British Journal of Pharmacology (2008) 153, 1054–1061
 0007–1188/08 $30.00
www.brjpharmacol.org

RESEARCH PAPER

α7 nicotinic acetylcholine receptor agonist properties of tilorone and related tricyclic analogues

CA Briggs, MR Schrimpf, DJ Anderson, EJ Gubbins, JH Grønlien, M Håkerud, H Ween, K Thorin-Hagene, J Malysz, J Li, WH Bunnelle, M Gopalakrishnan and MD Meyer

Neuroscience Research, Global Pharmaceutical Research and Development, Abbott Laboratories, Abbott Park, IL, USA

Background and purpose: The α7 nicotinic acetylcholine receptor (nAChR) has attracted considerable interest as a target for cognitive enhancement in schizophrenia and Alzheimer's Disease. However, most recently described α7 agonists are derived from the quinuclidine structural class. Alternatively, the present study identifies tilorone as a novel α7-selective agonist and characterizes analogues developed from this lead.
Experimental approach: Activity and selectivity were determined from rat brain α7 and α4β2 nAChR binding, recombinant nAChR activation, and native α7 nAChR mediated stimulation of ERK1/2 phosphorylation in PC12 cells.
Key results: Tilorone bound α7 nAChR (IC_{50} 110 nM) with high selectivity relative to α4β2 (IC_{50} 70 000 nM), activated human α7 nAChR with an EC_{50} value of 2.5 μM and maximal response of 67% relative to acetylcholine, and showed little agonist effect at human α3β4 or α4β2 nAChRs. However, the rat α7 nAChR maximal response was only 34%. Lead optimization led to 2-(5-methyl-hexahydro-pyrrolo[3,4-c]pyrrol-2-yl)-xanthen-9-one (A-844606) with improved binding (α7 IC_{50} 11 nM, α4β2 $IC_{50} > 30\,000$ nM) and activity at both human and rat α7 nAChR (EC_{50}s 1.4 and 2.2 μM and apparent efficacies 61 and 63%, respectively). These compounds also activated native α7 nAChR, stimulating ERK1/2 phosphorylation in PC12 cells.
Conclusions and implications: Tilorone, known as an interferon inducer, is a selective α7 nAChR agonist, suggesting utility of the fluorenone pharmacophore for the development of α7 nAChR selective agonists. Whether α7 stimulation mediates interferon induction, or whether interferon induction may influence the potential anti-inflammatory properties of α7 nAChR agonists remains to be elucidated.
British Journal of Pharmacology (2008) **153,** 1054–1061; doi:10.1038/sj.bjp.0707649; published online 24 December 2007

Keywords: nicotinic acetylcholine receptor; α7 nicotinic receptor; *Xenopus* oocytes; cognition; tilorone

Abbreviations: ACh, acetylcholine; FLIPR, fluorescence light-imaging plate reader; MLA, methyllycaconitine; nAChR, nicotinic acetylcholine receptor

Introduction

The past 15 years have witnessed the cloning of 17 different nicotinic acetylcholine receptor (nAChR) subunits, demonstrating nAChR expression not only in muscle and autonomic ganglia but also in sensory ganglia, throughout the central nervous system, and in a variety of non-neuronal cells (Jensen *et al.*, 2005; Gahring and Rogers, 2006). Over half of these subunits are expressed in brain, and often coexpressed in the same cells. As nAChRs are pentameric, there is potential for a considerable variety of functionally and pharmacologically distinct nAChRs. Among the more prominent nAChR in brain are the α4β2 heteromeric and α7 homomeric subtypes, both of which have been implicated in sensory gating, cognitive processes and other physiological functions (Martin *et al.*, 2004; D'Andrea and Nagle, 2006). Additionally, there is increasing evidence that α7 nAChR may play important roles in non-neuronal processes, such as angiogenesis (Heeschen *et al.*, 2002) and inflammation (Tracey, 2005; Ulloa, 2005).

To address these targets, subtype-selective compounds continue to be developed, particularly to avoid adverse effects mediated by α3-containing nAChR in autonomic ganglia. The α7 nAChR is a target of considerable interest, due to the critical disease processes in which it is implicated (for example, schizophrenia and Alzheimer's disease) and the idea that novel selective agonists could be developed given the pharmacological distinction exemplified by the selective antagonists α-bungarotoxin and the alkaloid methyllycaconitine (MLA).

Correspondence: Dr CA Briggs, Neuroscience Research, Abbott Laboratories, R47W, Bldg, AP9A-3, 100 Abbott Park Road, Abbott Park, IL 60064, USA.
E-mail: clark.briggs@abbott.com
Received 4 September 2007; revised 26 October 2007; accepted 29 November 2007; published online 24 December 2007

The first compound advanced as an α7-selective agonist was GTS-21, also known as DMXBA (Briggs *et al*., 1995; Kem, 2000; Papke *et al*., 2004). However, the selectivity of GTS-21 is context dependent. Although it was found to activate rat α7 nAChR selectively, it also binds α4β2 nAChR and it was found to elicit a low maximal response at recombinant human α7 nAChR *in vitro* (Briggs *et al*., 1995; Meyer *et al*., 1998). Whether the α4β2 ligand properties contribute towards or detract from the physiological effects of GTS-21 is not yet clear. Additionally, active metabolites of GTS-21 have been identified, and may contribute effects *in vivo* (Kem *et al*., 2004).

Recently, more efficacious α7 nAChR-selective ligands have been found, such as AR-R 17779 (Van Kampen *et al*., 2004), PNU-282987 (Bodnar *et al*., 2005), PHA-543613 (Wishka *et al*., 2006), SSR-180711 (Biton *et al*., 2007; Pichat *et al*., 2007), ABBF (Boess *et al*., 2007) and others (Tatsumi *et al*., 2006). However, except for GTS-21 and SSR-180711, all these compounds share a quinuclidine pharmacophore.

A seemingly unrelated compound, tilorone, has been known as an interferon inducer since 1970 (Krueger and Mayer, 1970; Mayer and Krueger, 1970). We found that tilorone is a selective α7 nAChR ligand. Other tricyclic compounds have been found to inhibit α4β2 and other β2-containing nAChR at micromolar concentrations (Connolly *et al*., 1992). Surprisingly, however, tilorone acted as a highly selective agonist for α7 nAChR. Thus, tilorone appeared to represent a novel structural scaffold for discovery of α7-selective agonists. Here, we describe the *in vitro* functional properties of tilorone and three related analogues.

Materials and methods

Rat α7 nAChR binding

Binding to rat α7 nAChR was measured by [^{3}H]MLA displacement as described by Davies *et al*. (1999). Membrane-enriched fractions from rat brain minus cerebellum (ABS Inc., Wilmington, DE, USA) were slowly thawed at 4 °C, washed and resuspended in 30 volumes of BSS-Tris buffer (120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$ and 50 mM Tris-Cl, pH 7.4, 22 °C). Samples containing 100–200 μg of protein, 5 nM [^{3}H]MLA (25 Ci mmol^{-1}) and 0.1% BSA were incubated in a final volume of 500 μl for 60 min at 22 °C. Seven log-dilution concentrations of each compound were tested in duplicate. Nonspecific binding was determined in the presence of 10 μM MLA. Bound radioactivity was isolated by vacuum filtration onto glass fibre filter plates pre-wetted with 2% BSA using a 96-well filtration apparatus (Packard Instruments, Meriden, CT, USA) and were then rapidly rinsed with 2 ml of ice-cold BSS; 40 μl per well of Packard MicroScint-20 was added and radioactivity was determined using a Packard TopCount. The IC_{50} values were determined by nonlinear regression in Microsoft Excel.

Binding to rat α4β2 nAChR was measured by [^{3}H]cytisine displacement as modified from Pabreza *et al*. (1991). Procedures were similar to those described above, except that 0.75 nM [^{3}H]cytisine (30 Ci mmol^{-1}) was used and nonspecific binding was determined in the presence of 10 μM (−)-nicotine.

Human and rat recombinant α7 nAChR functional evaluation

Female *Xenopus laevis* frogs obtained from Nasco (Fort Atkinson, WI, USA) and from Blades Biological Ltd. (Cowden, Edenbridge, Kent, UK) were maintained and treated using standard protocols approved by Abbott's Institutional Animal Care and Use Committee. The preparation and maintenance of *X. laevis* oocytes and their injection with cDNA or cRNA prepared by standard techniques followed procedures similar to those described previously (Briggs *et al*., 1995; Trumbull *et al*., 2003). Oocytes were injected within 24 h of their preparation and were used 2–7 days after injection.

α7 nAChR responses were measured by two-electrode voltage clamp (−60 mV) at room temperature in OR-2 solution (pH 7.4) containing 90 mM NaCl, 2.5 mM KCl, 2.5 mM $CaCl_2$, 1.0 mM $MgCl_2$, 5 mM Na-HEPES buffer and 0.5 μM atropine to block endogenous muscarinic receptors. Agonists were applied using the robotic Gilson pipettor of the POETs apparatus (Trumbull *et al*., 2003) and responses were measured as the compound-induced peak (maximal) inward current relative to the baseline-holding current and normalized to the acetylcholine (ACh) response determined in the same oocyte. In each oocyte, 10 mM ACh, which elicited a maximal α7 nAChR response (Briggs and McKenna, 1998; Briggs *et al*., 1999), was applied before and after test compound to test response stability and for the purpose of normalizing test compound's response to the maximal ACh response. Typically, ACh was applied several times initially to ensure stability, followed by two applications of test compound, followed by two applications of ACh, followed by two applications of test compound, etc. The intervals between applications were 5 min, which were sufficient for full recovery of the ACh response, or longer. High concentrations of some compounds can produce a prolonged after-inhibition of the nAChR response, perhaps due to slow washout or channel block. This could be detected as a reduction in the control ACh response. In such instances, the interval between applications was increased or control ACh applications were added, thereby increasing the test compound washout duration. Responses affected by prolonged after-inhibition or other forms of instability reflected by ACh controls were discarded. Otherwise, responses to test compound were normalized to an average of the 10 mM ACh responses determined before and after test compound. Concentration–response parameters were determined using the nonlinear curve fitting in Graphpad Prism and the built-in variable slope sigmoidal curve (Hill equation); fitting parameters were not constrained except that the bottom of the curve was set equal to 0.

Rat native α7 nAChR signalling via phospho-ERK

Activation of native α7 nAChR signalling in rat PC12 cells was assessed by stimulation of ERK1/2 phosphorylation (Gubbins *et al*., 2006). Briefly, PC12 cells obtained from American Type Culture Collection (Manassas, VA, USA) were cultured in poly-D-lysine coated 96-well plates containing Ham's F12K supplemented with 15% horse serum (heat inactivated), 2.5% fetal bovine serum and 2 mM glutamine. At 24 h before the experiment, culture medium was replaced

with non-supplemented F12K to reduce the basal level of ERK phosphorylation. Cells were transferred to Hank's balanced salt solution (HBSS) with or without nAChR antagonist for a 60 min preincubation (37 °C), then 3 μM PNU-120596 (Hurst *et al*., 2005) to reduce α7 nAChR desensitization, followed 10 min later by nAChR agonist and a final 7-min incubation in a final volume of 100 μl. Activity was terminated by transferring the incubation plate to ice, aspirating the medium and lysing the cells on ice for 15 min with 5 μl Insect Cell Lysis Buffer containing 1% sodium deoxycholate, 0.1% sodium dodecylsulphate, 1 mM sodium orthovanadate, 1% protease inhibitor cocktail and 100 U ml^{-1} benzonase. Samples were stored at −80 °C prior to assay of total ERK and phospho-ERK by western blot using fluorescent antibodies. Secondary antibodies were fluorescently labelled with Alexafluor-680 or IRDye800. Signals were measured by Li-Cor Odyssey Infrared Imaging System.

Non-α7 recombinant nAChR functional evaluation

The effects of compounds on non-α7 nAChR were measured as described previously (Grønlien *et al*., 2007) using fluorescence light-imaging plate reader (FLIPR), Ca^{2+}-sensitive fluorescent probes and human neuroblastoma IMR-32 or HEK293 cell lines expressing recombinant human α3* or α4* nAChR. Responses were normalized to the response to 100 μM (−)-nicotine determined in the same 96-well plate.

Additionally, the ability of tilorone and A-844606 to activate human α4β2 nAChR was tested by whole-cell patch clamp using standard techniques and the same HEK293 cell line as used in FLIPR. The cells were maintained in polystyrene culture flasks (175 mm^2) with Dulbecco's modified Eagle's medium plus Glutamax supplemented with 10% fetal bovine serum, 100 mg ml^{-1} zeocin and 0.5 mg ml^{-1} geneticin and a humidified atmosphere of 5% CO_2 in air at 37 °C. For transfer, cells were rinsed with 4 ml phosphate-buffered saline, incubated for 2 min in 1 ml of TrypLE Select, resuspended in 25 ml culture medium with trituration, and plated onto 12 mm diameter coverslips coated with poly-D-lysine and mouse laminin.

Patch-clamp recordings were obtained 1–2 days after plating with standard borosilicate glass electrodes (2–5 MΩ) and HEKA EPC-9 amplifier. The extracellular solution contained 130 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM Na-HEPES buffer (pH 7.4) and 0.5 μM atropine. The intracellular (pipette) solution contained 130 mM KCl, 5 mM NaCl, 2 mM K-EGTA, 2 mM $MgCl_2$ and 10 mM K-HEPES (pH 7.4). Serial resistance was <10 MΩ and was uncompensated.

Compounds were applied using theta-tube liquid filament and Burleigh PZ-150M piezo-electric device for position switching. For applying different compounds to the same cell, the solution in one barrel of the theta tube could be changed using a manifold valve (Type C25Z-3180; Valco Instruments Co. Inc., Schenkon, Switzerland). Agonist was applied for 2 s with 180 s between applications.

Materials

The following materials were used: [^{3}H]MLA and [^{3}H]cytisine (Perkin Elmer/NEN Life Science Products, Boston, MA, USA); BSA (Millipore, Bedford, MA, USA); Ham's F12K (Gibco); 15% horse serum (heat inactivated; Sigma Chemical Co., St Louis, MO, USA); Insect Cell Lysis Buffer (Pharmingen, San Diego, CA, USA); 1% protease inhibitor cocktail (Sigma Chemical Co.) and benzonase (Novagen, Madison, WI, USA). Primary antibodies for ERK1/2 and diphosphorylated ERK1/2 were obtained from Cell Signaling (Beverly, MA, USA) and Sigma Chemical Co., respectively. Cyclophilin A antibody was from Upstate Biotechnology (Lake Placid, NY, USA); Alexafluor-680 (Invitrogen, Carlsbad, CA, USA); IRDye800 (Rockland Immunochemicals, Gilbertsville, PA, USA); Glutamax (Gibco/Invitrogen, Paisley, UK); fetal bovine serum (Gibco); zeocin (Invitrogen); geneticin and TrypLE Select (Gibco) and mouse laminin (BD BioCoat; BD Bioscience Europe, Erembodegem-Drop, Belgium).

Results

Binding to α7 and α4β2 nAChR was determined in rat brain membranes by displacement of radioligands selective for the respective nAChR subtypes. Tilorone displaced [^{3}H]MLA binding to α7 nAChR with an IC_{50} value of 110 nM (67–180 nM at 95% confidence interval (CI), $n=4$) while exhibiting little displacement of [^{3}H]cytisine binding to α4β2 nAChR ($IC_{50}\sim 70$ μM, 19–260 μM CI, $n=5$).

While the relatively large size of tilorone compared with other α7 nAChR agonists initially suggested that it may be an antagonist, in fact, it was found to be a highly selective agonist. Tilorone activated human α7 nAChR expressed in oocytes with an EC_{50} value of 2.5 μM and maximal response of 67% relative to 10 mM ACh, as shown in Figure 1 and Table 1. Tilorone activated rat α7 nAChR with similar potency ($EC_{50}=0.94$ μM), but with a smaller maximal response (34%) compared with human α7 nAChR (Figure 1 and Table 1).

Among the analogues developed through lead optimization were 2,7-bis(hexahydropyrrolo[3,4-*c*]pyrrol-2(1*H*)-yl)-9*H*-fluoren-9-one (A-746050) and the 'one-armed' analogues exemplified by 2-amino-7-(hexahydropyrrolo[3,4-*c*]pyrrol-2(1*H*)-yl)-9*H*-fluoren-9-one (A-795723) and 2-(5-methyl-hexahydro-pyrrolo[3,4-*c*]pyrrol-2-yl)-xanthen-9-one (A-844606). Structures and concentration–response data are shown in Figure 1 and Table 1.

A-746050 bound α7 nAChR with an IC_{50} value of 0.87 nM (0.47–1.6 nM CI, $n=3$) compared with α4β2 nAChR where the IC_{50} value was 6000-fold higher ($IC_{50}=9.3$ μM, 6.2–14 μM CI, $n=6$). A-746050 functioned as a rat α7 nAChR agonist with an EC_{50} value of 0.22 μM and maximal response of 59%. However, A-746050 elicited a somewhat smaller maximal response (41%) at human α7 nAChR.

Reducing one of the diazabicyclo[3.3.0]octane arms to an amino group in A-795723 (Figure 1) had little impact on binding, as the α7 nAChR IC_{50} was 1.4 nM (0.70–2.9 nM CI, $n=5$) and selectivity against α4β2 nAChR was maintained ($IC_{50}=26$ μM, 21–31 μM CI, $n=3$). This compound functioned as an α7 agonist with low EC_{50} (0.3 μM), comparable potencies at rat and human α7 nAChR, but low maximal response (28–33%) (Table 1).

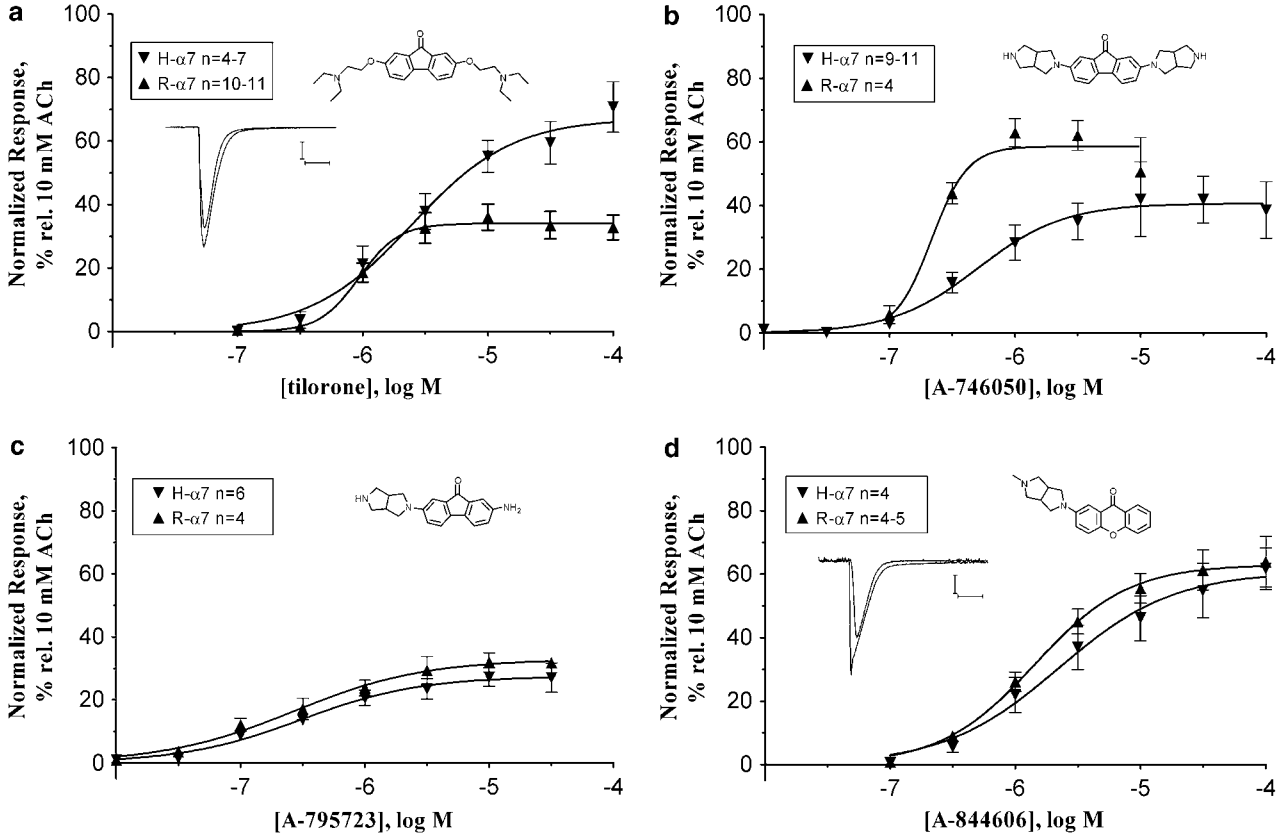

Figure 1 Activation of human and rat α7 nAChR by tilorone and analogues. Concentration–response data (mean ± s.e.mean) and structures are shown for tilorone (**a**), A-746050 (**b**), A-795723 (**c**) and A-844606 (**d**) as agonists at recombinant human and rat α7 nAChR expressed in *Xenopus* oocytes. Curves show the Hill equation fit to the data by nonlinear regression. The concentration–response parameters derived and the number of measurements (*n*) per data point are shown in Table 1. Also shown are sample responses to 100 μM tilorone and 10 mM ACh from one oocyte (**a**) and 100 μM A-844606 and 10 mM ACh from another oocyte (**d**). In each case, the response to ACh is the larger one. Scale bars represent 500 nA and 500 ms for all.

Table 1 α7 nAChR concentration–response parameters for tilorone and analogues

Compounds	*nAChR*	*EC_{50} (μM)*	*Hill coefficient*	*Maximum (%)*	n
Tilorone	Human α7	2.5 (1.4–4.4)	1.1 ± 0.3	67 ± 6	4–7
	Rat α7	0.94 (0.7–1.3)	2.8 ± 1.6	34 ± 2	10–11
A-746050	Human α7	0.50 (0.22–1.2)	1.3 ± 0.6	41 ± 4	9–11
	Rat α7	0.22 (0.14–0.33)	3.0 ± 1.2	59 ± 4	4
A-795723	Human α7	0.31 (0.16–0.60)	0.91 ± 0.22	28 ± 2	6
	Rat α7	0.27 (0.13–0.56)	0.83 ± 0.19	33 ± 3	4
A-844606	Human α7	2.2 (0.98–0.51)	0.95 ± 0.28	61 ± 6	4
	Rat α7	1.4 (0.95–2.2)	1.2 ± 0.2	63 ± 3	4–5

Concentration–response parameters were determined by nonlinear fitting of the Hill equation to the values depicted in Figure 1. Data are shown as mean (95% confidence interval) for EC_{50} and mean ± s.e.mean for Hill coefficient and maximal response relative to ACh (10 mM). The values for *n* are the number of determinations per data point.

Further development of these analogues included modification of the fluorenone pharmacophore, leading to the compound A-844606 (Figure 1). A-844606 had somewhat lower affinity for rat brain α7 nAChR (IC_{50} = 11 nM, 4–29 nM CI, n = 4) compared with the above compounds, but was about 30-fold more potent than tilorone and retained selectivity against α4β2 nAChR (IC_{50} > 30 μM, n = 4). Further, as an α7 agonist, A-844606 was potent and efficacious, without the apparent rat/human species selectivity evident in tilorone (Table 1).

Functional selectivity of A-844606 across other nAChR subtypes was evaluated using Ca^{2+} imaging in neuroblastoma and transfected cell lines. At concentrations at or above the α7 nAChR EC_{50} value, tilorone and A-844606 (3 μM) had

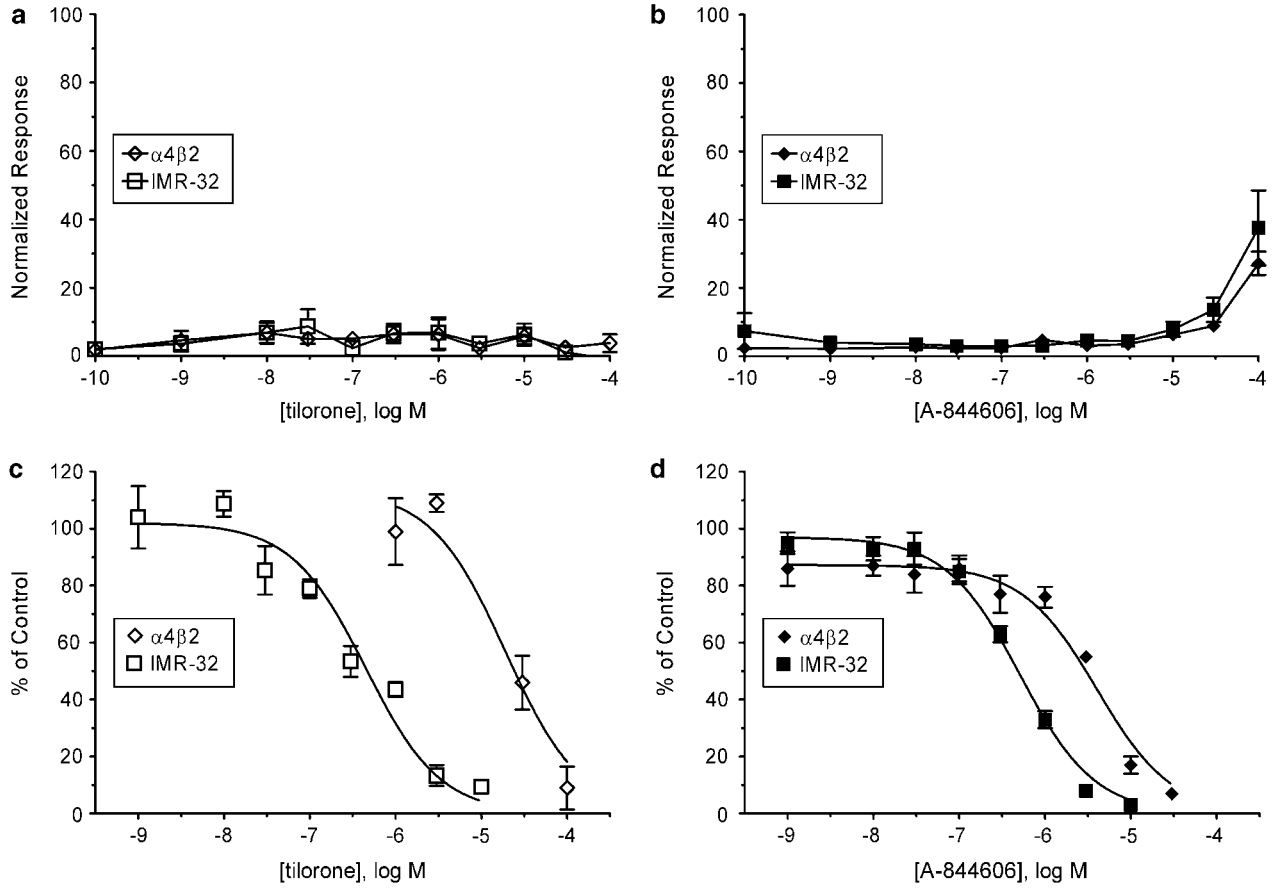

Figure 2 nAChR selectivity of tilorone and A-844606. Responses to tilorone (**a**, **c**) and A-844606 (**b**, **d**) were measured using a human α4β2 nAChR stably-transfected HEK293 cell line and in the human neuroblastoma IMR-32 using fluorescence light-imaging plate reader (FLIPR) Ca^{2+} imaging. Data from the agonist assay (**a**, **b**) ($n = 7$–11 per data point) were normalized to the response to 100 μM nicotine determined in each plate. A-844606 elicited a small apparent increase in fluorescence at 100 μM, whereas tilorone elicited no significant response. The compounds were also evaluated as antagonists of the response to 100 μM nicotine (**c**, **d**). Tilorone (**c**) inhibited α4β2 nAChR with an IC_{50} value of 19 μM (8.7–42 μM CI, $n = 3$) and IMR-32 α3* nAChR with an IC_{50} value of 0.45 μM (0.31–0.66 μM CI, $n = 6$). A-844606 (**d**) inhibited α4β2 nAChR with an IC_{50} value of 4.0 μM (3.0–5.3 μM CI, $n = 9$) and IMR-32 α3* nAChR with an IC_{50} value of 0.52 μM (0.41–0.65 μM CI, $n = 10$).

little agonist effect on human nAChR other than α7, as shown for α4β2 expressed in HEK293 (Figure 2a) and the native α3* expressed by IMR-32 cells (Figure 2b). Only at 100 μM was there an apparent small effect of A-844606 as an agonist. However, both compounds did weakly inhibit human α4β2 and α3* responses to nicotine (Figures 2c and d).

The selectivity of these compounds for activation of human α7 versus α4β2 nAChR was consistent with their selectivity for α7 versus α4β2 binding in rat brain membranes. For further confirmation, responses to tilorone (10 μM), A-844606 (10 μM) and ACh (100 μM) were measured in the human α4β2 HEK293 cell line using patch-clamp electrophysiology. In four cells responding to ACh, neither tilorone nor A-844606 elicited a measurable response (Figure 3).

To evaluate the abilities of tilorone and A-844606 to activate rat native α7 nAChR and associated signalling cascades, stimulation of ERK1/2 phosphorylation in PC12 cells was determined. As shown in Figure 4a, both tilorone and A-844606 stimulated ERK1/2 phosphorylation, with EC_{50} values of 0.59 and 0.047 μM, respectively. Compared with α7 nAChR responses measured electrophysiologically in oocytes, these EC_{50} values are lower and responses are larger because of the inclusion of an α7-selective positive allosteric modulator in the ERK1/2 assay. A-844606 was more potent than tilorone or nicotine and was similar in potency to a reference α7 nAChR agonist, PNU-282987, in this assay (Gubbins *et al.*, 2006).

Blockade experiments with MLA suggested that the increase in ERK1/2 phosphorylation was due to stimulation of α7 nAChR, at least in part (Figures 4b and c). A-844606 (1 μM) increased phospho-ERK1/2 to 95 ± 5% of the response to 1 μM PNU-282987 ($n = 3$). This was reduced to 58 ± 5% in the presence of 10 nM MLA, and 8 ± 0.9% in the presence of 50 nM MLA. Likewise, tilorone (10 μM) increased phospho-ERK1/2 to 83 ± 12% ($n = 3$) and this was reduced to 11 ± 11% in 10 nM MLA, and −1.0 ± 1.7% in 50 nM MLA. While MLA appeared somewhat less potent against A-844606 than against tilorone, it is not clear whether that is due to a non-α7 effect of A-844606 or to the larger response of rat α7 nAChR to A-844606 than to tilorone. In either case, the results suggest that A-844606 is a potent agonist at rat native α7 nAChR as well as recombinant human and rat α7 nAChR.

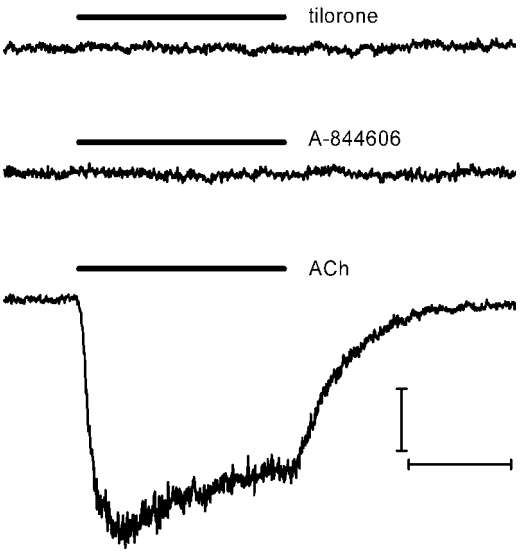

Figure 3 Human α4β2 nAChR response measured electrophysiologically. Whole-cell patch-clamp recordings were obtained from the HEK293 cell line also used in Figure 2. Tilorone (10 μM), A-844606 (10 μM) and ACh (100 μM) were applied to each of four cells. Reponses to ACh were 80, 130, 370 and 900 pA at peak amplitude, but no responses to tilorone or A-844606 could be detected. Sample traces from one cell are shown; scale bars represent 100 pA and 1 s.

Discussion

Screening of an in-house compound library identified tilorone as a novel ligand that bound and activated α7 nAChR. Surprisingly, tilorone has been known for over 30 years as an orally active interferon inducer (Levin and Albrecht, 1981). To our knowledge, never before has tilorone been evaluated as a ligand for a neurotransmitter receptor. The mechanism by which tilorone acts as an interferon inducer is not clear, and there is no direct evidence to implicate α7 nAChR in the process. However, from a structural perspective, tilorone was a novel α7 nAChR ligand. Lead optimization explored the structure–activity relationship around tilorone, leading to the identification of potent and selective analogues, as for example A-844606.

Further studies explored the selectivity and function of tilorone and related compounds at recombinant and native nAChR *in vitro*. Tilorone and analogues including A-844606 functioned as selective α7 nAChR agonists with little or no agonist activity at recombinant α4β2, α3β2 and α3β4 nAChR or at α3* nAChR expressed natively in IMR-32 neuroblastoma cells as measured by FLIPR. Failure to activate α4β2 was confirmed by patch-clamp recordings. Nicotine and numerous other compounds demonstrating potent binding to α4β2 sites in rat brain do activate human α4β2 nAChR and IMR-32 nAChR in the FLIPR assay (Frost *et al*., 2006; Bunnelle *et al*., 2007; Ji *et al*., 2007a, b). Rather, micromolar concentrations of tilorone and A-844606 inhibited α4β2 and IMR-32 nAChR, perhaps similar to the inhibition of β2-containing nAChR by other tricyclics (Connolly *et al*., 1992). Thus, interaction of these compounds with recombinant human α4β2 nAChR and with native IMR-32 nAChR was characterized by inhibition of the response to nicotine rather than activation.

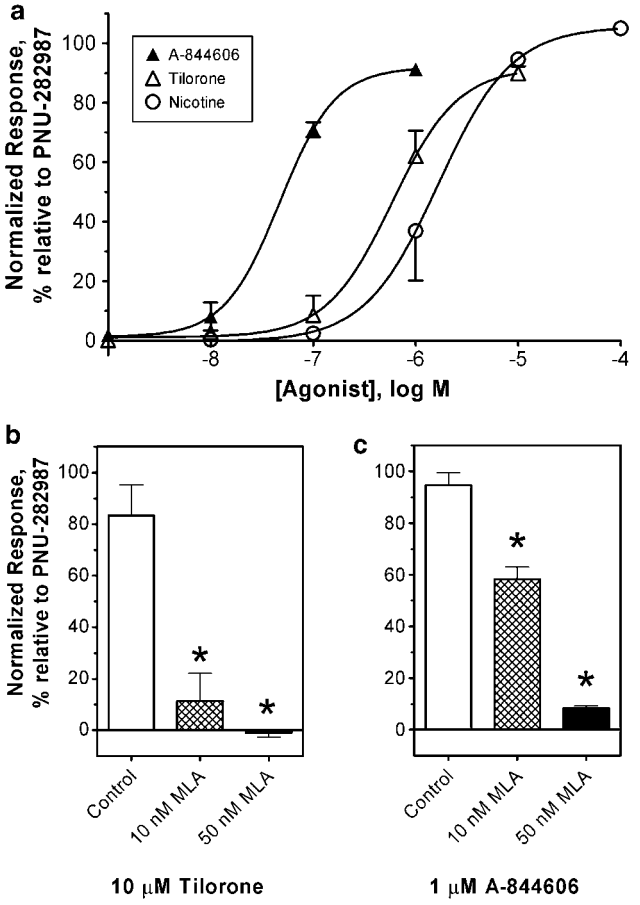

Figure 4 Tilorone and A-844606 activated rat native α7 nAChR. Stimulation of ERK1/2 phosphorylation in rat PC12 cells was used to determine activation of native α7 nAChR. Responses were normalized to the maximal response to PNU-282987 (1 μM). (**a**) Concentration–response parameters ($n=3$) for A-844606 were $EC_{50}=0.047$ μM (0.036–0.063 μM CI), plateau = 92 ± 3%, and Hill coefficient = 1.6 ± 0.2. For tilorone, these values were $EC_{50}=0.59$ μM (0.35–0.99 μM CI), plateau = 92 ± 6%, and Hill coefficient = 1.4 ± 0.4. For nicotine, these values were $EC_{50}=1.7$ μM (0.80–3.5 μM CI), plateau = 106 ± 8%, and Hill coefficient = 1.2 ± 0.5. (**b**, **c**) Inhibition of the responses to A-844606 and tilorone by MLA was measured in separate experiments ($n=3$). Responses were reduced by 10 nM and blocked by 50 nM MLA. *Data significantly ($P<0.01$, Dunnett's test) different from control response measured in the absence of MLA.

At α7 nAChR, the agonist potency of A-844606 was about 30-fold higher than that of (−)-nicotine and similar to that of epibatidine. Furthermore, tilorone and A-844606 were active as agonists in the native α7 nAChR ERK1/2 signalling expressed by PC12 cells as well as at recombinant α7 nAChR expressed in *Xenopus* oocytes and measured electrophysiologically. Thus, these compounds represent potent, efficacious and highly selective α7 nAChR agonists that could be used as tool compounds for comparison versus α7-selective agonists from other structural classes.

Tilorone displayed relatively lower maximal responses at rat compared with human α7 nAChR, limiting its potential utility in rodent behavioural models. Additionally, tilorone may have a number of nonspecific adverse effects, such as binding to glycosaminoglycans and DNA intercalation, at

high concentrations (Bispinck *et al*., 1998; Alcaro *et al*., 2004). Such effects are thought to be promoted by the amphiphilic structure of tilorone, with a central negative region and positive charge at each end of the molecule contributed by the tertiary amines protonated at physiological pH. Thus, A-844606 represents an optimized analogue as it is not only more potent than tilorone but also displayed no species selectivity between rat and human α7 nAChR activation and, with its one amine 'arm', is presumed to have reduced affinity for nonspecific sites.

Localization of α7 nAChR in hippocampus and cortex (Hogg *et al*., 2003; Alkondon and Albuquerque, 2004), high levels of α7 nAChR expression during brain development (Adams *et al*., 2002; Falk *et al*., 2003; Tribollet *et al*., 2004) and apparent neuroprotective properties of α7 nAChR stimulation *in vitro* (Belluardo *et al*., 2000; O'Neill *et al*., 2002) led to the idea that α7 nAChR may be involved in cognitive processing and perhaps synapse formation or stabilization. In α7 nAChR knockout animals, effects on behavioural performance and hippocampal anatomy were subtle, as revealed from initial studies (Orr-Urtreger *et al*., 1997). However, more recent studies have pointed to performance deficits in the α7 nAChR knockout animals (Young *et al*., 2006). In wild-type animals, nicotine and other nAChR agonists have been found to enhance cognitive performance, an effect that may involve α4β2 as well as α7 activation (Levin *et al*., 2002; Newhouse *et al*., 2004; Mazurov *et al*., 2006). GTS-21 also enhances cognitive performance, but this compound binds to α4β2 as well as α7 nAChR (de Fiebre *et al*., 1995; Briggs *et al*., 1997; Kem *et al*., 2004; Olincy *et al*., 2006). Pharmacological evidence to support the idea that α7 nAChR activation alone may be sufficient to enhance cognitive performance has come largely from the quinuclidine class of compounds. Thus, it will be of significant interest to determine whether α7 agonists from other structural classes are able to replicate the cognitive enhancement and other positive behavioural effects of the foregoing compounds.

Conflict of interest

This study was supported by Abbott Laboratories.

References

Adams CE, Broide RS, Chen YL, Winzer-Serhan UH, Henderson TA, Leslie FM *et al*. (2002). Development of the α7 nicotinic cholinergic receptor in rat hippocampal formation. *Brain Res Dev Brain Res* **139**: 175–187.

Alcaro S, Arena A, Neri S, Ottana R, Ortuso F, Pavone B *et al*. (2004). Design and synthesis of DNA-intercalating 9-fluoren-beta-*O*-glycosides as potential IFN-inducers, and antiviral and cytostatic agents. *Bioorg Med Chem* **12**: 1781–1791.

Alkondon M, Albuquerque EX (2004). The nicotinic acetylcholine receptor subtypes and their function in the hippocampus and cerebral cortex. *Prog Brain Res* **145**: 109–120.

Belluardo N, Mudo G, Blum M, Fuxe K (2000). Central nicotinic receptors, neurotrophic factors and neuroprotection. *Behav Brain Res* **113**: 21–34.

Bispinck F, Fischer J, Lullmann-Rauch R, von Witzendorff B (1998). Lysosomal glycosaminoglycan storage as induced by dicationic amphiphilic drugs: investigation into the mechanisms underlying the slow reversibility. *Toxicology* **128**: 91–100.

Biton B, Bergis OE, Galli F, Nedelec A, Lochead AW, Jegham S *et al*. (2007). SSR180711, a novel selective α7 nicotinic receptor partial agonist: (1) binding and functional profile. *Neuropsychopharmacology* **32**: 1–16.

Bodnar AL, Cortes-Burgos LA, Cook KK, Dinh DM, Groppi VE, Hajos M *et al*. (2005). Discovery and structure–activity relationship of quinuclidine benzamides as agonists of α7 nicotinic acetylcholine receptors. *J Med Chem* **48**: 905–908.

Boess FG, De Vry J, Erb C, Flessner T, Hendrix M, Luithle J *et al*. (2007). The novel α7 nicotinic acetylcholine receptor agonist *N*-[(3*R*)-1-azabicyclo[2.2.2]oct-3-yl]-7-[2-(methoxy)phenyl]-1-benzofuran-2-carboxamide improves working and recognition memory in rodents. *J Pharmacol Exp Ther* **321**: 716–725.

Briggs CA, Anderson DJ, Brioni JD, Buccafusco JJ, Buckley MJ, Campbell JE *et al*. (1997). Functional characterization of the novel neuronal nicotinic acetylcholine receptor ligand GTS-21 *in vitro* and *in vivo*. *Pharmacol Biochem Behav* **57**: 231–241.

Briggs CA, McKenna DG (1998). Activation and inhibition of the human α7 nicotinic acetylcholine receptor by agonists. *Neuropharmacology* **37**: 1095–1102.

Briggs CA, McKenna DG, Monteggia LM, Touma E, Roch J-M, Arneric SP *et al*. (1999). Gain of function mutation of the α7 nicotinic acetylcholine receptor: distinct pharmacology of the human α7V274T variant. *Eur J Pharmacol* **366**: 301–308.

Briggs CA, McKenna DG, Piattoni-Kaplan M (1995). Human α7 nicotinic acetylcholine receptor responses to novel ligands. *Neuropharmacology* **34**: 583–590.

Bunnelle WH, Daanen JF, Ryther KB, Schrimpf MR, Dart MJ, Gelain A *et al*. (2007). Structure–activity studies and analgesic efficacy of *N*-(3-pyridinyl)-bridged bicyclic diamines, exceptionally potent agonists at nicotinic acetylcholine receptors. *J Med Chem* **50**: 3627–3644.

Connolly J, Boulter J, Heinemann SF (1992). α4-2β2 and other nicotinic acetylcholine receptor subtypes as targets of psychoactive and addictive drugs. *Br J Pharmacol* **105**: 657–666.

D'Andrea MR, Nagle RG (2006). Targeting the α7 nicotinic acetylcholine receptor to reduce amyloid accumulation in Alzheimer's disease pyramidal neurons. *Curr Pharm Des* **12**: 677–684.

Davies ARL, Hardick DJ, Blagbrough IS, Potter BVL, Wolstenholme AJ, Wonnacott S (1999). Characterisation of the binding of [^{3}H]methyllycaconitine: a new radioligand for labelling α7-type neuronal nicotinic acetylcholine receptors. *Neuropharmacology* **38**: 679–690.

de Fiebre CM, Meyer EM, Henry JC, Muraskin SI, Kem WR, Papke RL (1995). Characterization of a series of anabaseine-derived compounds reveals that the 3-(4)-dimethylaminocinnamylidine derivative is a selective agonist at neuronal nicotinic α7/^{125}I-α-bungarotoxin receptor subtypes. *Mol Pharmacol* **47**: 164–171.

Falk L, Nordberg A, Seiger A, Kjaeldgaard A, Hellstrom-Lindahl E (2003). Higher expression of α7 nicotinic acetylcholine receptors in human fetal compared to adult brain. *Brain Res Dev Brain Res* **142**: 151–160.

Frost JM, Bunnelle WH, Tietje KR, Anderson DJ, Rueter LE, Curzon P *et al*. (2006). Synthesis and structure–activity relationships of 3,8-diazabicyclo[4.2.0]octane ligands, potent nicotinic acetylcholine receptor agonists. *J Med Chem* **49**: 7843–7853.

Gahring LC, Rogers SW (2006). Neuronal nicotinic acetylcholine receptor expression and function on nonneuronal cells. *AAPS J* **7**: E885–E894.

Grønlien JH, Haakerud M, Ween H, Thorin-Hagene K, Briggs CA, Gopalakrishnan M *et al*. (2007). Distinct profiles of α7 nAChR positive allosteric modulation revealed by structurally diverse chemotypes. *Mol Pharmacol* **72**: 715–724.

Gubbins EJ, Gopalakrishnan M, Li J (2006). α7 neuronal nicotinic acetylcholine receptor mediated activation of MAP kinase pathways. *Abstr Soc Neurosci* **32** no. 325.22.

Heeschen C, Weis M, Aicher A, Dimmeler S, Cooke JP (2002). A novel angiogenic pathway mediated by non-neuronal nicotinic acetylcholine receptors. *J Clin Invest* **110**: 527–536.

Hogg RC, Raggenbass M, Bertrand D (2003). Nicotinic acetylcholine receptors: from structure to brain function. *Rev Physiol Biochem Pharmacol* **147**: 1–46.

Hurst RS, Hajos M, Raggenbass M, Wall TM, Higdon NR, Lawson JA *et al*. (2005). A novel positive allosteric modulator of the α7 neuronal nicotinic acetylcholine receptor: *in vitro* and *in vivo* characterization. *J Neurosci* **25**: 4396–4405.

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

Ji J, Schrimpf MR, Sippy KB, Bunnelle WH, Li T, Anderson DJ *et al*. (2007a). Synthesis and structure–activity relationship studies of 3,6-diazabicyclo[3.2.0]heptanes as novel α4β2 nicotinic acetylcholine receptor selective agonists. *J Med Chem* **50**: 5493–5508.

Ji J, Bunnelle WH, Anderson DJ, Faltynek C, Dyhring T, Ahring PK *et al*. (2007b). 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.

Kem WR (2000). The brain α7 nicotinic receptor may be an important therapeutic target for the treatment of Alzheimer's disease: studies with DMXBA (GTS-21). *Behav Brain Res* **113**: 169–181.

Kem WR, Mahnir VM, Prokai L, Papke RL, Cao X, LeFrancois S *et al*. (2004). Hydroxy metabolites of the Alzheimer's drug candidate 3-[(2,4-dimethoxy)benzylidene]-anabaseine dihydrochloride (GTS-21): their molecular properties, interactions with brain nicotinic receptors, and brain penetration. *Mol Pharmacol* **65**: 56–67.

Krueger RE, Mayer GD (1970). Tilorone hydrochloride: an orally active antiviral agent. *Science* **169**: 1213–1214.

Levin ED, Bradley A, Addy N, Sigurani N (2002). Hippocampal α7 and α4β2 nicotinic receptors and working memory. *Neuroscience* **109**: 757–765.

Levin RH, Albrecht WL (1981). Tilorone and related bis-basic substituted polycyclic aromatic and heteroaromatic compounds. *Prog Med Chem* **18**: 135–190.

Martin LF, Kem WR, Freedman R (2004). α7 nicotinic receptor agonists: potential new candidates for the treatment of schizophrenia. *Psychopharmacology (Berl)* **174**: 54–64.

Mayer GD, Krueger RF (1970). Tilorone hydrochloride: mode of action. *Science* **169**: 1214–1215.

Mazurov A, Hauser T, Miller CH (2006). Selective α7 nicotinic acetylcholine receptor ligands. *Curr Med Chem* **13**: 1567–1584.

Meyer EM, Kuryatov A, Gerzanich V, Lindstrom J, Papke RL (1998). Analysis of 3-(4-hydroxy, 2-methoxybenzylidene)anabaseine selectivity and activity at human and rat α7 nicotinic receptors. *J Pharmacol Exp Ther* **287**: 918–925.

Newhouse PA, Potter A, Singh A (2004). Effects of nicotinic stimulation on cognitive performance. *Curr Opin Pharmacol* **4**: 36–46.

O'Neill MJ, Murray TK, Lakics V, Visanji NP, Duty S (2002). The role of neuronal nicotinic acetylcholine receptors in acute and chronic neurodegeneration. *Curr Drug Targets CNS Neurol Disord* **1**: 399–411.

Olincy A, Harris JG, Johnson LL, Pender V, Kongs S, Allensworth D *et al*. (2006). Proof-of-concept trial of an α7 nicotinic agonist in schizophrenia. *Arch Gen Psych* **63**: 630–638.

Orr-Urtreger A, Göldner FM, Saeki M, Lorenzo I, Goldberg L, De Biasi M *et al*. (1997). Mice deficient in the α7 neuronal nicotinic acetylcholine receptor lack α-bungarotoxin binding sites and hippocampal fast nicotinic currents. *J Neurosci* **17**: 9165–9171.

Pabreza LA, Dhawan S, Kellar KJ (1991). [^{3}H]Cytisine binding to nicotinic cholinergic receptors in brain. *Mol Pharmacol* **39**: 9–12.

Papke RL, Meyer EM, Lavieri S, Bollampally SR, Papke TAS, Horenstein NA *et al*. (2004). Effects at a distance in α7 nAChR selective agonists: benzylidene substitutions that regulate potency and efficacy. *Neuropharmacology* **46**: 1023–1038.

Pichat P, Bergis OE, Terranova JP, Urani A, Duarte C, Santucci V *et al*. (2007). SSR180711, a novel selective α7 nicotinic receptor partial agonist: (II) efficacy in experimental models predictive of activity against cognitive symptoms of schizophrenia. *Neuropsychopharmacology* **32**: 17–34.

Tatsumi R, Fujio M, Takanashi S, Numata A, Katayama J, Satoh H *et al*. (2006). (*R*)-3′-(3-methylbenzo[*b*]thiophen-5-yl)spiro[1-azabicyclo[2,2,2]octane-3,5′-oxazolidin]-2′-one, a novel and potent α7 nicotinic acetylcholine receptor partial agonist displays cognitive enhancing properties. *J Med Chem* **49**: 4374–4383.

Tracey KJ (2005). Fat meets the cholinergic antiinflammatory pathway. *J Exp Med* **202**: 1017–1021.

Tribollet E, Bertrand D, Marguerat A, Raggenbass M (2004). Comparative distribution of nicotinic receptor subtypes during development, adulthood and aging: an autoradiographic study in the rat brain. *Neuroscience* **124**: 405–420.

Trumbull JD, Maslana ES, McKenna DG, Nemcek TA, Niforatos W, Pan JY *et al*. (2003). High throughput electrophysiology using a fully automated, multiplexed recording system. *Receptors Channels* **9**: 19–28.

Ulloa L (2005). The vagus nerve and the nicotinic anti-inflammatory pathway. *Nat Rev Drug Discov* **4**: 673–684.

Van Kampen M, Selbach K, Schneider R, Schiegel E, Boess F, Schreiber R (2004). AR-R 17779 improves social recognition in rats by activation of nicotinic α7 receptors. *Psychopharmacology (Berl)* **172**: 375–383.

Wishka DG, Walker DP, Yates KM, Reitz SC, Jia S, Myers JK *et al*. (2006). Discovery of *N*-[(3*r*)-1-azabicyclo[2.2.2]oct-3-yl]furo[2,3-*c*]pyridine-5-carboxamide, an agonist of the α7 nicotinic acetylcholine receptor, for the potential treatment of cognitive deficits in schizophrenia: synthesis and structure–activity relationship. *J Med Chem* **49**: 4425–4436.

Young JW, Crawford N, Kelly JS, Kerr LE, Marston HM, Spratt C *et al*. (2006). Impaired attention is central to the cognitive deficits observed in α7 deficient mice. *Eur Neuropsychopharmacol* **17**: 145–155.