Psychopharmacology (2007) 190:157–170
DOI 10.1007/s00213-006-0596-8

ORIGINAL INVESTIGATION

Ligands selective for α4β2 but not α3β4 or α7 nicotinic receptors generalise to the nicotine discriminative stimulus in the rat

Janice W. Smith · Adrian Mogg · Elisiana Tafi · Eleanor Peacey · Ian A. Pullar · Philip Szekeres · Mark Tricklebank

Received: 30 January 2006 / Accepted: 19 September 2006 / Published online: 18 November 2006

Abstract
Rationale Nicotine produces behavioural effects that are potentially related to its interaction with diverse nicotinic acetylcholine receptor populations. Evidence from gene deletion studies suggests that the interoceptive stimulus properties of nicotine are mediated by heteromeric high-affinity receptors containing α4β2 subunits. Mice lacking β2 subunits do not discriminate nicotine (Shoaib et al., Neuropharmacology, 42:530–539, 2002), and nicotine does not elicit dopamine release in these animals (Grady et al., J Neurochem, 76:258–268, 2001). The stimulus properties of nicotine can be detected in rats using a two-lever operant drug discrimination paradigm, allowing them to be classified pharmacologically using ligands with selectivity for receptors containing α4β2, α3β4 or α7 subunits.
Materials and methods Rats trained to discriminate 0.4 mg/kg nicotine from vehicle were given the nicotinic receptor agonists, cytisine, varenicline, TC2559, ABT-594, A-85380 (all having high affinity but varying selectivity for α4β2-containing receptors), and WO 03/062224 and WO 01/60821A1 (selective for β4- and α7-containing receptors, respectively). In separate studies, WO 03/062224 was used as the training stimulus.
Results Nicotine, TC-2559, A-85380 and ABT-594 showed dose-dependent and complete stimulus substitution, whilst WO 03/062224 and WO 01/60821A1 were completely without effect. Cytisine and varenicline showed partial generalisation, consistent with their partial agonist activity at nicotinic receptors eliciting dopamine release in rat striatal slices. After almost 50 training sessions with WO 03/062224, there was no clear evidence that an α3β4 receptor agonist could sustain a discriminable stimulus.
Conclusion Substitution to the nicotine discriminative stimulus required high-affinity and high intrinsic activity at β2 but not at β4- or at α7-containing nicotinic receptors.

Keywords Drug discrimination · α4β2 receptor · α3β4 receptor · α7 receptor · Nicotine

J. W. Smith (✉) · A. Mogg · E. Tafi · E. Peacey · I. A. Pullar · P. Szekeres · M. Tricklebank
Eli Lilly & Co. Ltd., Lilly Research Centre,
Sunninghill Road, Windlesham,
Surrey GU20 6PH, UK
e-mail: Smith_Janice_W@lilly.com

Introduction

Neuronal nicotinic acetylcholine receptors (nAChRs) are located on nerve cell bodies, dendrites and axon terminals and are involved in modulation of release of multiple neurotransmitters such as dopamine (Cao et al. 2005), glutamate (Lambe et al. 2003), norepinephrine (Leslie et al. 2002) and GABA (Zhu and Chiappinelli 2002). Neuronal nicotinic receptors are found as pentameric assemblies that can be formed from combinations of at least 11 different receptor subunits (α2–α10 and β2–β4). In addition, the non-neuronal subunits, α1, β1, γ, δ, and ε, form the nAChR at the neuromuscular junction (Cooper et al. 1991; Le Novere et al. 2002; Corringer et al. 2000). High-affinity nAchRs are usually made up of a combination of α and β subunits per pentamer, with α4β2 being the predominant combination in the brain (e.g. Zoli et al. 1998), whilst α7 subunits preferentially form homo-pentamers (Couturier et al. 1990). Binding of nicotine to the agonist binding site causes a rotation in the receptor that leads to the opening of a cation channel (Unwin 2003).

The interoceptive stimulus properties of drugs—loosely their subjective effects—are often considered to result from the dynamic modulation of activity of a wide range of

neuronal systems. However, when the appropriate pharmacological tools are available, there are numerous examples where an interoceptive stimulus can be traced to the activation of a single neurotransmitter receptor subtype (http://www.dd-database.org/). Nicotine produces an interoceptive stimulus that is discriminated by both animals and humans (e.g. Hirschhorn and Rosecrans 1974; Kallman et al. 1982), which in the rat has been extensively studied using a two-lever operant drug discrimination paradigm. In such studies, an animal learns to associate the interoceptive stimulus induced by nicotine with reward delivered by pressing one of the two levers. This interoceptive stimulus has demonstrable pharmacological specificity, as agonists acting at nicotinic but not muscarinic acetylcholine receptors generalise to the nicotine cue (Pratt et al. 1983; Schechter 1995). Also, competitive antagonists of nAChRs, such as mecamylamine and dihydro-ß-erythroidine, will block this generalisation (Garcha et al. 1983; Stolerman et al. 1997; Shoaib et al. 2000). The lack of effect of ganglionic blocking agents such as hexamethonium or chlorisondamine when administered systemically can be explained by the presence of the quaternary ammonium ion that prevents rapid crossing of the blood brain barrier (Wiley et al. 1996): when administered centrally; ganglionic blocking agents will antagonise nicotine-mediated events (e.g. Kumar et al. 1987; Malin et al. 1997), consistent with mediation of the effect by nicotinic receptors located centrally. Recent research has indicated that the specific receptor subtypes may actually be crucial. For instance, several findings have indicated that α4β2-containing nicotinic receptors play a major role in the pharmacological properties of nicotine. Studies performed using brain sections from humans and rodents have shown that chronic exposure to nicotine increases the density of nicotine binding, mainly to the α4β2 nAChR type (Marks et al. 1992). Also, nicotine self-administration is reduced in animals given the competitive, and relatively selective (β2-preferring nAChR) antagonist, dihydro-ß-erythroidine (Watkins et al. 1999). More recently, technologies have allowed the selective deletion of different nicotinic receptor subunits to aid the delineation of nicotinic receptor subtype function. For example, self-administration of nicotine and nicotine-induced taste aversion is reduced in mice lacking the β2 subunit. These animals also fail to learn to discriminate nicotine from saline but succeed when morphine is used as the training drug (Picciotto et al. 1998; Shoaib et al. 2002). The β2 subunit has also been shown to be crucial in mediating dopamine release caused by nicotine (Grady et al. 2001), an action which may underlie its discriminative stimulus (Di Chiara and Imperato 1988; Corrigall et al. 1992, 1994).

Evidence for the involvement of other nicotinic receptors is either much weaker or has not yet been investigated. Thus, in contrast to the absence of stimulus control in β2 knockout mice, those lacking the α7 receptor showed normal acquisition of the nicotine discriminative stimulus (Stolerman et al. 2004). This finding is consistent with the failure of the α7-selective receptor antagonist, methyllycaconitine, to block the stimulus in intact animals (Brioni et al. 1996; Gommans et al. 2000). No group has previously reported the behavioural effects of selective agents targeting the nicotine α3β4-containing receptors, or any putative involvement of this receptor configuration in mediation of the interoceptive stimulus generated by nicotine.

Previous work has suggested the involvement of α4β2 receptors through selective deletion of receptors or through antagonist action; however, no selective agonists at β2, β4 or α7 receptors have been studied. In the present report, a range of recently characterised nicotinic receptor ligands with varying degrees of selectivity for and/or efficacy at α4β2, α3/α4/β4 and α7 subunit-containing receptors were assessed for their ability to generalise to or reverse the discriminative stimulus properties of nicotine. The degree of generalisation of the agonists tested to the nicotinic discriminative stimulus was directly compared with their relative efficacy to modulate dopamine release in rat striatal slices. The results indicate that the nicotine stimulus is mediated by receptors containing α4β2 subunits. Rats failed to acquire full stimulus control when a nicotinic agonist with high affinity and selectivity for receptors containing α3β4 subunits was used as the training drug.

Drugs

(−)-Nicotine hydrogen tartrate, (−)-cytisine, pargyline, nomifensine, mecamylamine and A-85380 (3-(2(*S*)-azetidinylmethoxy) pyridine dihydrochloride) were purchased from Sigma Aldrich. ABT594 tosylate ((*R*)-5-(2-azetidinylmethoxy)-2-chloropyridine), TC2559 hemigalactarate ((*E*)-*N*-methyl-4-[3-(5-ethoxypyridin)yl]-3-buten-1-amine); WO 01/60821A1 ((*R*)-*N*-(1-azabicyclo[2.2.2]oct-3-yl)(5-(2-pyridyl)thiophene-2-carboxamide (WO 01/60821A1))) and WO 03/062224 (1-methyl-4-(2-chloro-4-hydroxyphenylthio)-piperidine (WO 03/062224)) were synthesised by Discovery Chemistry Research, Lilly Research Laboratories. [7,8-^{3}H] Dopamine (20–25 Ci/mmol) was purchased from Perkin Elmer. Pargyline, nomifensine and mecamylamine were purchased from Sigma (Poole, Dorset, UK). All other chemicals used were of analytical grade and obtained from standard commercial sources.

For administration to rats, all drug solutions were prepared on the day of training or test. Nicotine, cytisine, TC2559, ABT-594, A-85380 and WO 01/60821A1 were dissolved in 5% glucose. WO 03/062224 was dissolved in 1 M equivalent 1 M HCl and then made up to volume with 5% glucose. Adjustments to pH 7 were made as appropri-

ate. Compounds were administered subcutaneously in an injection volume of 1 ml/kg. All doses quoted are those of the free base.

Subjects

Male Lister hooded rats (200–250 g, Harlan, UK) were housed in groups of three or four in plastic cages containing sawdust. Each cage contained environmental enrichment (Jolly Balls™, Lillico, UK). They were maintained on a 12-h light/dark cycle with lights on at 0700 hours. Temperature and humidity were controlled. Animals undergoing drug discrimination training were maintained on a food-restricted diet (approximately 17 g per day per rat) that allowed for growth with ad libitum access to water.

Materials and methods

[^{3}H]Epibatidine binding assay

Membrane preparation

Cell pastes from large-scale production of HEK-293 cells expressing cloned human nicotinic receptors were homogenised in four volumes of buffer (50 mM Tris–HCl, 150 mM NaCl and 5 mM KCl, pH 7.4). The homogenate was centrifuged twice (40,000×*g*, 10 min, 4°C), and the pellets re-suspended in four volumes of Tris–HCl buffer after the first spin and eight volumes after the second spin. The resuspended homogenate was centrifuged (100×*g*, 10 min, 4°C) and the supernatant kept and re-centrifuged (40,000×g, 20 minutes, 4°C). 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 required. The protein concentration of the membrane preparation was determined using a BCA protein assay reagent kit.

Nicotinic receptor radioligand binding Scintillation Proximity Assay (SPA)

SPA radioligand binding 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: [^{3}H]-epibatidine (53 Ci/mmol; Amersham)—α4β2=1 nM, α3β4=2nM, α7=5 nM; WGA-coated PVT SPA beads (Amersham)—α4β2=1 mg/well, α3β4 and α7=1.5 mg/well; membrane protein = 30 μg/well for all three assay types. Non-specific binding (<10% for all three assay types) was determined using 10 μM epibatidine. Reactions were allowed to equilibrate for 2–4 h at room temperature before reading on a Trilux Scintillation counter (Perkin Elmer). Data were analysed using a standard four-parameter logistic equation (Multicalc, Perkin Elmer) to provide IC_{50} values that were converted to K_i values using the Cheng–Prusoff equation (Cheng and Prusoff 1973).

[^{3}H]Dopamine release from rat striatal slices

Male Lister hooded rats (250–350 g) were killed by exposure to CO_2 followed by cervical dislocation. Striata from the two rats were dissected and chopped three times at 150 μm using a McIlwain tissue chopper, at 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) 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 0.2 ml Solvable (Packard Biosciences) for 1 h followed by the addition of 0.5 ml isopropyl alcohol and 4.5 ml Hionic Fluor scintillation fluid (Packard Biosciences). In this case, radioactivity was then quantified using a Wallac 1410 scintillation counter (Wallac Oy, Turku, Finland, counting efficiency 35%). Release of [^{3}H]dopamine 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).

Drug discrimination

Apparatus

Sixteen standard operant chambers, housed in sound and light attenuating chambers, were used. Two retractable

levers were located either side of a recessed magazine where food pellets (Noyes, 45 mg, Formula P) were delivered from an automatic pellet dispenser. The onset of the house-light and lever extension into the operant chamber signalled the start of a session, and offset of lights and levers indicated the end of a session. Experimental sessions were controlled, and data were recorded using programs written in-house using MedPC IV© software, and data were prepared for analysis using an in-house data macro designed for the experiment.

Lever-press training

Animals were trained between 0800 and 1030 hours and fed at approximately 1600 hours each day. Principles of laboratory animal care were followed (http://www.nap.edu/readingroom/books/labrats); the studies complied with local ethical requirements and were carried out in accordance with the Animals (Experimental Procedures) Act, 1986 under licence from the UK Home Office (PPL 70/5492).

Animals were given access to food pellets in their home cage to prevent food neophobia. The following day, the animals were placed in the operant chambers where food pellets were freely delivered on a fixed interval 30-s schedule. On the two following days, the animals were trained to lever press for food pellets on an FR1 (fixed ratio, i.e. every lever press resulted in delivery of a food pellet). Subsequently, the response requirement was increased until the animals were routinely responding on FR10 to obtain food pellets. At this time, discrimination training began.

Discrimination training

Animals were injected with 0.4 mg/kg nicotine (training dose—nicotine; Hirschhorn and Rosecrans 1974; Pratt et al. 1983) or 1 mg/kg WO 03/062224 (training dose for WO 03/062224 discrimination) and placed in the operant chambers 30 min later. Both levers were extended into the operant chamber, but pressing one (the drug-associated lever) resulted in food reinforcement after drug treatment on a tandem variable interval 30-s, FR10 schedule. Under this tandem schedule, the tenth lever press after a randomly determined variable interval of time was reinforced (range of variable interval was 7–53 s). Pressing the second lever (the vehicle-associated lever) was recorded but had no programmed consequences. During separate sessions, animals were administered with vehicle (5% glucose) and placed in operant chambers 30 min later. Both levers were extended into the operant chamber, but in this instance, pressing the second lever would result in food reinforcement on a tandem variable interval 30-s, FR10 schedule. Lever presses made on the drug-associated lever were recorded but had no programmed consequences. Half of the animals were required to press the left lever for food pellets after drug administration and the right lever after 5% glucose. In the remaining animals, the response requirement was reversed. Injections of nicotine or 5% glucose were given daily in a pseudo-randomised order where animals never received more than three consecutive administrations of nicotine or 5% glucose vehicle. Daily experimental sessions lasted for 15 min.

Substitution test

After the appropriate pre-treatment time, the animals were placed in the operant chamber, and the session began. During tests, both levers were reinforced on independent tandem variable interval 30-s, FR10 schedules. Test sessions lasted for 15 min. Before the test, the animals were divided into groups counterbalanced for stimulus control and response rates. When necessary, the animals were excluded from substitution tests on the basis of poor performance, i.e. not meeting criterion performance of greater than 80% responding on appropriate levers during the previous week's discrimination training. Compounds were determined to be drug like if animals made 80% or more responses on the drug-associated lever during substitution testing. Similarly, they were determined to be vehicle like when 20% or fewer responses were made on the drug-associated lever. This measurement was calculated from total lever presses made over the whole 15-min session.

Each substitution test experiment consisted of groups of animals treated with 0.4 mg/kg nicotine or 5% glucose (as positive and negative controls, respectively) together with a range of doses of the compounds under test. Pre-treatment times of compounds were chosen to reflect previous findings or in-house pharmacokinetic analysis indicating that compounds were present at significant levels at the time point chosen. Nicotine, A-85380, TC-2559, WO 03/062224 or WO 01/60821A1 and 5% glucose (vehicle) were administered 30 min before the test. ABT594 was given 15 min before the test based on the findings that the drug was less efficacious by 30 min after injection (Bannon et al. 1998). The nicotinic receptor antagonist, dihydro-ß-erythroidine, was given 15 min before the test. This pre-treatment time was an average time based on literature examples where some researchers used 30 min and others used 10 min as pre-treatment times (e.g. Buckley et al. 2004; Watkins et al. 1999). Some tests were repeated for quality control (e.g. nicotine, cytisine). Other tests were repeated if no minimal or maximal effective dose was obtained in the first study (e.g. TC2559, A85380).

WO 03/062224 discrimination training

Animals were treated with 1 mg/kg WO 03/062224 or 5% glucose. Thirty minutes later, the animals were placed in the operant chamber, and the training session began. Three separate discrimination substitution tests were conducted after 39, 43 and 47 training sessions.

Statistical analysis

Results were expressed as the percentage of total lever presses made on the nicotine-associated lever. The rates of responding were calculated from the sum of lever presses made on both the nicotine-associated and the vehicle-associated levers on the test session divided by 15 min (lever presses per minute). As all studies were of between-subjects design, treatment groups were counterbalanced for performance before each test. In all studies, there was a 0.4-mg/kg nicotine-treated group (positive control) and a 5% glucose-treated group (negative control) against which the other treatments were compared. Percentage responses on the drug lever were analysed using a one-way analysis of variance with between-subjects factor of treatment. Where studies were repeated, a main effect of order of treatment was investigated. Planned comparisons (tests between sample means that are planned before collecting data are called a priori comparisons or planned comparisons) using least squares means were conducted comparing with vehicle and with nicotine as negative and positive controls.

Analysis of WO 03/062224 discrimination data was conducted using a one-way analysis of variance. Planned comparisons (a priori contrasts) of the percentage responses on the drug lever were conducted between vehicle- and drug-treated animals.

Results

Affinity of drugs for human recombinant nicotinic receptor subtypes

Table 1 shows the binding affinities of all molecules tested at α4β2 and α3β4 nAChRs expressed in HEK-293 cells. A85380, varenicline, cytisine and ABT-594 showed highest affinity for α4β2-containing receptors with K_i values of approximately 1 nM. TC-2559 had somewhat lower affinity for α4β2 (K_i=21.8 nM) but failed to displace the radioligand at α3β4- or α7-containing receptors at concentrations of 10 μM. Although not tested within this body of work, TC-2559 was found to have no affinity for α7 receptors up to 100 μM (Chen et al. 2003). WO 03/062224 had a high affinity for α3β4 (K_i=1.5 nM), and WO 01/60821A1 had similar affinity for α7-containing receptors (K_i=1.2 nM,), but both had very low or negligible affinity for those containing α4β2 receptors. ABT-594 showed least selectivity between α4β2 and α3β4 (eightfold), whilst all other compounds with high affinity for α4β2 had selectivities of between 80- and 190-fold. Nicotine itself had its highest affinity for α4β2 (K_i=22 nM), a value 22-fold greater than its affinity for α3β4-containing receptors.

Table 1 Binding affinities for compounds at α4β2, α3β4 and α7 nAChR expressed in HEK-293 cells and effect of compounds on [^{3}H]dopamine release from rat striatal slices

Compound	Binding			[^{3}H]DA release	
	α4β2 (K_i/nM)	α3β4 (K_i/nM)	α7 (K_i/nM)	EC_{50}/nM	Efficacy (% of 10 μM nicotine)
WO 03/062224	413.4	1.5	ND[e]	8% increase at 10,000 nM	8% increase at 10,000 nM
WO 01/60821A1	2.3% change at 10,000 nM	−14.4% change at 10,000 nM	1.2	Inactive	Inactive
TC-2559	21.8	−23% change at 10,000 nM	>100,000[a]	192	104%
A-85380	0.53	44.7	148[b]	11.1	155%
ABT-594	0.846	6.8	13,800[c]	3.44	136%
Cytisine	2.92	560	4,600	15.5	51%
Nicotine	29	651	2,000[b]	73.3	100%
Varenicline	1.15	205	617[d]	9.91	70%

[a] Chen et al. 2003
[b] Sullivan et al. 1998
[c] Donnelly-Roberts et al. 1998
[d] Coe et al. 2005
[e] Not determined using receptor binding; however, this compound was assessed in a functional assay in cells expressing α7 receptors and found to be completely inactive at 10,000 nM, but experiments to assess receptor binding were not carried out.

Effects on dopamine release in rat striatal slices

Both WO 03/062224 and WO 01/60821A1 were essentially inactive at concentrations up to 10 μM in stimulating dopamine release from rat striatal slices (Table 1). All other nicotinic receptor agonists examined increased dopamine release in a concentration-dependent manner with EC_{50} values that correlated significantly (r^2=0.8) with K_i values for displacement of [^{3}H] epibatidine from membranes expressing receptors containing α4β2 subunits (Fig. 1). The maximum stimulation of dopamine release elicited by varenicline and cytosine was significantly lower than that seen with nicotine, whilst that induced by A-85380 and ABT-594 was significantly higher (Table 1).

Nicotine drug discrimination

Approximately 30 sessions were required to train animals to discriminate 0.4 mg/kg nicotine from 5% glucose (vehicle) using the two-lever operant procedure (Fig. 2). Once reliable discrimination was obtained, drug substitution testing began. In the first experiment (Fig. 3), four doses of nicotine (0.05, 0.1, 0.2 and 0.8 mg/kg) were substituted for the training dose of nicotine. This experiment was conducted twice, and the data were collated. The results were analysed for effect of order of test on choice of nicotine-associated lever [$F(1,71)=0.22$, $P>0.1$] or on response rates [$F(1,71)=0.12$, P>0.1]. As there was no significant effect of treatment order, the results were collapsed across order, and the effect of treatment was analysed. Nicotine dose-dependently induced nicotine-appropriate responding [$F(5,67)=18.43$, $P<0.001$] without effecting response rate [$F(5,67)=0.73$, $P>0.1$].

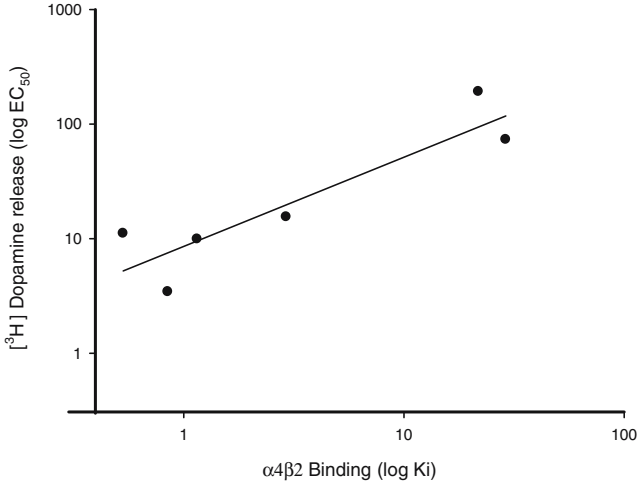

Fig. 1 Correlation between binding affinity to human α4β2-containing nicotinic receptors and potency to induce dopamine release in rat striatal slices for a number of nicotinic receptor agonists

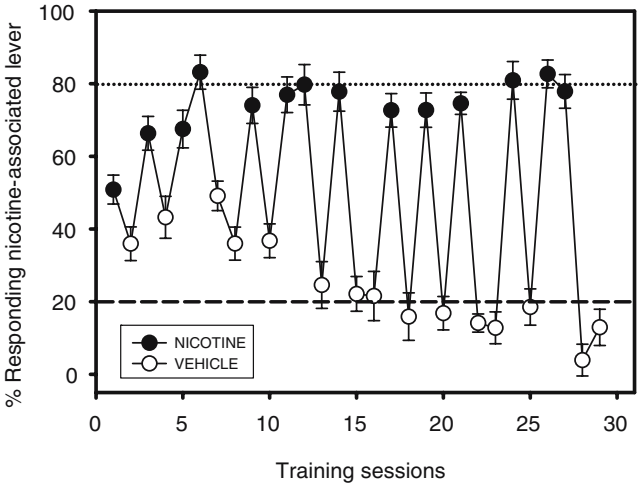

Fig. 2 Discriminative stimulus effects of nicotine (*filled circles*) or vehicle (*empty circles*) in rats during training to discriminate 0.4 mg/kg nicotine from 5% glucose. *Horizontal broken line* shows criterion for 5% glucose alone; the *horizontal dotted line* shows criterion for nicotine alone. All results are presented as mean±standard error of the mean (of 16 animals) for a 15-min training session where responses on only treatment-appropriate levers were reinforced on tandem variable interval 30-s, FR10 schedules beginning 30 min after injection

In the second experiment (Fig. 3), the effects of dihydro-ß-erythroidine in combination with the training dose of nicotine were tested. Dihydro-ß-erythroidine dose-dependently reduced nicotine-appropriate responding [$F(4,76)=67.37$, $P<0.001$] without effecting response rates [$F(4,43)=1.37$, $P>0.1$].

In the third experiment, the effects of cytisine were investigated (Fig. 4). Again, this experiment was repeated twice, and, as with the nicotine dose response test, an effect of order of test was investigated and was found to be non-significant both on choice of nicotine-associated lever [$F(1,83)=0.49$, $P>0.1$] and on rates of responding [$F(1,83)=0.09$, $P>0.1$]. Consequently, order was discounted as a factor, and the results collapsed over the two experiments. Cytisine dose-dependently induced nicotine-appropriate responding [$F(5,79)=12.84$, $P<0.001$], but the maximum degree of generalisation was only 56% at 3 mg/kg, a dose that also significantly depressed response rate [$F(5,79)=10.58$, $P<0.001$].

Investigation of the effects of varenicline also consisted of two separate experiments with two overlapping doses in the two studies as well as inclusion of the positive and negative controls. No effect of order was seen on the selection of the nicotine lever [$F(1,49)=0.33$, $P>0.1$] or on response rates [$F(1,49)=0.17$, $P>0.1$], and, therefore, the data were collapsed across order (Fig. 4). Varenicline dose-dependently induced nicotine-appropriate responding [$F(5,64)=11.39$, $P<0.001$]. Planned comparisons showed that doses from 1 to 5 mg/kg differed significantly from vehicle-treated animals, and doses of 0.3, 1.0 and 5.0 mg/kg

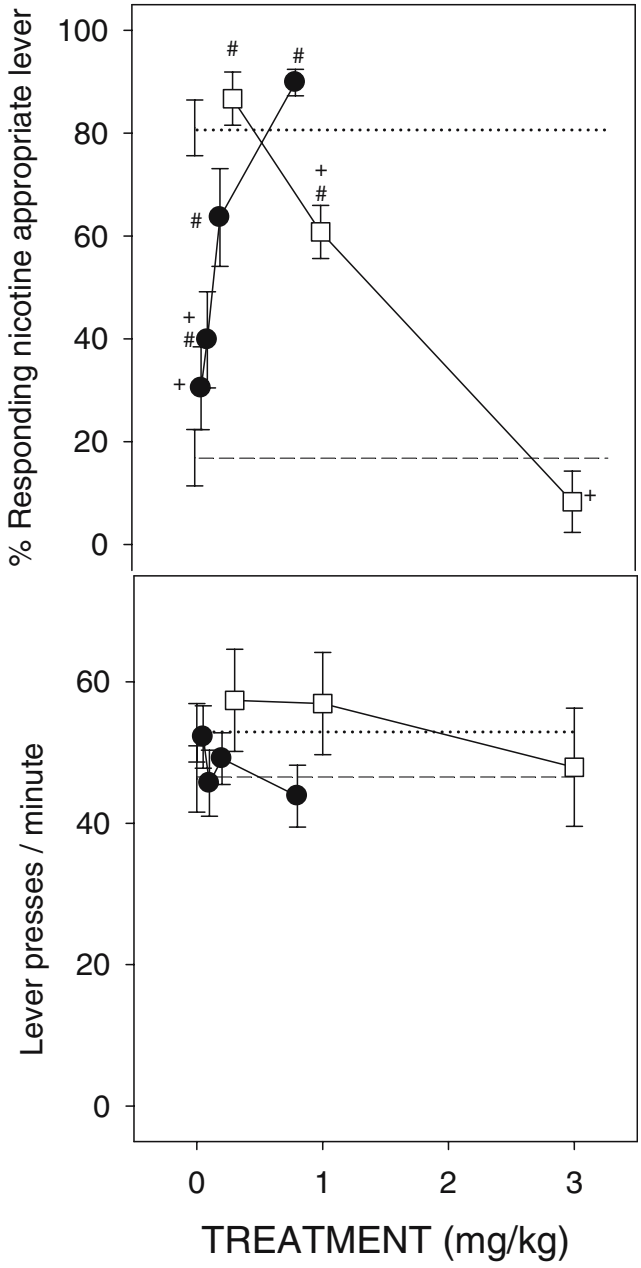

Fig. 3 Discriminative stimulus (*top*) and response rate (*bottom*) effects of nicotine (*filled circles*) alone or dihydro-beta-erythroidine (*empty square*) on 0.4 mg/kg nicotine in rats trained to discriminate 0.4 mg/kg nicotine from 5% glucose. *Horizontal broken line* shows results after 5% glucose alone; the *horizontal dotted line* shows results from nicotine alone. All results are presented as mean±standard error of the mean for a 15-min test where responses on both levers are reinforced on independent tandem variable interval 30-s, FR10 schedules beginning 30 min after injection of nicotine (s.c.). Nicotine discrimination group sizes: vehicle—13 animals, nicotine—13 animals, 0.05 mg/kg—13 animals, 0.1 mg/kg—8 animals, 0.2 mg/kg—13 animals, 0.8 mg/kg—13 animals. Dihydro-beta-erythroidine experiment group sizes: vehicle—12 animals, nicotine—14 animals, 0.3 mg/kg—8 animals, 1.0 mg/kg—8 animals, 3.0 mg/kg—6 animals. *Number symbol* indicates that $P<0.05$ compared with 5% glucose alone; *Plus symbol* indicates that $P<0.05$ compared with 0.4 mg/kg nicotine alone

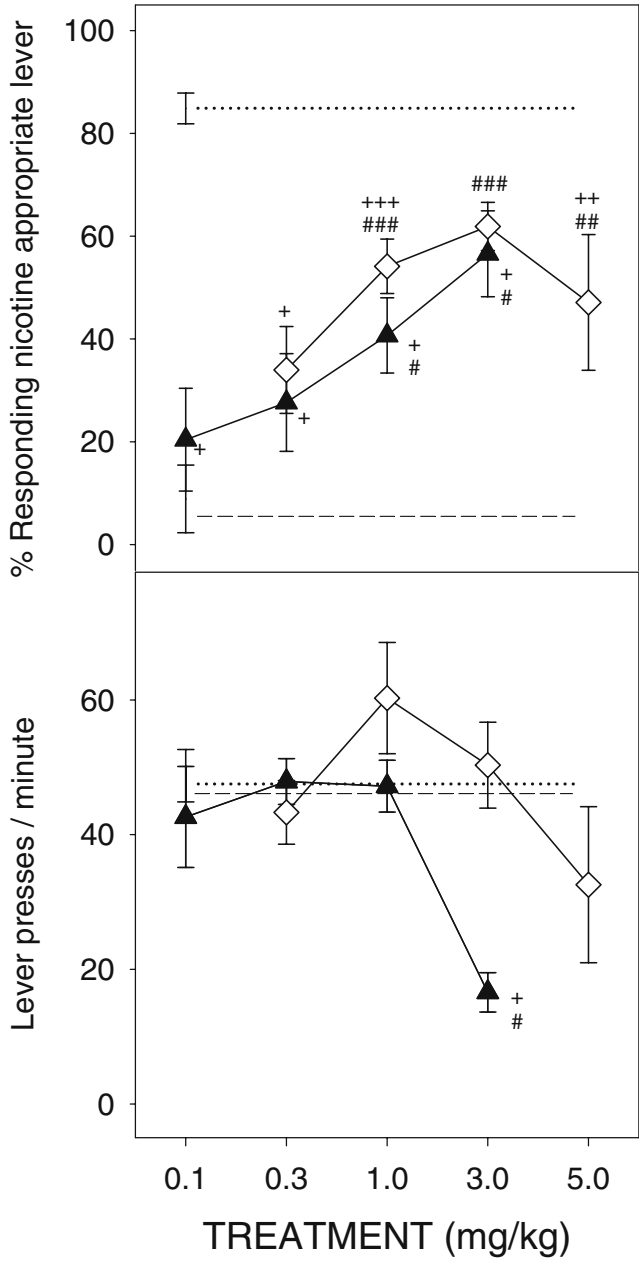

Fig. 4 Discriminative stimulus (*top*) and response rate (*bottom*) effects of cytisine (*filled triangle*) or varenicline (*empty diamond*) in rats trained to discriminate 0.4 mg/kg nicotine from 5% glucose. *Horizontal broken line* shows results after 5% glucose alone; the *horizontal dotted line* shows results from nicotine alone. All results are presented as mean±standard error of the mean for a 15-min test where responses on both levers are reinforced on independent tandem variable interval 30-s, FR10 schedules beginning 30 min after injection of cytisine (s.c.). Cytisine group sizes: vehicle—15 animals, nicotine—15 animals, 0.1 mg/kg—7 animals, 0.3 mg/kg—16 animals, 1.0 mg/kg—16 animals, 3.0 mg/kg—16 animals. Varenicline group sizes: vehicle—14 animals, nicotine—15 animals, 0.3 mg/kg—14 animals, 1.0 mg/kg—14 animals, 3.0 mg/kg—14 animals, 5.0 mg/kg—7 animals. *Number symbol* indicates that $P<0.05$ compared with 5% glucose alone; *Plus symbol* indicates that $P<0.05$ compared with 0.4 mg/kg nicotine alone

differed significantly from nicotine treatment. There was no overall effect of treatment on response rates [$F(5,64)=2.03$, $P=0.08$]. As with cytisine, the maximum degree of generalisation was significantly less than that achieved with the training dose of nicotine and was less marked at 3.0 mg/kg (45%) than at 1 mg/kg (60%) with the plateau occurring without any significant change in response rate.

The effects of TC2559 and A85380 were each examined in two separate studies consisting of three drug doses and positive and negative controls. For both TC2559 and A85380, there were two overlapping doses in each study, and, consequently, the examination of the effect of order of experiment was limited to these doses as well as the positive and negative controls. There was no effect of order of treatment with TC2559 or A85380 on either selection of nicotine-associated lever [$F(1,62)=0.14$, $P>0.1$; $F(1,60)=1.11$, $P>0.1$] or on response rates [$F(1,62)=0.10$, $P>0.1$; $F(1,60)=0.8$, $P>0.1$]. Consequently, the data were collapsed across order, and only the effect of treatment was investigated (see Fig. 5). Both TC2559 and A85380 showed highly significant generalisation to the nicotine stimulus [$F(5,75)=39.08$, $P<0.001$; $F(5,71)=30.30$, $P<0.001$]. However, this effect was not accompanied by any changes in response rates with either drug [$F(5,75)=1.73$, $P>0.1$; $F(5,71)=0.81$, $P>0.1$]. TC2559 showed full substitution with 3 and 10 mg/kg, and A85380 with 0.03 and 0.1 mg/kg.

In contrast, ABT594 (Fig. 5) was not repeated as the clear dose-dependent generalisation [$F(5,38)=21.86$, $P<0.001$] was established in a single study with full substitution occurring with 0.03 mg/kg. No effects on response rates were observed in the study [$F(5,38)=1.73$, $P>0.1$].

No experiments with WO 03/062224 or with WO 01/60821A1 were repeated (Fig. 6). Two animals were excluded from the final analyses (both from the 30-mg/kg WO 03/062224-treated group) as they failed to make sufficient lever presses in the test to result in the delivery of one food pellet and therefore did not meet the criteria for making a choice. There was a significant effect of treatment both on choice of drug lever [$F(4,42)=141.6$, $P<0.001$] and on response rates [$F(4,42)=10.2$, $P<0.001$]. All animals treated with WO 03/062224 chose the vehicle lever significantly more than nicotine lever. At the highest dose (3 mg/kg), there was an effect of treatment on response rates. There was a significant effect of treatment with WO 01/60821A1 both on choice of drug lever [$F(4,42)=266.46$, $P<0.001$] and on response rates [$F(4,42)=3.2$, $P<0.05$]. All animals treated with WO 01/60821A1 chose the vehicle lever significantly more than the nicotine lever. As with WO 03/062224, at the highest dose of WO 01/60821A1 (30 mg/kg), there was a significant decrease in response rates.

Animals did not acquire stimulus control with WO 03/062224 (Fig. 7a,b). Although a significant difference in lever selection was obtained in each of the three substitution-type tests (test 1: $F(1,14)=2.22$; test 2: $F(1,14)=2.28$; test 3: $F(1,14)=2.18$; all $P<0.05$), animals treated with vehicle never responded less than 40% on the drug lever regardless of treatment.

Discussion

Nicotine exerts its pharmacological effects in both humans and animals through its interactions with receptors found both centrally and peripherally. The discriminative stimulus that nicotine can generate may play a crucial role in tobacco addiction by serving as a conditional cue for responding in smoking behaviour. It is also thought that discriminative stimulus properties of a drug are the expression of its subjective effects (Di Chiara 2000). The operant, two-lever drug discrimination paradigm with reward delivered on a tandem variable interval 30-s, FR10 schedule provided a robustly parametric means of quantifying the ability of rats to discriminate the administration of nicotine from that of vehicle. After about 30 training sessions, the percentage of lever presses on the nicotine-associated lever increased dose-dependently under the conditions of a stimulus generalisation test (i.e. with both levers reinforced). In fact, more than 80% responding on the nicotine-associated lever was achieved with a dose of nicotine that was twofold greater than the training dose.

The presently used methodology has clear advantages over the frequently used, FR schedule of reinforcement with a binomial outcome (percentage of animals choosing the nicotine-associated lever), particularly in quantitatively assessing intermediate levels of discrimination.

Animals lacking the β2 receptor have been shown to be insensitive to the nicotine cue, yet are still able achieve stimulus control with morphine (Shoaib et al. 2002). In contrast, animals lacking the α7 receptor did not differ from wild-types in acquisition of the nicotine discrimination task. However, data from gene deletion studies do not absolutely prove that a receptor is or is not involved in mediation of the response to acute drug treatment as numerous adaptive changes may occur during foetal and neonatal development. For example, animals lacking the HMG CoA reductase enzyme do not survive past birth (Ohashi et al. 2003), whereas inhibitors of the enzyme (the statin class of medicines, e.g. Lipitor) are used widely in the clinic to lower cholesterol levels and are considered safe drugs. Consequently, determination of effects with selective agonists and antagonists is a critical adjunct to the story.

More than 80% of nicotine-appropriate responding was induced by the nicotinic receptor agonists, ABT594, A85380 and TC2559. In radioligand binding experiments, each of these compounds had high affinity for α4β2-

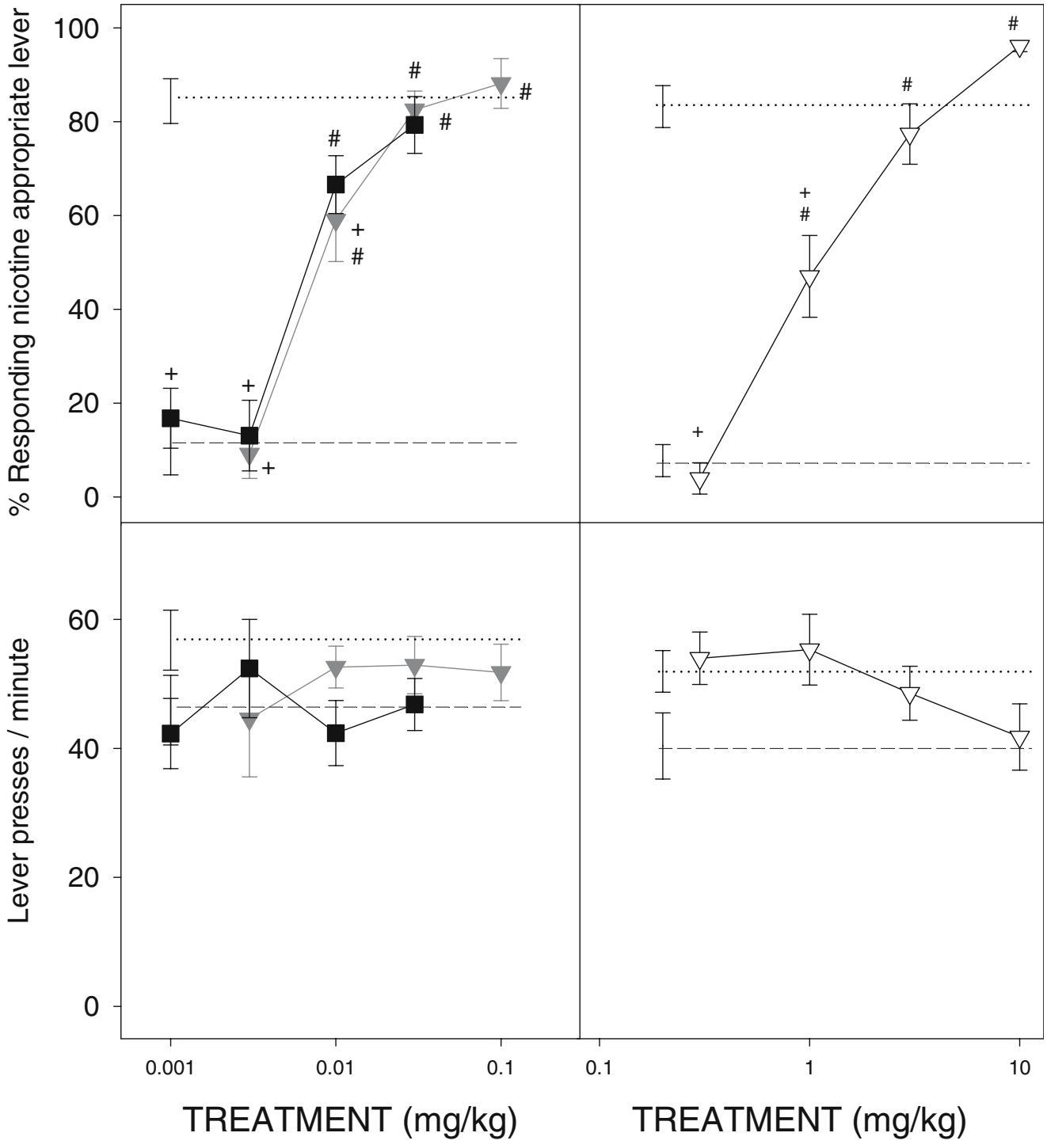

Fig. 5 Discriminative stimulus (*left top*) and response rate (*left bottom*) effects of A85380 (*inverted filled triangle*) or ABT-594 (*filled square*) in rats trained to discriminate 0.4 mg/kg nicotine from 5% glucose. Discriminative stimulus (*right top*) and response rate (*right bottom*) effects of TC2559 hemigalactarate (*inverted empty triangle*) in rats trained to discriminate 0.4 mg/kg nicotine from 5% glucose. *Horizontal broken line* shows results after 5% glucose alone; the *horizontal dotted line* shows results from nicotine alone. All results are presented as mean±standard error of the mean for a 15-min test where responses on both levers are reinforced on independent tandem variable interval 30-s, FR10 schedules beginning 30 min after injection of A-85380 (s.c.). A-85380 group sizes: vehicle—15 animals, nicotine—15 animals, 0.003 mg/kg—8 animals, 0.01 mg/kg—16 animals, 0.03 mg/kg—16 animals, 0.1 mg/kg—8 animals. ABT-594 group sizes: vehicle—8 animals, nicotine—7 animals, 0.001 mg/kg—8 animals, 0.003 mg/kg—7 animals, 0.01 mg/kg—7 animals, 0.03 mg/kg—7 animals. TC2559 group sizes: vehicle—16 animals, nicotine—17 animals, 0.3 mg/kg—8 animals, 1.0 mg/kg—16 animals, 3.0 mg/kg—15 animals, 10.0 mg/kg—9 animals. *Number symbol* indicates that $P<0.05$ compared with 5% glucose alone; *Plus symbol* indicates that $P<0.05$ compared with 0.4 mg/kg nicotine alone

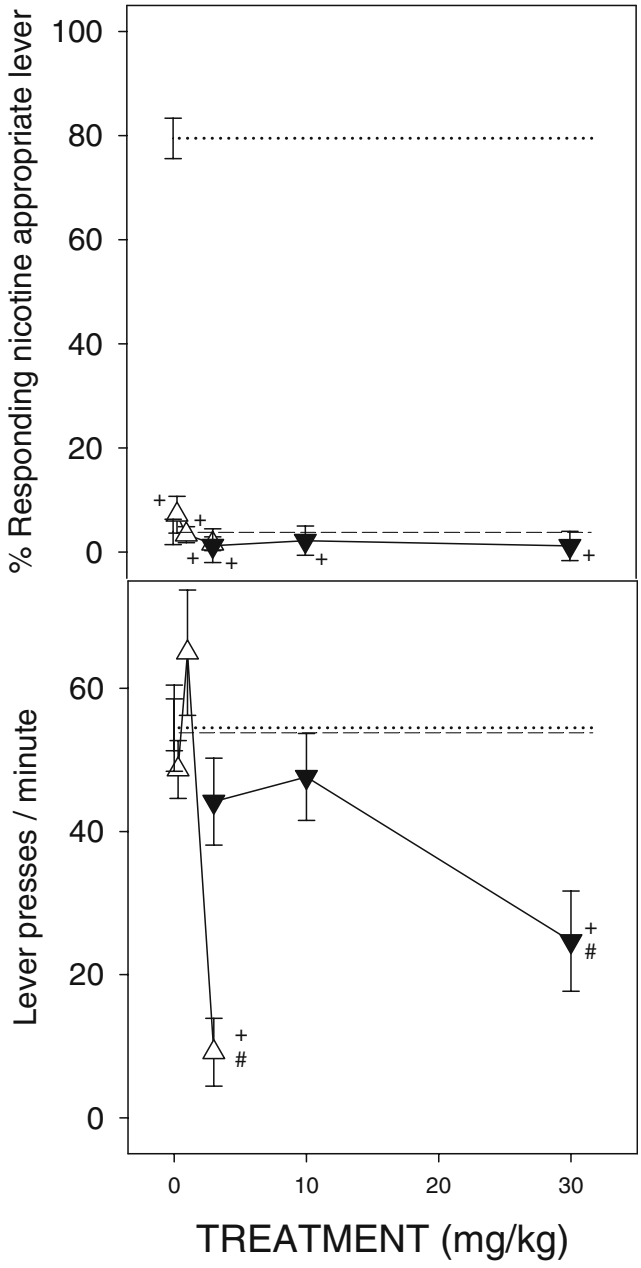

Fig. 6 Discriminative stimulus (*top*) and response rate (*bottom*) effects of WO 03/062224 (*empty triangle*) or WO 01/60821A1 (*inverted filled triangle*) in rats trained to discriminate 0.4 mg/kg nicotine from 5% glucose. *Horizontal broken line* shows results after 5% glucose alone; the *horizontal dotted line* shows results from nicotine alone. All results are presented as mean±standard error of the mean for a 15-min test where responses on both levers are reinforced on independent tandem variable interval 30-s, FR10 schedules beginning 30 min after injection of WO 03/062224 (s.c.). WO 03/062224 group sizes: vehicle—12 animals, nicotine—12 animals, 0.3 mg/kg—8 animals, 1 mg/kg—8 animals, 3 mg/kg—8 animals. WO 01/60821A1 group sizes: vehicle—13 animals, nicotine—12 animals, 3 mg/kg—9 animals, 10 mg/kg—8 animals, 30 mg/kg—6 animals. *Number symbol* indicates that $P<0.05$ compared with 5% glucose alone; *Plus symbol* indicates that $P<0.05$ compared with 0.4 mg/kg nicotine alone

containing nicotinic receptors and some (ABT594, eight-fold) or very large degrees of selectivity (TC2559, more than 1,000-fold; A-85380, 84-fold) over receptors containing $\alpha3\beta4$ subunits. Furthermore, each of these compounds acted as a full agonist (i.e. had a maximum efficacy that was fully comparable to that of nicotine) at native rat nicotinic receptors controlling dopamine release in striatal slices. In contrast, varenicline, which also has very high affinity for $\alpha4\beta2$-containing receptors and about 200-fold selectivity over receptors containing $\alpha3\beta4$ subunits, failed to enhance dopamine release beyond 70% of the maximum response to nicotine. Consistent with partial agonist activity in vitro, varenicline dose-dependently induced nicotine-appropriate responding in the drug discrimination paradigm but only to a maximum of 60% and more than that seen at the highest dose tested when response rate also declined. Similarly, cytisine has high affinity and almost 200-fold selectivity for $\alpha4\beta2$- over $\alpha3\beta4$-containing receptors but was capable of enhancing dopamine release to only 50% of the maximum seen with nicotine in vitro. Again, generalisation to the nicotine discriminative stimulus was dose dependent, but the maximum degree of nicotine-appropriate responding was only 60% and achieved at a dose that also induced a significant decrease in overall response rate. Thus, nicotinic compounds capable of enhancing striatal dopamine release will generalise to the nicotine discriminative stimulus to a degree that reflects their efficacy in vitro.

Cytisine and varenicline showed different levels of partial generalisation. It is possible that full substitution was not achieved simply because the compounds could not be given at high enough doses rather than because the maximum possible stimulation of $\alpha4\beta2$ receptors had been achieved. The validity of the partial agonist hypothesis could be tested by determining the dose–response relationship to nicotine in the presence of such a compound: the curve should be shifted to the left with the percentage maximum nicotine-appropriate responding occurring below that of nicotine but above that of the partial agonist alone. It remains to be seen whether the drug discrimination methodology is sufficiently sensitive and devoid of confounds to be able to adequately test this theory. In addition, neither cytisine nor varenicline is a clean $\alpha4\beta2$ agonist, and action at receptors containing the $\beta4$ subunit may be the restricting factor. However, this is perhaps unlikely as ABT594 and A85380 also show appreciable affinity for the $\alpha3\beta4$ receptor, and they demonstrate full substitution.

Further evidence that receptors containing $\alpha4\beta2$ subunits mediate the discriminative stimulus properties of nicotine is provided by the failure of the $\alpha3\beta4$-selective agonist, WO 03/062224, and $\alpha7$-selective agonist, WO 01/60821A1, to induce nicotine-appropriate responding even at doses that markedly reduced overall response rate. The results are consistent with the inability of $\beta2$-subunit

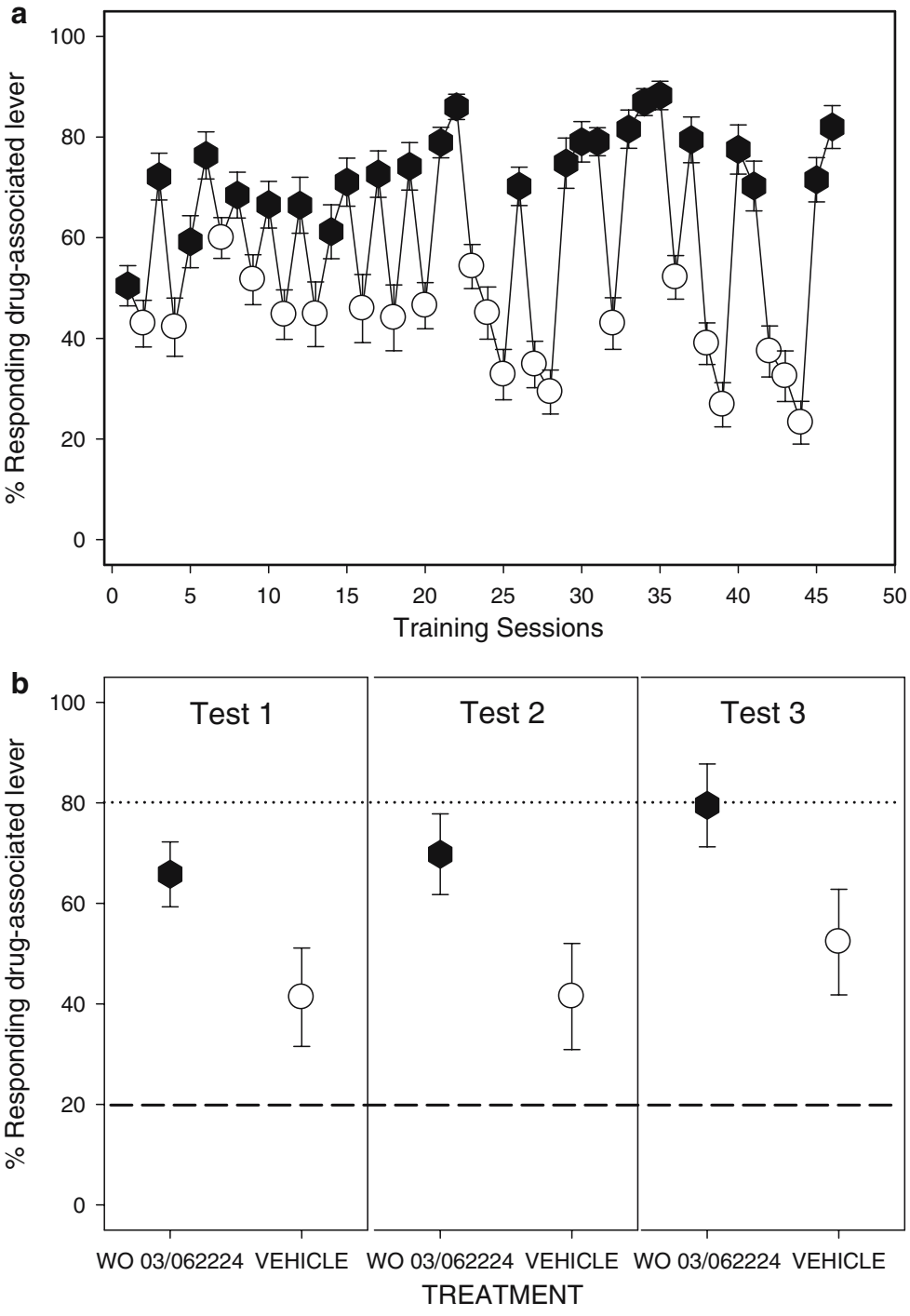

Fig. 7 **a** Discriminative stimulus effects of WO 03/062224 (*filled hexagon*) or vehicle (*empty circle*) in rats during training to discriminate 1 mg/kg WO 03/062224 from 5% glucose. *Horizontal broken line* shows criterion for 5% glucose alone; the *horizontal dotted line* shows criterion for WO 03/062224 alone. All results are presented as mean±standard error of the mean of 15 animals for a 15-min training session where responses on only treatment-appropriate levers were reinforced on tandem variable interval 30-s, FR10 schedules beginning 30 min after injection. **b** Discriminative stimulus effects of WO 03/062224 (*filled hexagon*) or vehicle (*empty circle*) in rats during training to discriminate 1 mg/kg WO 03/062224 from 5% glucose. All results are presented as mean±standard error of the mean of 15 animals for a 15-min test where responses on both levers are reinforced on independent tandem variable interval 30-s, FR10 schedules beginning 30 min after injection

knockout mice to learn to discriminate nicotine from vehicle (Shoaib et al. 2002) and with the failure of WO 03/062224 to induce a robust discriminative stimulus in rats at behaviourally active doses. The interoceptive stimulus properties of α7 receptor agonists are currently unknown, but the failure of WO 01/60821A1 to generalise to nicotine and the ability of nicotine to support nicotine discrimination in α7 knockout mice (Stolerman et al. 2004) suggest that if they do exist, they are substantially different from those of agonists of α4β2-containing receptors. This is particularly important to note as receptors expressing α7 subunits are expressed somato-dendritically by midbrain dopaminergic neurons and have been suggested to be important to nicotine reinforcement and dependence (Wu et al. 2004).

The similarity in efficacy of compounds to induce nicotine-appropriate responding and enhance striatal dopamine release, and the insensitivity of both processes to WO

03/062224 and WO 01/60821A1 suggest not only the common involvement of α4β2 receptors but also that dopaminergic systems may contribute to the generation of the nicotine discriminative stimulus. Indeed, Grady et al. (2001) reported that striatal dopamine release is not sensitive to nicotine in β2 knockout mice. Furthermore, nicotine is reported to fully substitute for the dopamine-releasing agent amphetamine in trained animals (Cunningham et al. 2006), whereas amphetamine only partially substitutes for nicotine in animals trained to discriminate nicotine (Stolerman et al. 1984). However, nicotine fully generalises to the cytisine cue (Chandler and Stolerman 1997). In generating a nicotine-like stimulus, cytisine appears to be reproducing specifically the dopamine-related component of this discrimination (Di Chiara 2000). However, local injection studies suggest that these effects are probably not generated in striatal tissue per se but more likely in prefrontal cortex with some contributions from ventral striatum and dorsal hippocampus (Miyata et al. 1999, 2002; Shoaib and Stolerman 1996).

Cytisine is not a selective agent at the α4β2 receptor, and at the highest dose tested, there was a significant effect on the motor output of the animals. It is possible that α3β4 receptors may be involved in some of the "side effects" ascribed to nicotine treatment, including decreases in activity after acute treatment. This has also been suggested by Salas et al. (2004) who showed that animals lacking either β4 subunits or were heterozygous for α3 were less sensitive to nicotine with respect to both hypolocomotion and nicotine-elicited seizures. The nicotinic β4 subunit is widely expressed in the mouse peripheral nervous system (PNS), whereas in the central nervous system (CNS), it is detected only in the olfactory bulb (Olf), medial habenula (MHb), pineal gland, interpeduncular nucleus, and inferior colliculus (Salas et al. 2003; Xu et al. 1999b). Due to its restricted expression pattern and the previous lack of very specific ligands, the β4 subunit has not been extensively studied. Similarly to β4, the α3 subunit is highly expressed in the PNS (Xu et al. 1999a), but in the CNS, α3 is detected only in the certain cells of Olf, the accessory Olf, MHb, substantia nigra/ventral tegmental area and superior and inferior colliculi. De Biasi et al. have shown that α3-containing nAChRs are necessary for normal autonomic function, (e.g. De Biasi 2002) but the role of α3 in the CNS is as yet unclear. Null α3 (−/−) mice rarely survive past weaning age and so have been of limited use in behavioural studies (Xu et al. 1999a). It is interesting to note that nicotine-elicited norephinephrine release appears to be mediated through α3β4 receptors (Fu et al. 1999; Luo et al. 1998), whereas nicotine-elicited dopamine release appears to be mediated through α4β2-containing receptors. Further evidence from Grady et al. (2001) showed that nicotine-elicited dopamine release was lost in β2 knockout mice but that nicotine-elicited norepinephrine remained no different from that in wild-type animals. As discussed previously, it is thought that dopamine release is critical in mediating its discriminative stimulus (Di Chiara and Imperato 1988; Corrigall et al. 1992, 1994).

Despite evidence of functional relevance, there was no evidence of the selective α3β4 receptor agonist, WO 03/062224, inducing an interoceptive stimulus even after 47 training sessions. Examination of the discrimination training and test data suggested that the animals primarily responded on the lever, which had been reinforced on the most recent training session. In training, this would not necessarily be reinforced, and, consequently, the animals learned to adapt and select the alternative lever when appropriate. On test, however, responding on both levers was reinforced, and, consequently, animals continued to press the "incorrect" lever. This strongly suggests that even though this drug has been shown to affect response rates, and that α3β4 receptors are involved in generating some of the "side-effects" of nicotine including respiratory system effects and locomotor depression, stimulation of α3β4 receptors is neither necessary nor sufficient to produce a discriminable stimulus that an animal can use to guide its behaviour. The dose chosen to train the animals lacked overt side effects but was known to produce levels in the brain of approximately 2 μM (Whiffin et al., unpublished data)—several times higher than the measured binding affinity of the agonist.

Although there are currently no reports of α7-selective receptor agonists supporting drug discrimination training, animals lacking the α7 nAChR learn to discriminate nicotine as well as wild-type animals (Stolerman et al. 2004). The selective α7 receptor agonist, WO 01/60821A1, failed to substitute for nicotine. As with α3β4-containing receptors, despite evidence of functional relevance, stimulation of α7 receptors does not induce an interoceptive stimulus nor does it play any role in the stimulus induced by nicotine.

In conclusion, the present work confirms and extends previous findings that have suggested that α4β2 receptors play a key, if not unique role, in generating the discriminative stimulus properties of nicotine that has been suggested through gene deletion, antagonism at the receptor or less selective pharmacology. Uniquely, agonists at the nicotinic α3β4 and α7 receptors were assessed in these studies and have definitively shown that these nicotinic receptor subtypes are not involved in the discriminative stimulus properties of nicotine. These findings suggest that the α7 receptor may not be involved in nicotine reinforcement. Furthermore, these studies have shown for the first time that an agonist at nicotinic α3β4 receptors do not themselves produce an interoceptive stimulus that enables a discrimination to be maintained.

Acknowledgements This work would not have been made possible without the technical assistance of Louise Wallace, Anne-Helene

Clugery, Daniel Kumpik, Kimberley Smith, Ellen Colvin and John Boot.

All experiments conducted within this paper comply with the current laws of the country in which they were performed.

References

Bannon AW, Decker MW, Kim DJB, Campbell JE, Arneric SP (1998) ABT-594, a novel cholinergic channel modulator, is efficacious in nerve ligation and diabetic neuropathy models of neuropathic pain. Brain Res 801:158–163

Brioni JD, Kim DJB, O'Neill AB (1996) Nicotine cue: lack of effect of the alpha7 nicotinic receptor antagonist methyllycaconitine. Eur J Pharmacol 301:1–5

Buckley MJ, Surowy C, Meyer M, Curzon P (2004) Mechanism of action of A-85380 in an animal model of depression. Prog Neuropsychopharmacol Biol Psychiatry 28:723–730

Cao YJ, Surowy CS, Puttfarcken PS (2005) Different nicotinic acetylcholine receptor subtypes mediating striatal and prefrontal cortical [3H]dopamine release. Neuropharmacology 48:72–79

Chandler CJ, Stolerman IP (1997) Discriminative stimulus properties of the nicotinic agonist cytisine. Psychopharmacology 129: 257–264

Chen Y, Sharples TJW, Phillips KG, Benedetti G, Broad LM, Zwart R, Sher E (2003) The nicotinic alpha4beta2 receptor selective agonist, TC-2559, increases dopamine neuronal activity in the ventral tegmental area of rat midbrain slices. Neuropharmacology 45:334–344

Cheng YC, Prusoff WH (1973) Relationship between the inhibition constant (Ki) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. Biochem Pharmacol 22:3099–3108

Coe JW, Brooks PR, Wirtz MC, Bashore CG, Bianco KE, Vetelino MG, Arnold EP, Lebel LA, Fox CB, Tingley FD 3rd, Schulz DW, Davis TI, Sands SB, Mansbach RS, Rollema H, O'Neill BT (2005) 3,5-Bicyclic aryl piperidines: a novel class of alpha4beta2 neuronal nicotinic receptor partial agonists for smoking cessation. Bioorg Med Chem Lett 15:4889–4897

Cooper E, Couturier S, Ballivet M (1991) Pentameric structure and subunit stoichiometry of a neuronal acetylcholine receptor. Nature 350:235–238

Corrigall WA, Franklin KBJ, Coen KM, Clarke PBS (1992) The mesolimbic dopaminergic system is implicated in the reinforcing effects of nicotine. Psychopharmacology 107:285–289

Corrigall WA, Coen KM, Adamson KL (1994) Self-administered nicotine activates the mesolimbic dopamine system through the ventral tegmental area. Brain Res 653:278–284

Corringer PJ, Le Novere N, Changeux J-P (2000) Nicotinic receptors at the amino acid level. Annu Rev Pharmacol Toxicol 40:431–458

Couturier S, Bertrand D, Matter JM, Hernandez MC, Bertrand S, Millar N, Valera S, Barkas T, Ballivet M (1990) A neuronal nicotinic acetylcholine receptor subunit (alpha 7) is developmentally regulated and forms a homo-oligomeric channel blocked by alpha-BTX. Neuron 5:847–856

Cunningham CS, Polston JE, Jany JR, Segert IL, Miller DK (2006) Interaction of lobeline and nicotinic receptor ligands with the discriminative stimulus properties of cocaine and amphetamine. Drug Alcohol Depend 84:211–222

De Biasi M (2002) Nicotinic mechanisms in the autonomic control of organ systems. J Neurobiol 53:568–579

Di Chiara G (2000) Role of dopamine in the behavioural actions of nicotine related to addiction. Eur J Pharmacol 393:295–314

Di Chiara G, Imperato A (1988) Drugs abused by humans preferentially increase synaptic dopamine concentrations in the mesolimbic system of freely moving rats. Proc Natl Acad Sci USA 85:5274–5278

Donnelly-Roberts D, Puttfarcken PS, Kuntzweiler TA, Briggs CA, Anderson DJ, Campbell JE, Piattoni-Kaplan M, McKenna DG, Wasicak JT, Holladay MW, Williams M, Arneric SP (1998) ABT-594 [(R)-5-(2-azetidinylmethoxy)-2-chloropyridine]: a novel, orally effective analgesic acting via neuronal nicotinic acetylcholine receptors: I. In vitro characterisation. J Pharmacol Exp Ther 285:777–786

Fu Y, Matta SG, Sharp BM (1999) Local alpha-bungarotoxin-sensitive nicotinic receptors modulate hippocampal norepinephrine release by systemic nicotine. J Pharmacol Exp Ther 289:133–139

Garcha HS, Giardini V, Pratt JA, Stolerman IP (1983) Investigation of the effects of ganglion-blocking drugs and methergoline on the nicotine cue in rats. Br J Pharmacol 78:9P

Gommans J, Stolerman IP, Shoaib M (2000) Antagonism of the discriminative and aversive stimulus properties of nicotine in C57BL/6J mice. Neuropharmacology 39:2840–2847

Grady SR, Meinerz NM, Cao J, Reynolds AM, Picciotto MR, Changeux J-P, McIntosh JM, Marks MJ, Collins AC (2001) Nicotinic agonists stimulate acetylcholine release from mouse interpeduncular nucleus: a function mediated by a different nAChR than dopamine release from striatum. J Neurochem 76:258–268

Hirschhorn ID, Rosecrans JA (1974) Studies on the time course and the effect of cholinergic and adrenergic receptor blockers on the stimulus effect of nicotine. Psychopharmacologia 40:109–120

Kallman WM, Rosecrans JA, Kallman MJ, Harry GJ, Woodson PT (1982) Nicotine as a discriminative stimulus in human subjects. Psychopharmacology 76:A8

Kumar R, Reavill C, Stolerman IP (1987) Nicotine cue in rats: effects of central administration of ganglion-blocking drugs. Br J Pharmacol 90:239–246

Lambe EK, Picciotto MR, Aghajanian GK (2003) Nicotine induces glutamate release from thalamocortical terminals in prefrontal cortex. Neuropsychopharmacology 28:216–225

Le Novere N, Corringer P-J, Changeux J-P (2002) The diversity of subunit composition in nAChRs: evolutionary origins, physiologic and pharmacologic consequences. J Neurobiol 53:447–456

Leslie FM, Gallardo KA, Park MK (2002) Nicotinic acetylcholine receptor-mediated release of [3H]norepinephrine from developing and adult rat hippocampus: direct and indirect mechanisms. Neuropharmacology 42:653–661

Luo S, Kulak JM, Cartier GE, Jacobsen RB, Yoshikami D, Olivera BM, McIntosh JM (1998) alpha-conotoxin AuIB selectively blocks alpha3 beta4 nicotinic acetylcholine receptors and nicotine-evoked norepinephrine release. J Neurosci 18:8571–8579

Malin DH, Lake JR, Schopen CK, Kirk JW, Sailer EE, Lawless BA, Upchurch TP, Shenoi M, Rajan N (1997) Nicotine abstinence syndrome precipitated by central but not peripheral hexamethonium. Pharmacol Biochem Behav 58:695–699

Marks MJ, Pauly JR, Gross SD, Deneris ES, Hermans-Borgmeyer I, Heinemann SF, Collins AC (1992) Nicotine binding and nicotinic receptor subunit RNA after chronic nicotine treatment. J Neurosci 12:2765–2784

Miyata H, Ando K, Yanagita T (1999) Medial prefrontal cortex is involved in the discriminative stimulus effects of nicotine in rats. Psychopharmacology 145:234–236

Miyata H, Ando K, Yanagita T (2002) Brain regions mediating the discriminative stimulus effects of nicotine in rats. Ann N Y Acad Sci 965:354–363

Ohashi K, Osuga J-I, Tozawa R, Kitamine T, Yagyu H, Sekiya M, Tomita S, Okazaki H, Tamura Y, Yahagi N, Iizuka Y, Harada K, Gotoda T, Shimano H, Yamada N, Ishibashi S (2003) Early embryonic lethality caused by targeted disruption of the 3-

hydroxy-3-methylglutaryl-CoA reductase gene. J Biol Chem 278:42936–42941

Picciotto MR, Zoli M, Rimondini R, Lena C, Marubio LM, Pich EM, Fuxe K, Changeux J-P (1998) Acetylcholine receptors containing the beta2 subunit are involved in the reinforcing properties of nicotine. Nature 391:173–177

Pratt JA, Stolerman IP, Garcha HS, Giardini V, Feyerabend C (1983) Discriminative stimulus properties of nicotine: further evidence for mediation at a cholinergic receptor. Psychopharmacology 81:54–60

Salas R, Orr-Urtreger A, Broide RS, Beaudet A, Paylor R, De Biasi M (2003) The nicotinic acetylcholine receptor subunit a5 mediates short-term effects of nicotine in vivo. Mol Pharmacol 63:1059–1066

Salas R, Cook KD, Bassetto L, De Biasi M (2004) The a3 and b4 nicotinic acetylcholine receptor subunits are necessary for nicotine-induced seizures and hypolocomotion. Neuropharmacology 47:401–407

Schechter MD (1995) Scopolamine–physostigmine combination does not substitute for nicotine. Prog Neuropsychopharmacol Biol Psychiatry 19:499–508

Shoaib M, Stolerman IP (1996) Brain sites mediating the discriminative stimulus effects of nicotine in rats. Behav Brain Res 78:183–188

Shoaib M, Zubaran C, Stolerman IP (2000) Antagonism of stimulus properties of nicotine by dihydro-b-erythroidine (DHbE) in rats. Psychopharmacology 149:140–146

Shoaib M, Gommans J, Morley A, Stolerman IP, Grailhe R, Changeux J (2002) The role of nicotinic receptor beta-2 subunits in nicotine discrimination and conditioned taste aversion. Neuropharmacology 42:530–539

Stolerman IP, Garcha HS, Pratt JA, Kumar R (1984) Role of training dose in discrimination of nicotine and related compounds by rats. Psychopharmacology 84:413–419

Stolerman IP, Chandler CJ, Garcha HS, Newton JM (1997) Selective antagonism of behavioural effects of nicotine by dihydro-beta-erythroidine in rats. Psychopharmacology 129:390–397

Stolerman IP, Chamberlain S, Bizarro L, Fernandes C, Schalkwyk L (2004) The role of nicotinic receptor alpha7 subunits in nicotine discrimination. Neuropharmacology 46:363–371

Sullivan JP, Donnelly-Roberts D, Briggs CA, Anderson DJ, Gopalakrishnan M, Piattoni-Kaplan M, Campbell JE, McKenna DG, Molinari E, Hettinger A-M, Garvey DS, Wasicak JT, Holladay MW, Williams M, Arneric SP (1998) A-85380 [3-(2 (S)-azetidinylmethoxy) pyridine]: in vitro pharmacological properties of a novel, high affinity alpha4beta2 nicotinic acetylcholine receptor ligand. Neuropharmacology 35:725–734

Unwin N (2003) Structure and action of the nicotinic acetylcholine receptor explored by electron microscopy. FEBS Lett 555:91–95

Watkins SS, Epping-Jordan MP, Koob GF, Markou A (1999) Blockade of nicotine self-administration with nicotinic antagonists in rats. Pharmacol Biochem Behav 62:743–751

Wiley JL, James JR, Rosecrans JA (1996) Discriminative stimulus properties of nicotine: approaches to evaluating potential nicotinic receptor agonists and antagonists. Drug Dev Res 38:222–230

Wu J, George AA, Schroeder KM, Xu L, Marxer-Miller S, Lucero L, Lukas RJ (2004) Electrophysiological, pharmacological and molecular evidence for α7-nicotinic acetylcholine receptors in rat midbrain dopamine neurons. J Pharmacol Exp Ther 311:80–91

Xu W, Orr-Urtreger A, Nigro F, Gelber S, Sutcliffe CB, Armstrong D, Patrick JW, Role LW, Beaudet AL, De Biasi M (1999a) Multiorgan autonomic dysfunction in mice lacking the beta2 and the beta4 subunits of neuronal nicotinic acetylcholine receptors. J Neurosci 19:9298–9305

Xu W, Gelber S, Orr-Urtreger A, Armstrong D, Lewis RA, Ou C, Patrick J, Role L, De Biasi M, Beaudet AL (1999b) Megacystis, mydriasis, and ion channel defect in mice lacking the alpha3 neuronal nicotinic acetylcholine receptor. Proc Natl Acad Sci USA 96:5746–5751

Zhu PJ, Chiappinelli VA (2002) Nicotinic receptors mediate increased GABA release in brain through a tetrodotoxin-insensitive mechanism during prolonged exposure to nicotine. Neuroscience 115:137–144

Zoli M, Lena C, Picciotto MR, Changeux JP (1998) Identification of four classes of brain nicotinic receptors using beta2 mutant mice. J Neurosci 18:4461–4472