjin Ding, Beverly A. Heinz and Emanuele Sher

Eli Lilly & Company, Lilly Research Centre, Windlesham, Surrey, U.K. (RZ, AJM, EFA, LMB, ACW, ES); Eli Lilly & Company, Lilly Corporate Center, Indianapolis, IN (DZ, CD, BAH); School of Life Sciences, Oxford Brookes University, Oxford, U.K. (ALC, MM, IB).

Molecular Pharmacology Fast Forward. Published on March 26, 2008 as DOI: 10.1124/mol.108.045104
This article has not been copyedited and formatted. The final version may differ from this version.

Running title: Activation of α4β2 nAChRs by sazetidine-A

Corresponding author: Dr. Ruud Zwart

Eli Lilly and Company

Lilly Research Centre

Sunninghill Road

Windlesham

Surrey, GU20 6PH

United Kingdom

Tel. +44.1276.483554

Fax. +44.1276.483525

Email: Zwart_Ruud@Lilly.com

Number of text pages:	28
Number of tables:	0
Number of figures:	4
Number of references:	37
Number of words in abstract:	202
Number of words in introduction:	610
Number of words in discussion:	786

Abbreviations: nAChR, nicotinic acetylcholine receptor

Molecular Pharmacology Fast Forward. Published on March 26, 2008 as DOI: 10.1124/mol.108.045104
This article has not been copyedited and formatted. The final version may differ from this version.

ABSTRACT

Sazetidine-A has been recently proposed to be a "silent desensitizer" of α4β2 nicotinic acetylcholine receptors (nAChRs), implying that it desensitizes α4β2 nAChRs without first activating them. This unusual pharmacological property of sazetidine-A makes it, potentially, an excellent research tool to distinguish between the role of activation and desensitization of α4β2 nAChRs in mediating the CNS effects of nicotine itself, as well as those of new nicotinic drugs. Surprisingly, we found that sazetidine-A potently and efficaciously stimulates nAChR-mediated dopamine release from rat striatal slices, which is mediated by α4β2* and α6β2* subtypes of nAChR. The agonist effects on native striatal nAChRs prompted us to re-examine the effects of sazetidine-A on recombinant α4β2 nAChRs in more detail. We expressed the two alternative stoichiometries of α4β2 nAChR in *Xenopus* oocytes and investigated the agonist properties of sazetidine-A on both α4(2)β2(3) and α4(3)β2(2) nAChRs. We found that sazetidine-A potently activated both stoichiometries of α4β2 nAChR: it was a full agonist on α4(2)β2(3) nAChRs, whereas it had an efficacy of only 6% on α4(3)β2(2) nAChRs. In contrast to what has been published before, we therefore conclude that sazetidine-A is an agonist of native and recombinant α4β2 nAChRs while showing differential efficacy on α4β2 nAChRs subtypes.

Molecular Pharmacology Fast Forward. Published on March 26, 2008 as DOI: 10.1124/mol.108.045104
This article has not been copyedited and formatted. The final version may differ from this version.

Neuronal nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels, expressed throughout the central and peripheral nervous system. Molecular cloning has provided evidence for the existence of at least nine different types of nAChR α subunits (α2-α10) and three different types of nAChR β subunits (β2-β4), in the nervous system. Some of these subunits form homo-pentameric receptors when expressed in heterologous expression systems (α7, α8 and α9), while other subunits assemble into hetero-pentameric structures with various combinations of α and β subunits. Different subunit combinations yield functional nAChRs that differ considerably in their functional and pharmacological properties (Luetje and Patrick, 1991; Papke, 1993; Chavez-Noriega et al., 1997). The predominant subtype of nAChR in the central nervous system contains α4 and β2 subunits (α4β2*) (Flores et al., 1992; Piciotto et al., 2001). The asterisk indicates that besides α4 and β2 subunits there may be additional subunits co-assembled in native α4β2 receptors. These α4β2* nAChRs are possible targets for drugs to treat pain, nicotine addiction, attention deficit disorders and diseases such as Alzheimer's and Parkinson's (Lloyd and Williams, 2000; Jensen et al., 2005). The exact subunit composition of α4β2* nAChRs in the brain is still largely unknown.

Epibatidine, a chemical originally isolated from the skin of the Equadorian poison-arrow frog *Epipedobates tricolor*, has been shown to act as a nAChR agonist and to cause profound analgesic effects (Badio and Daly, 1994). However, apart from showing strong analgesic effects, epibatidine also causes a wide range of severe side effects, possibly because of its lack of subtype specificity, which makes it far too toxic to be considered for clinical use (Rupniak et al., 1994; Sullivan et al., 1994a,b; Boyce et al., 2000). In the last few years, significant efforts have been directed towards the discovery of new nAChR-based analgesics with improved safety profiles.

One focus has been on selectivity, i.e. trying to identify nAChR agonists that selectively activate those nAChR subtypes that are involved in causing the positive analgesic effect (mainly $\alpha4\beta2^*$), but don't activate those nAChRs (e.g. $\alpha3\beta4$, $\alpha1\beta1\gamma\varepsilon\delta$) that are involved in mediating the most severe side effects (Ji et al., 2007). Also, variations in potency and efficacy of selective $\alpha4\beta2$ nAChR agonists have been explored. On these lines, sazetidine-A, has been recently reported to bind selectively and with high affinity to $\alpha4\beta2$ nAChRs (Xiao et al., 2006). The reported functional properties of this compound are, however, uncommon. It has been shown that sazetidine-A can potently desensitize $\alpha4\beta2$ nAChRs without activating them. It has been suggested that sazetidine-A could represent a new class of nicotinic cholinergic drugs with a novel mechanism of action, branded as "silent desensitizers". On the other hand, recent reports suggest *in vivo* efficacy strikingly similar to more classical $\alpha4\beta2$ agonists. For example, in rats that were trained to discriminate nicotine from saline it was shown that sazetidine-A fully substitutes for nicotine as discriminative stimulus in a drug discrimination assay (Xiao et al., 2007). Furthermore, sazetidine-A was found to be more potent and efficacious than epibatidine in the formalin test for persistent pain (Cucchiaro et al., 2007). Because of the intriguing discrepancy between the *in vitro* and *in vivo* reports, we decided to characterize sazetidine's *in vitro* pharmacological properties in more detail. Surprisingly, we found that sazetidine-A is a potent agonist on native $\alpha4\beta2^*$ nAChRs mediating dopamine release from rat striatal slices. Further *in vitro* characterization of the compound on distinct human recombinant $\alpha4\beta2$ nAChR stoichiometries revealed that the receptors with the $\alpha4(2)\beta2(3)$ stoichiometry, are also potently and efficaciously activated by sazetidine-A. Our results, showing that sazetidine-A is a potent agonist at native and

Molecular Pharmacology Fast Forward. Published on March 26, 2008 as DOI: 10.1124/mol.108.045104
This article has not been copyedited and formatted. The final version may differ from this version.

recombinant α4β2 nAChRs, reconcile the apparent discrepancy between *in vitro* and *in vivo* results previously reported.

Molecular Pharmacology Fast Forward. Published on March 26, 2008 as DOI: 10.1124/mol.108.045104
This article has not been copyedited and formatted. The final version may differ from this version.

Materials and Methods

Sazetidine-A. Sazetidine-A (Fig. 1) was synthesized as described (Xiao et al., 2006) with modification on the step of Sonogashira coupling reaction. Thus, a mixture of (*s*)-5-bromo-3-{[1-(*t*-butoxycarbonyl)-2-azetinyl]-methoxy}pyridine (1.213 g, 3.53 mmol), potassium carbonate (1.22 g, 8.84 mmol), triphenylphosphine (148 mg, 0.57 mmol), copper(I) bromide (81 mg, 0.57 mmol) and palladium on carbon (100 mg) in 1,2-dimethoxyethane (14 ml) and water (14 ml) was stirred at room temperature for 30 min under N_2, then 5-hexyn-1-ol (867mg, 8.84 mmol) was added to the mixture. The reaction was heated to reflux for 68 hrs. The reaction was diluted with EtOAc, the organic layer was separated and the aqueous layer was extracted with EtOAc twice. The combined organic layers were washed with brine, dried over Na_2SO_4, filtered and concentrated. The residue was purified by chromatography (silica gel) eluting with EtOAc/hexanes 50-80% to afford (*s*)-5-(5-hexyn-1-ol)-3-{[1-t-(butoxycarbonyl)-2-azetidinyl]-methoxy}pyridine as a colorless oil (1.162 g, 91.2%). ^{1}H NMR data match the reported data on this compound. Deprotection of (*s*)-5-(5-hexyn-1-ol)-3-{[1-t-(butoxycarbonyl)-2-azetidinyl]-methoxy}pyridine provided Sazetidine-A. The purity of the final product is 100% as measured by liquid chromatography-mass spectrometry. An alternative batch of sazetidine-A was purchased from Alexis Biochemicals (Nottingham, U.K.).

[^{3}H]Epibatidine Binding to α4β2 and α3β4 nAChRs stably expressed in HEK-293 cells. Membrane Preparation: HEK293 cell lines stably expressing the nicotinic α4β2 or α3β4 receptors were maintained in D-MEM/F-12 3:1 (3 part of Dulbecco's Modified Eagle Medium in 1 part of Nutrient Mix F-12 medium; Invitrogen,

Molecular Pharmacology Fast Forward. Published on March 26, 2008 as DOI: 10.1124/mol.108.045104
This article has not been copyedited and formatted. The final version may differ from this version.

Carlsbad, CA) supplemented with 5% fetal bovine serum (Invitrogen), 250 µg/ml geneticin and 20 mM Hepes (Invitrogen). Membranes from large-scale cell production were prepared as described (Smith et al., 2007). Briefly, cell pastes were homogenized in 4 volumes of buffer (50 mM Tris.HCl , 150 mM NaCl and 5 mM KCl, pH 7.4). The homogenate was centrifuged twice (40,000 x g, 10 minutes, 4°C), and the pellets were re-suspended in 4 volumes of Tris-HCl buffer after the first spin and 8 volumes after the second spin. The resuspended homogenate and the supernatant were centrifuged again (100 x g, 10 minutes, 4°C and 40,000 x g, 20 minutes, 4°C, respectively), and the pellet was resuspended in Tris.HCl buffer supplemented with 10% w/v sucrose. The membrane preparation was stored in 1 ml aliquots at -80°C until tested. The protein concentration of the membrane preparation was determined using a BCA protein assay reagent kit (Pierce, Rockford, IL).

Nicotinic receptor radioligand binding Scintillation Proximity Assay (SPA). SPA radioligand binding assays were modified from methods described previously (Badio and Daly, 1994; Gerzanich et al, 1995). Both $\alpha4\beta2$ and $\alpha3\beta4$ assays were performed in 96-well plates in a final volume of 250 µl Tris-HCl buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM KCl, pH 7.4) using the following conditions: 2 nM of [^{3}H]-epibatidine (53 Ci/mmol; Amersham, Piscataway, NJ); 1 mg/well of wheatgerm agglutinin scintillation proximity assay (WGA-SPA) beads (GE Health Sciences, Piscataway , NJ); and 5 µg/well of membrane protein. Non-specific binding (< 10% for both assay types) was determined using 100 µM of unlabeled epibatidine. Reactions were allowed to equilibrate for 3 h at room temperature prior to reading on a Wallac counter (Perkin Elmer). Data were analyzed as Ki values using a four-parameter logistic curve fitting program (ActivityBase v5.3.1.22).

Molecular Pharmacology Fast Forward. Published on March 26, 2008 as DOI: 10.1124/mol.108.045104
This article has not been copyedited and formatted. The final version may differ from this version.

[^{3}H]Dopamine and [^{3}H]noradrenaline release from rat native tissue. Male Lister hooded rats (250–350 g) were killed by exposure to CO_2 followed by cervical dislocation. Striata from two rats or hippocampi from three rats were dissected and chopped three times at 150 μm using a McIlwain tissue chopper, each time rotating the tissue through 60°. Slices were dispersed in Krebs bicarbonate buffer (118 mM NaCl, 2.4 mM KCl, 2.4 mM $CaCl_2 \cdot 2H_2O$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4 \cdot 7H_2O$, 25 mM $NaHCO_3$, 10 mM glucose, 1 mM ascorbic acid, gassed with 5% CO_2/95% O_2 for 1 h, pH 7.4), including 10 μM pargyline, and were incubated with [^{3}H]dopamine (20–23 μCi/ml; 50 nM) or [^{3}H]noradrenaline (56-60 μCi/ml; 100 nM) for 30 min at 37°C. After loading, slices were washed with Krebs buffer containing 1 μM nomifensine and 10 μM pargyline. After the final wash, slices were resuspended in Krebs buffer, and a 100 μl aliquot was placed in each well of a 96-well GF/C filter plate (Millipore). Buffer was removed to waste, and 70 μl of buffer was added to each well before incubation at 37°C for 5 min, after which the buffer was removed into a 96-well collection plate. Slices were then stimulated for 5 min with agonist (70 μl/well), after which the stimulating buffer was removed into another 96-well collection plate. Optiphase Supermix (Wallac) scintillation fluid (170 μl) was added to each well of the collection plates before the plates were heat-sealed and radioactivity was quantified using a Wallac 1450 Microbeta 96-well plate counter (Wallac Oy, Turku, Finland, counting efficiency 25%). Radioactivity remaining in the slices was measured by digestion of the tissue in 1 M HCl for 1 h with release quantified as above. Release of [^{3}H]dopamine or [^{3}H]noradrenaline was expressed as a fraction of the total radioactivity contained within the slices at the time of stimulation. Data points are shown as mean ± SEM of at least three independent experiments (each with four or more replicates). Curves were fitted using the four-

parameter Hill equation using Sigmaplot 9.0. α-Conotoxin MII was purchased from Tocris (Bristol, U.K.), dihydro-β-erythroidine and mecamylamine were purchased from Sigma-Aldrich (Poole, U.K.).

***Xenopus* oocyte expression and electrophysiological recordings.** Stage V and VI *Xenopus* oocytes were prepared using standard procedures (Chávez-Noriega et al., 1997). Human α4 and β2 subunit cDNAs, ligated into the pCI (Promega) expression vector, were dissolved in distilled water at a concentration of 1 μg/μl (spectrophotometric and agarose gel electrophoresis determinations). Mixtures of α4 and β2 cDNA at 1:10, 10:1 ratios were injected into the nuclei of oocytes in a volume of 18.4 nl/oocyte, using a Nanoject Automatic Oocyte Injector (Drummond, Broomall, PA). The total amount of cDNA injected per oocyte was kept constant at 2 ng. After injection, oocytes were incubated at 18 °C for 2 - 5days in a modified Barth's solution containing 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.3 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 15 mM Hepes and 5 mg/l neomycin (pH 7.6). Recordings were performed 3 - 5 days post-injection. Oocytes were placed in a 0.1-ml recording chamber and perfused with modified Ringer solution (in mM: NaCl 150, KCl 2.8, Hepes 10, $BaCl_2$ 1.8; pH 7.2, adjusted with NaOH) at a rate of 10 ml/min. We chose a nominally Ca^{2+}–free solution in order to minimize the contribution to the response of Ca^{2+}–gated chloride channels that are endogenous to the *Xenopus* oocyte and may be activated by Ca^{2+} entry through the nAChRs. Oocytes were impaled by two agarose-cushioned microelectrodes filled with 3 M KCl (0.5-2.0 MΩ) and voltage-clamped at –60 mV using a Geneclamp 500B amplifier and PCLAMP 6 software (Axon Instruments, CA, U.S.A.). Typically, traces were filtered at 1kHz during recording and digitized at 0.5 - 5kHz using the DigiData 1200

interface (Axon Instruments, CA). All experiments were carried out at room temperature. Agonist concentration-response curves were obtained by normalizing agonist-induced responses to the control responses induced by 1 mM ACh (a near-maximum effective concentration at receptors obtained with 10:1 α4 to β2 cDNA transfecting ratios and an EC_{100} concentration at receptors expressed by oocytes injected with 1:10 α4:β2 cDNA ratios). A minimum interval of 4 minutes was allowed between agonist applications, as this was found to be sufficient to ensure reproducible recordings.

Concentration-response curves were fitted by a non-linear least-squares algorithm according to the equation:

$$i = i_{max} / (1 + \{EC50/[conc]\}^{n}) \quad [1]$$

in which i_{max} is the maximum obtainable peak current, EC50 is the concentration of the agonist that elicits 50% of the maximum obtainable peak current, and n is the slope factor. Results are expressed as mean ± S.D.

Molecular Pharmacology Fast Forward. Published on March 26, 2008 as DOI: 10.1124/mol.108.045104
This article has not been copyedited and formatted. The final version may differ from this version.

Results

Radioligand binding assays. The binding selectivity of sazetidine-A for human recombinant α4β2 versus human recombinant α3β4 nAChRs was evaluated in competition experiments utilizing [^{3}H]epibatidine as the radioligand. Using standard saturation binding methods, the Kd values of epibatidine for α4β2 and α3β4 membranes were determined to be 0.24 nM and 0.37 nM, respectively. Sazetidine-A displaced [^{3}H]epibatidine binding to α4β2 with a mean relative IC50 of 2.6 ± 1.2 nM, a calculated mean Ki of 0.26 ± 0.11 nM, and a mean Hill coefficient of 0.85 ± 0.03. On the other hand, sazetidine-A displaced [^{3}H]epibatidine binding to α3β4 with a mean relative IC50 of 365 ± 59 nM, a calculated mean Ki of 54 ± 5 nM, and a mean Hill coefficient of 0.85 ± 0.11 (Fig. 2). We therefore confirmed previously published binding results (Xiao et al., 2006) showing that sazetidine-A binds with significantly higher affinity to α4β2 than α3β4, and extended this finding to human receptors. It is worth noting, though, that the selectivity ratios between human receptors (this paper) seem to be smaller than that for rat receptors (Xiao et al. 2006). Species specificity issues, specifically between human and rat α3β4 nAChRs, have recently been highlighted by our laboratory (Young et al., 2007).

Neurotransmitter release assays. Binding assays per se cannot predict if a ligand acts as an agonist or an antagonist at a particular receptor. Functional assays with sazetidine-A on native rat nAChRs have therefore been performed in order to answer this specific question. Dopamine release from rat striatal slices is mediated by the activation of native α4β2* and α6β2* nAChRs (Mogg et al., 2004; Salminen et al., 2004; Smith et al., 2007; Grady et al., 2007). We found that sazetidine-A acts as a

Molecular Pharmacology Fast Forward. Published on March 26, 2008 as DOI: 10.1124/mol.108.045104
This article has not been copyedited and formatted. The final version may differ from this version.

potent and efficacious agonist in evoking [^{3}H]-dopamine release from rat striatal slices (EC50 = 1.1 ± 0.3 nM, and Emax = 96 ± 6 %; n=3; Fig. 3A). Both the nicotinic antagonists mecamylamine (n=3) and dihydro-β-erythrodine (n=3) inhibited sazetidine-A-evoked dopamine release, demonstrating the nicotinic nature of the sazetidine-A-evoked response. α-Conotoxin MII is a nicotinic antagonist that distinguishes between α4β2* and α6β2* nAChRs. α4β2* nAChRs are resistant to 100 nM of the toxin, whereas α6β2* nAChRs are fully blocked by the same toxin concentration (Salminen et al., 2004). At the concentration of 100 nM α-conotoxin MII reduced the Emax, but had no significant effect on the EC50 of sazetidine-A. The estimates of EC50 and Emax values in the presence of 100 nM α-contotoxin MII are 0.27 ± 0.5 nM and 49 ± 1 % (n=3), respectively, indicating that 52% of the sazetidine-A induced DA release is mediated by α-conotoxin MII-resistant (α4β2*) nAChRs and that 48% of the sazetidine-A-induced DA release is mediated by α-conotoxin MII-sensitive (α6β2*) nAChRs.

Noradrenaline release from rat hippocampal slices is mediated by the activation of native α3β4* nAChRs (Luo et al., 1998; Anderson et al., 2000). We found that sazetidine-A also evoked noradrenaline release, however with much lower potency and lower efficacy (EC50 = 4.5 ± 2.6 μM, and Emax = 55 ± 7 %; n=3) than it evoked dopamine release from striatal slices. The nicotinic antagonists mecamylamine and dihydro-β-erytroidine both inhibited sazetidine-A-evoked [^{3}H]-dopamine release (Fig. 3B), confirming that sazetidine-A effects are mediated by nAChR activation, rather than by other means. These neurotransmitter release experiments from native rat tissues demonstrate that sazetidine-A is a potent agonist

Molecular Pharmacology Fast Forward. Published on March 26, 2008 as DOI: 10.1124/mol.108.045104
This article has not been copyedited and formatted. The final version may differ from this version.

of native rat α4β2* and α6β2* receptors, but a weak agonist of native α3β4* nAChRs.

In order to verify that the agonist effects of sazetidine-A observed are not due to the specific batch of sazetidine-A used, we also tested another, commercially available sazetidine-A. The EC50 and Emax values obtained with this new source of sazetidine-A were 0.85 ± 0.28 nM and 94 ± 3% (n=3) in the dopamine release assay from striatal slices, and 24 ± 6 μM and 38 ± 3% (n=3) in the noradrenaline release assay from hippocampal slices (data not shown), respectively. These data are consistent with the data obtained with the sazetidine-A we synthesized in house.

Oocyte expression experiments.

Based on the intriguing new finding described above, we decided to revisit the functional properties of sazetidine-A with the aim of a) confirming that the agonist effects on α4β2* nAChRs we saw were not specific to the rat but also translate to the human receptors, and b) understand better the biophysical basis for the absence of agonism previously reported (Xiao et al. 2006). We chose to use the *Xenopus* oocyte expression system, combined with the two-electrode voltage clamp technique, because this is a very sensitive technique that can also detect very partial agonism. In addition, the oocyte expression system can be used to study different subunit stoichiometries of α4β2 nAChR simply by varying the α4 and β2 subunit cDNA ratio that is injected prior to the experiments. α4(2)β2(3) and α4(3)β2(2) nAChRs can then be studied in isolation and, importantly, have previously been shown to have distinct pharmacology (Zwart et al. 2006; Moroni et al., 2006). Fig. 4a shows the concentration-dependence of the peak amplitudes of currents induced by sazetidine-A on both subtypes of α4β2 nAChR. Various concentrations of sazetidine-A, and the

maximally effective concentration of 1 mM ACh (Moroni et al, 2006) were alternately applied in order to control for rundown of the response. Sazetidine-A-induced current amplitudes were normalized to the amplitude of 1 mM ACh-induced currents. Mean concentration-response curves calculated from the parameters obtained by fitting eqn. [1] to the data of individual experiments are depicted in Fig. 4a. The mean EC50, slope factor and Emax of the concentration-response curves for sazetidine-A (n = 3) are 6.1 ± 1.2 nM, 1.0 ± 0.1 and 98 ± 9 % for α4(2)β2(3) nAChRs, and 2.4 ± 1.2 nM, 1.3 ± 0.4 and 5.8 ± 1.1 % for α4(3)β2(2) nAChRs, respectively. Fig. 4b shows examples of currents induced by 1 mM ACh and 100 nM sazetidine-A, which are maximally effective concentrations for the two agonists at α4(2)β2(3) nAChRs. Clearly, sazetidine-A is able to evoke currents of approximately the same size as those induced by ACh. On the other hand, Fig. 4c shows examples of currents induced by 1 mM ACh and 1 μM sazetidine-A, which are maximally effective concentrations for the two agonists at α4(3)β2(2) nAChRs. These traces highlight that sazetidine-A is a very poorly efficacious agonist at α4(3)β2(2) nAChRs.

Molecular Pharmacology Fast Forward. Published on March 26, 2008 as DOI: 10.1124/mol.108.045104
This article has not been copyedited and formatted. The final version may differ from this version.

Discussion

The most striking finding of this investigation is that, contrary to previous reports (Xiao et al. 2006), sazetidine-A is a potent and efficacious agonist of both human recombinant and rat native α4β2* nAChRs.

Addition of sazetidine-A to rat striatal slices potently and efficaciously stimulated dopamine release, an effect blocked by the nicotinic antagonists dihydro-β-erythroidine and mecamylamine. Interestingly, 100 nM of the nicotinic antagonist α-conotoxin MII, which distinguishes α4β2* nAChRs from α6β2* nAChRs, reduced the Emax of the concentration-response curve of sazetidine-A by approximately 48%, indicating that about half of the sazetidine-A-induced dopamine release is mediated by α6 subunit-containing nAChRs. Thus, besides being an agonist of α4β2* nAChRs, sazetidine-A is also a potent agonist on α6 subunit-containing nAChRs.

Especially the agonistic effects on α4β2* nAChRs were quite surprising, specifically because in SH-EP1 cells, stably transfected with recombinant human α4β2 nAChRs, sazetidine-A does not induce any measurable agonistic effect (Xiao et al., 2006). In contrast, prolonged (but not acute) applications of low concentrations of sazetidine-A potently desensitized α4β2 nAChRs expressed in the same cells, an effect more typically associated with agonists. This led the authors to suggest that sazetidine-A is a "silent-desensitizer" of α4β2 nAChRs (Xiao et al., 2006).

In order to identify the molecular basis for this apparent discrepancy we performed more electrophysiological experiments. In the *Xenopus laevis* oocyte expression system the expression of α4β2 nAChRs subtypes with high- and low-sensitivity to ACh can be manipulated by transfecting the oocytes with cDNAs encoding the α4 and β2 subunits in different ratios (Zwart and Vijverberg, 1998;

Zwart et al., 2006; Moroni et al., 2006). When α4 and β2 subunits are injected in the 1:10 α4:β2 ratio the oocytes will express a homogeneous population of α4β2 nAChRs with high sensitivity to ACh, whereas injection of α4 and β2 subunits in the 10:1 ratio results in the expression of a homogeneous population of α4β2 nAChRs with low sensitivity to ACh. When sazetidine-A was tested on both α4β2 nAChR subtypes, it was found to be a potent and efficacious agonist of the α4β2 nAChRs that have high sensitivity to ACh. It must be stressed, however, that sazetidine-A also potently activated the second isoform of α4β2 nAChRs with low sensitivity to ACh, but with very low efficacy.

The question then arises as to why Xiao et al. (2006) did not observe any agonist effect of sazetidine-A on their heterologously expressed α4β2 nAChRs. First of all, the fact that recombinant α4 and β2 nAChR subunits assemble in more than one α4β2 nAChR stoichiometry was totally neglected and the subtypes of α4β2 nAChRs expressed in SH-EP1 cells were not verified. This is an important factor, because potencies and efficacies of α4β2 nAChR ligands depend heavily on the subunit stoichiometry of α4β2 nAChRs (Zwart and Vijverberg, 1998; Nelson et al., 2003; Zhou et al., 2003; Khiroug et al., 2004; Lopez-Hernandez et al., 2004; Karadsheh et al, 2004; Briggs et al., 2006; Moroni et al., 2006; Zwart et al., 2006; Tapia et al., 2007). One possibility is that the SH-EP1 cells used by Xiao et al. (2006) express mainly α4(3)β2(2) nAChRs that have low affinity for ACh. In combination with the poorly sensitive biochemical rubidium efflux assay utilized, the very partial agonist effect of sazetidine-A on the low affinity subtype of α4β2 nAChR might have been missed. However, two reports from another laboratory show concentration-response curves for ACh in the same human α4β2 SH-EP1 cell line using a rubium

efflux assay with EC50 values of about 1 μM (Eaton et al., 2003; Kuo et al., 2005), suggesting that these cells might express α4(2)β2(3) nAChRs which have high affinity for ACh. A patch clamp study on the same cells revealed an intermediate EC50 for ACh of about 10 μM and a shallow slope factor of 0.6, suggesting that these cells express a mixture of α4β2 nAChRs with high- and low-sensitivity for ACh (Wu et al., 2004). All together, it looks like that different labs find different pharmacological properties with the same cell line. An explanation for this could be that cell culture conditions vary between labs. It is known that the proportion of α4β2 nAChRs with high- and low-affinity for ACh depends on cell culture conditions, like e.g. temperature during incubation of the cells (Nelson et al., 2002; Zwart et al., 2006). Along these lines, it might be possible that the major difference between the present findings on α4β2 nAChRs expressed in *Xenopus* oocytes and the previously published data with this compound (Xiao et al., 2006) on α4β2 nAChRs expressed in a mammalian cell line might be due to differences in expression system used.

Our results help to understand and reconcile the recent reports on *in vivo* effects of sazetidine-A showing that it acts as a quite typical α4β2 nAChR agonist in both drug discrimination (Xiao et al., 2007) and analgesic (Cuchiaro et al., 2007) assays. Although the contribution of additional subunits in native α4β2* nAChRs has not been excluded, the results are consistent with the hypothesis that *in vivo* effects of α4β2 nAChR agonists are mediated by native a4b2* nAChRs that have an α4(2)β2(3) stoichiometry.

Acknowledgments: The authors thank Brian G. Getman for his technical support.

References

Anderson DJ, Puttfarcken PS, Jacobs I and Faltynek C (2000) Assessment of nicotinic acetylcholine receptor-mediated release of [3H]-norepinephrine from rat brain slices using a new 96-well format assay. *Neuropharmacology* **39:**2663-2672.

Badio B and Daly JW (1994) Epibatidine, a potent analgesic and nicotinic agonist. *Mol Pharmacol* **45:**563-569.

Boyce S, Webb JK, Shepheard SL, Russell MGN, Hill RG and Rupniak NMJ (2000) Analgesic and toxic effects of ABT-594 resemble epibatidine and nicotine in rats. *Pain* **85:**443-450.

Briggs CA, Gubbins EJ, Marks MJ, Putman B, Thimmapaya R, Meyer MD and Surowy CS (2006) Untranslated region-dependent exclusive expression of high-sensitivity subforms of α4β2 and α3β2 nicotinic acetylcholine receptors. *Mol Pharmacol* **70:**227-240.

Chavez-Noriega LE, Crona JH, Washburn MS, Urrutia A, Elliott KJ and Johnson EC (1997) Pharmacological characterization of recombinant human neuronal nicotinic acetylcholine receptors hα2β2, hα2β4, hα3β2, hα3β4, hα4β2, hα4β4 and hα7 expressed in *Xenopus* oocytes. *J Pharmacol Exp Ther* **280:**346-356.

Cucchiaro G, Gonzalez-Sulser A, Kozikowski AP, Kellar KJ and Xiao Y (2007) Analgesic effects of sazetidine-A, a new nicotinic cholinergic drug. *Anesthesiology* **107:**A1348.

Eaton JB, Peng JH, Schoeder KM, George AA, Fryer JD, Krishnan C, Buhlman L, Kuo YP, Steinlein O and Lukas RJ (2003) Characterization of human α4β2 nicotinic acetylcholine receptors stably and heterologously expressed in native nicotinic receptor-null SH-EP1 human epithelial cells. *Mol Pharmacol* **64:**1283-1294.

Flores CM, Rogers SW, Pabreza LA, Wolfe BB and Kellar KJ (1992) A subtype of nicotinic cholinergic receptors in rat brain is composed of α4 and β2 subunits and is upregulated by chronic nicotine treatment. *Mol Pharmacol* **41:**31-37.

Gerzanich V, Peng X, Wang F, Wells G, Anand R, Fletcher S and Lindstrom J (1995) Comparative pharmacology of epibatidine: a potent agonist for neuronal nicotinic acetylcholine receptors. *Mol Pharmacol* **48:** 774-782.

Grady SR, Salminen O, Laverty DC, Whiteaker P, McIntosh JM, Collins AC and Marks MJ (2007) The subtypes of nicotinic acetylcholine receptors on dopaminergic terminals of mouse striatum. *Biochem Pharmacol* **74:**1235-1246.

Jensen AA, Frolund B, Liljefors T and Krogsgaard-Larsen P (2005) Neuronal nicotinic acetylcholine receptors: structural revelations, target identifications, and therapeutic inspirations. *J Med Chem* **48:**4705-4745.

Ji J, Bunnelle WH, Anderson DJ, Faltynek C, Dyhring T, Ahring PK, Rueter LE, Curzon P, Buckley MJ, Marsch KC, Kempf-Grote A and Meyer MD (2007) A-366833: A novel nicotinonitrile-substituted 3,6-diazabicyclo[3.2.0]-heptane α4β2 nicotinic acetylcholine receptor selective agonist: synthesis, analgesic efficacy and tolerability profile in animal models. *Biochem Pharmacol* **74:**1253-1262.

Karadsheh MS, Shah MS, Tang X, MacDonald RL and Stitzel JA (2004) Functional characterization of mouse α4β2 nicotinic acetylcholine receptors stably expressed in HEK293T cells. *J Neurochem* **91:**1138-1150.

Khiroug SS, Khiroug L and Yakel JL (2004) Rat nicotinic acetylcholine receptor α2β2 channels comparison of functional properties with α4β2 channels in *Xenopus* oocytes. *Neuroscience* **124:**817-822.

Kuo YP, Xu L, Eaton JB, Zhao L, Wu J and Lukas RJ (2005) Roles for nicotinic acetylcholine receptor subunit large cytoplasmic loop sequences in receptor expression and function. *J Pharmacol Exp Ther* **314:**455-466.

Lloyd GK and Williams M (2000) Neuronal nicotinic acetylcholine receptors as novel drug targets. *J Pharmacol Exp Ther* **292:**461-467.

Lopez-Hernandez GY, Sanchez-Padilla J, Ortiz-Acevedo A, Lizrdi-Ortiz JE, Salas-Vincenty J, Rojas LV and Lasalde-Dominicci JA (2004) Nicotine-induced upregulation and desensitization of α4β2 neuronal nicotinic receptors depend on subunit ratio. *J Biol Chem* **279:**38007-38015.

Luetje CW and Patrick J (1991) Both α- and β-subunits contribute to the agonist sensitivity of neuronal nicotinic acetylcholine receptors. *J Neurosci* **11:**837-845.

Luo S, Kulak JM, Cartier GE, Jacobsen RB, Yoshikami D, Olivera BM and McIntosh JM (1998) α-Conotoxin AuIB selectively blocks α3β4 nicotinic acetylcholine receptors and nicotine-evoked norepinephrine release. *J Neurosci* **18:**8571-8579.

Mogg, A.J., Jones, F.A., Pullar, I.A., Sharples, C.G.V. and Wonnacott, S. (2004) Functional responses and subunit composition of presynaptic nicotinic receptor subtypes explored using the novel agonist 5-iodo-A-85380. *Neuropharmacology* **47:**848-859.

Moroni M, Zwart R, Sher E, Cassels BK and Bermudez I (2006) α4β2 Nicotinic receptors with high and low acetylcholine sensitivity: pharmacology, stoichiometry, and sensitivity to long-term exposure to nicotine. *Mol Pharmacol* **70:**755-768.

Nelson ME, Kuryatov A, Choi CH, Zhou Y and Lindstrom J (2003) Alternate stoichiometries of α4β2 nicotinic acetylcholine receptors. *Mol Pharmacol* **63:**332-341.

Papke RL (1993) The kinetic properties of neuronal nicotinic receptors: genetic basis of functional diversity. *Prog Neurobiol* **41:**509-531.

Piciotto MR, Caldarone BJ, Brunzell DH, Zachariou V, Stevens TR and King SL (2001) Neuronal nicotinic acetylcholine receptor subunit knockout mice: physiological and behavioral phenotypes and possible clinical implications. *Pharmacol Ther* **92:**89-108.

Rupniak NMJ, Patel S, Marwood R, Webb J, Traynor JR, Elliott J, Freedman SB, Fletcher SR and Hill RG (1994) Antinociceptive and toxic effects of (+)-epibatidine oxalate attributable to nicotinic agonist activity. *Br J Pharmacol* **113:**1487-1493.

Salminen O, Murphy KL, McIntosh JM, Drago J, Marks MJ, Collins AC and Grady SR (2004) Subunit composition and pharmacology of two classes of striatal presynaptic nicotinic acetylcholine receptors mediating dopamine release in mice. *Mol Pharmacol* **65:**1526-1535.

Smith JW, Mogg A, Tafi E, Peacey E, Pullar IA, Szekeres P and Tricklebank M. (2007) Ligands selective for α4β2 but not α3β4 or α7 nicotinic receptors generalise to the nicotine discriminative stimulus in the rat. *Psychopharmacology* **190**:157–170.

Sullivan JP, Briggs CA, Decker MW, Brioni JD, Donnelly-Roberts D, Brioni JD, Radek RJ, McKenna DG, Campbell JE, Arneric SP, Decker MW and Bannon AW (1994a) (±)-Epibatidine can differentially evoke responses mediated by putative subtypes of nicotinic acetylcholine receptors (nAChRs). *Med Chem Res* **4:**502-516.

Sullivan JP, Decker MW, Brioni JD, Roberts-Donnelly D, Anderson DJ, Bannon A, Gopalakrishnan M, Piattoni-Kaplan M, Adams P, Buchley MJ, Kang CH, Williams M

and Arneric SP (1994b) (±)-Epibatidine elicits a diversity of in vitro and in vivo effects mediated by nicotinic acetylcholine receptors. *J Pharmacol Exp Ther* **271:**624-631.

Tapia L, Kuryatov A and Lindstrom J (2007) Ca^{2+} permeability of the (α4)3(β2)2 stoichiometry greatly exceeds that of (α4)2(β2)3 human acetylcholine receptors. *Mol Pharmacol* **71:**769-776.

Xiao Y, Fan H, Musachio JL, Wei ZL, Chellappan SK, Kozikowski AP and Kellar KJ (2006) Sazetidine-A, a novel ligand that desensitizes α4β2 nicotinic acetylcholine receptors without activating them. *Mol Pharmacol* **70:** 1454-1460.

Xiao Y, Woolverton WL, Sahibzada N, Yasuda RP and Kellar KJ (2007) Pharmacological properties of sazetidine-A, a desensitizer of α4β2 nicotinic acetylcholine receptors. *Soc Neurosci Abstr* **37:** 574.6

Wu J, Kuo YP, George AA, Xu L, Hu J and Lukas RJ (2004) β-Amyloid directly inhibits human α4β2 nicotinic acetylcholine receptors heterologously expressed in human SH-EP1 cells. *J Biol Chem* **279:**37842-37851.

Young GT, Broad LM, Zwart R, Astles PC, Bodkin M, Sher E and Millar NS (2007) Species selectivity of a nicotinic acetylcholine receptor agonist is conferred by two adjacent extracellular β4 amino acids that are implicated in the coupling of binding to channel gating. *Mol Pharmacol* **71:**389-397.

Zhou Y, Nelson ME, Kuryatov A, Choi C, Cooper J and Lindstrom J (2003) Human α4β2 acetylcholine receptors formed from linked subunits. *J Neurosci* **23:**9004-9015.

Zwart R, Broad LM, Xi Q, Lee M, Moroni M, Bermudez I and Sher E (2006) 5-I A-85380 and TC-2559 differentially activate heterologously expressed α4β2 nicotinic receptors. *Eur J Pharmacol* **539:**10-17.

Zwart R and Vijverberg HPM (1998) Four pharmacologically distinct subtypes of α4β2 nicotinc acetylcholine receptors expressed in *Xenopus laevis* oocytes. *Mol Pharmacol* **54:** 1124-1131.

Molecular Pharmacology Fast Forward. Published on March 26, 2008 as DOI: 10.1124/mol.108.045104
This article has not been copyedited and formatted. The final version may differ from this version.

Figure legends

Fig. 1. Chemical structure of sazetidine-A.

Fig. 2. Receptor binding experiments. Sazetidine-A displaces the radioligand [3H]epibatidine from its binding sites on α4β2 nAChRs (filled circles) and α3β4 nAChRs (open circles). Data are expressed as mean ± S.E.M. for three experiments.

Fig. 3. Nicotine and Sazetidine-A-induced neurotransmitter release. A) Nicotine evokes dopamine release from rat striatal slices in a concentration-dependent manner (EC50 = 36 ± 8 nM; Emax 104 ± 3%; n=3). B) Sazetidine-A evokes dopamine release from rat striatal slices in a concentration-dependent manner (squares). Sazetidine-A-evoked dopamine release is prevented in the presence of the nicotinic receptor antagonists dihydro-β-erythroidine (100 μM; diamonds) and mecamylamine (10 μM; triangles). α-Conotoxin MII (0.1 μM; circles) reduces the Emax of Sazetidine-A by 48%, but does not affect its EC50. C) Nicotine evokes noradrenaline release from rat hippocampal slices in a concentration-dependent manner (EC50 = 6.0 ± 2.4 μM; Emax 101 ± 5%; n=3). D) Sazetidine-A evokes noradrenaline release from rat hippocampal slices in a concentration-dependent manner (squares). Sazetidine-A evoked noradrenaline release is prevented in the presence of the nicotinic receptor antagonists dihydro-β-erythroidine (100 μM; diamonds) and mecamylamine (10 μM; triangles).

Fig. 4. Agonist effects of sazetidine-A on oocytes heterologously expressing α4β2 nAChRs that have high sensitivity to ACh (α4(2)β2(3)) and α4β2 nAChRs that have

low sensitivity to ACh (α4(3)β2(2)). A. Concentration-response curves of sazetidine-A to evoke ion currents in oocytes expressing α4(2)β2(3) nAChRs (squares) and in oocytes expressing α4(3)β2(2) nAChRs (circles). The inset shows the agonist concentration-response curve of sazetidine-A on α4(3)β2(2) nAChRs, showing that sazetidine-A is a potent, but partial agonist on this subtype of α4β2 nAChR. B. Ion currents evoked by maximum-effective concentrations of ACh and sazetidine-A in oocytes expressing α4(2)β2(3) nAChRs, showing that sazetidine-A is a full agonist on this subtype of α4β2 nAChR. C. Ion currents evoked by maximum-effective concentrations of ACh and sazetidine-A in oocytes expressing α4(3)β2(2) nAChRs, showing that sazetidine-A is a partial agonist on this subtype of α4β2 nAChR.

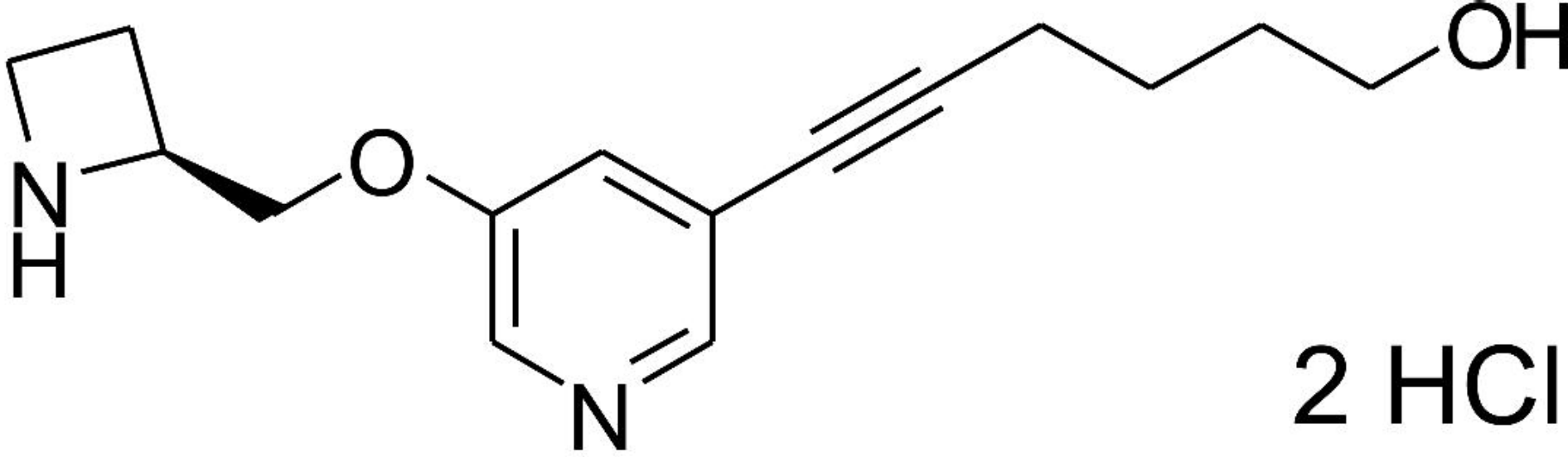

Figure 1

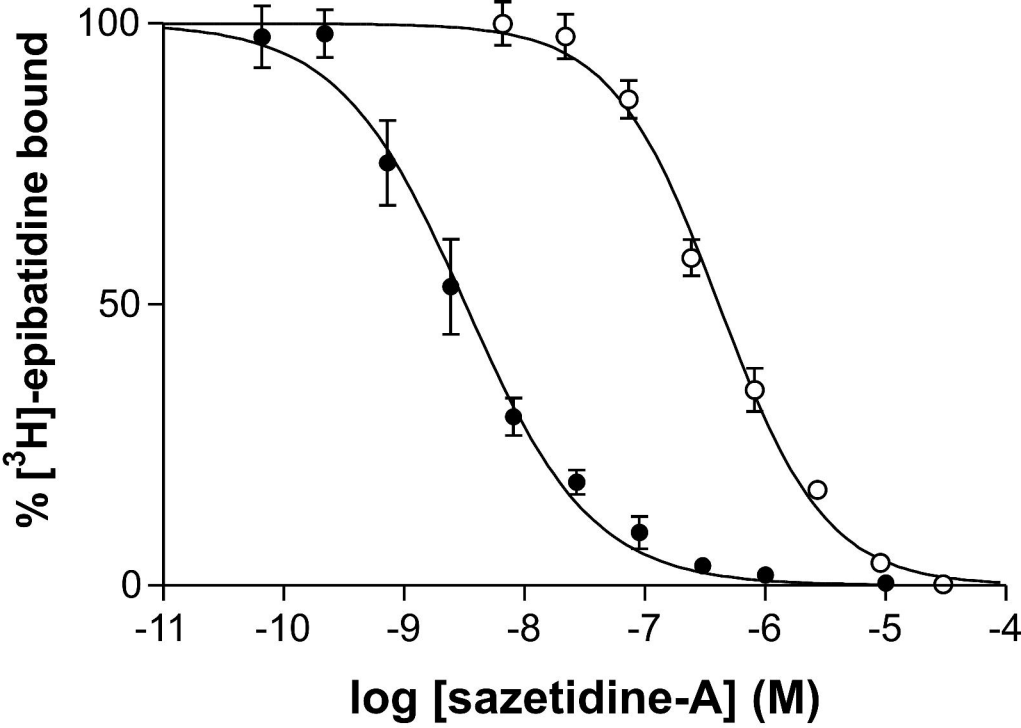

Figure 2

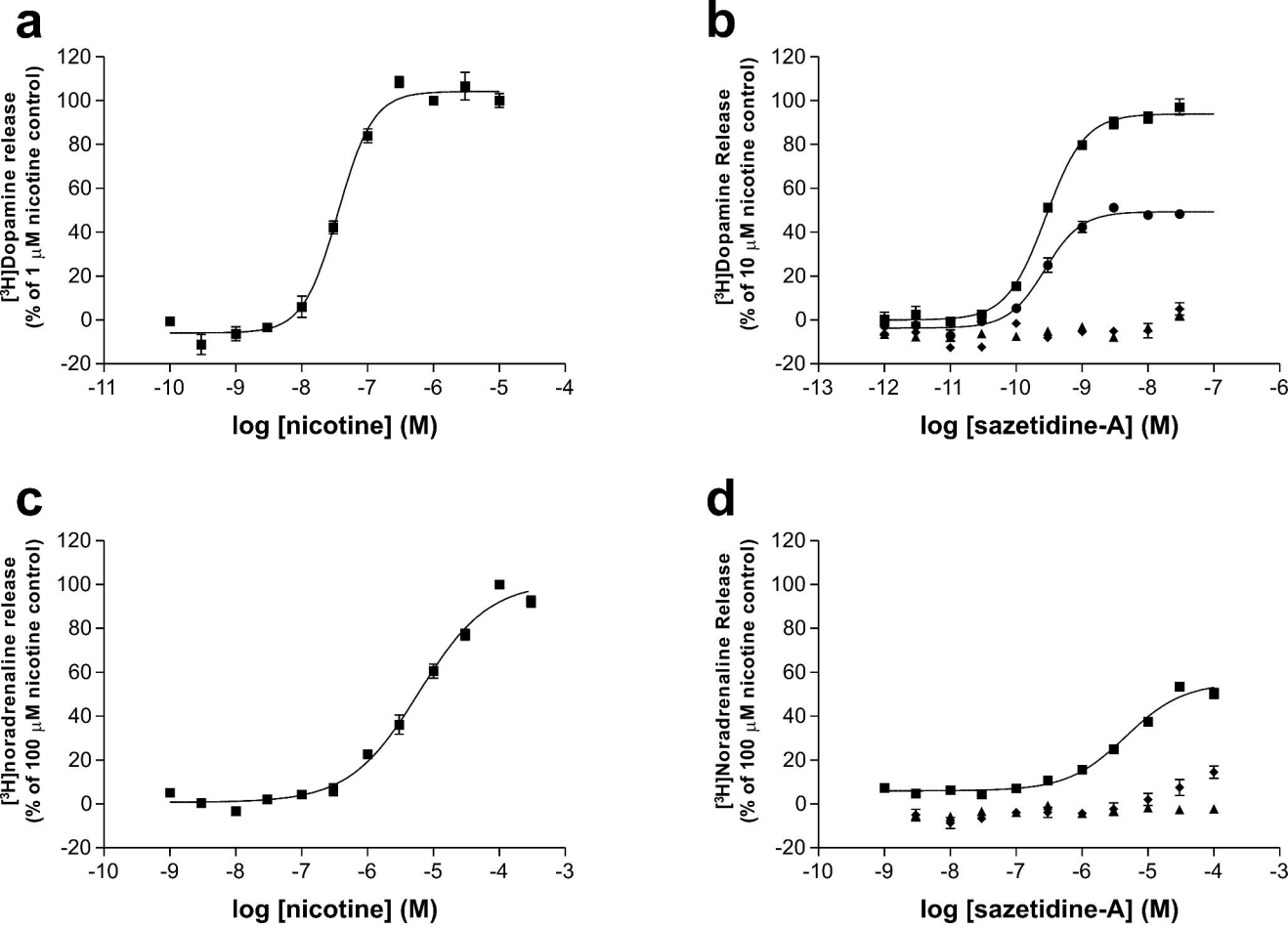
a
[3H]Dopamine release
(% of 1 μM nicotine control)
log [nicotine] (M)
b
[3H]Dopamine Release
(% of 10 μM nicotine control)
log [sazetidine-A] (M)
c
[3H]noradrenaline release
(% of 100 μM nicotine control)
log [nicotine] (M)
d
[3H]Noradrenaline Release
(% of 100 μM nicotine control)
log [sazetidine-A] (M)

Figure 3

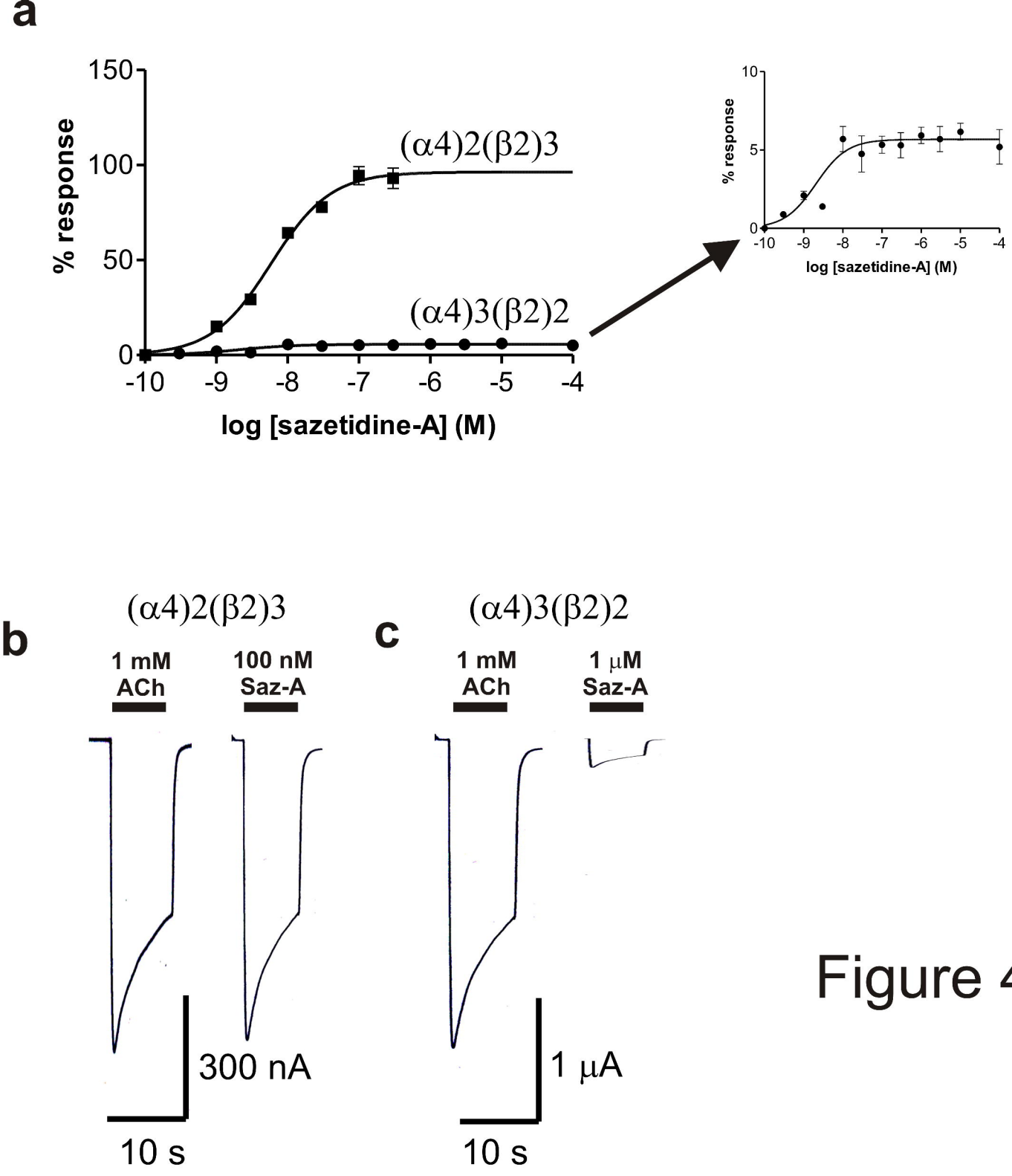
a
150
100
50
0
% response
(α4)2(β2)3
(α4)3(β2)2
-10
-9
-8
-7
-6
-5
-4
log [sazetidine-A] (M)
10
5
0
% response
-10
-9
-8
-7
-6
-5
-4
log [sazetidine-A] (M)
b
(α4)2(β2)3
1 mM
ACh
100 nM
Saz-A
300 nA
10 s
c
(α4)3(β2)2
1 mM
ACh
1 μM
Saz-A
1 μA
10 s

Figure 4