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EVP-6124, a novel and selective α7 nicotinic acetylcholine receptor partial agonist, improves memory performance by potentiating the acetylcholine response of α7 nicotinic acetylcholine receptors

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ABSTRACT

EVP-6124, (*R*)-7-chloro-*N*-quinuclidin-3-yl)benzo[b]thiophene-2-carboxamide, is a novel partial agonist of α7 neuronal nicotinic acetylcholine receptors (nAChRs) that was evaluated here *in vitro* and *in vivo*. In binding and functional experiments, EVP-6124 showed selectivity for α7 nAChRs and did not activate or inhibit heteromeric α4β2 nAChRs. EVP-6124 had good brain penetration and an adequate exposure time. EVP-6124 (0.3 mg/kg, p.o.) significantly restored memory function in scopolamine-treated rats (0.1 mg/kg, i.p.) in an object recognition task (ORT). Although donepezil at 0.1 mg/kg, p.o. or EVP-6124 at 0.03 mg/kg, p.o. did not improve memory in this task, co-administration of these sub-efficacious doses fully restored memory. In a natural forgetting test, an ORT with a 24 h retention time, EVP-6124 improved memory at 0.3 mg/kg, p.o. This improvement was blocked by the selective α7 nAChR antagonist methyllycaconitine (0.3 mg/kg, i.p. or 10 μg, i.c.v.). In co-application experiments of EVP-6124 with acetylcholine, sustained exposure to EVP-6124 in functional investigations in oocytes caused desensitization at concentrations greater than 3 nM, while lower concentrations (0.3–1 nM) caused an increase in the acetylcholine-evoked response. These actions were interpreted as representing a co-agonist activity

Abbreviations: A-582941, 2-methyl-5-(6-phenyl-pyridazin-3-yl)-octahydro-pyrrolo[3,4-c]pyrrole; $A\beta_{1-40}$, amyloid peptide; ABBF, *N*-[(3*R*)-1-azabicyclo[2.2.2]oct-3-yl]-7-[2-(methoxy)phenyl]-1-benzofuran-2-carboxamide; ABT-107, 5-(6-[(3*R*)-1-azabicyclo[2,2,2]oct-3-yloxy]pyridazin-3-yl)-1*H*-indole; ACh, acetylcholine; AChEI, acetylcholine esterase inhibitor; AD, Alzheimer's disease; AR-R17779, (−)-spiro[1-azabicyclo[2.2.2]octane-3,5′-oxazolidin-2′-one]; BSA, bovine serum albumin; AZD0328, (2′*R*)-spiro-[1-azabicyclo[2.2.2]octane-3,2′(3′H)-furo[2,3-b]pyridine] D-tartrate; B:P, brain to plasma; CREB, cAMP response element-binding protein; d2, discrimination during trial 2; ERK, extracellular-signal regulated kinase; EVP-6124, (*R*)-7-chloro-*N*-quinuclidin-3-yl)benzo[b]thiophene-2-carboxamide; fu, fraction unbound; GABA, γ-aminobutyric acid; GR65630, 3-(5-methyl-1*H*-imidazol-4-yl)-1-(1-methyl-1*H*-indol-3-yl)-1-propanone; GTS-21, 3-(2,4-dimethoxybenzylidene)anabaseine, (DMXB); 5-HT, 5-hydroxytryptamine; HTS, high-throughput screening; i.c.v., intracerebroventricular; LC-MS/MS, liquid chromatography and tandem mass spectrometry; LTP, long-term potentiation; MAPK, mitogen-activated protein kinase; MLA, methyllycaconitine; nAChR, nicotinic acetylcholine receptor; NMDA, *N*-methyl-D-aspartate; ORT, object recognition task; PBS, phosphate buffered saline; PC, sample concentration in protein-containing side; PF, sample concentration in protein free-side; PHA-543613, *N*-[(3*R*)-1-azabicyclo[2.2.2]oct-3-yl]furo[2,3-c]pyridine-5-carboxamide; PNU-282987, *N*-[(3*R*)-1-azabicyclo[2.2.2]oct-3-yl]-4-chlorobenzamide hydrochloride; RG3487, *N*-[(3*S*)-1-azabicyclo[2.2.2]oct-3-yl}-1*H*-indazole-3-carboxamide hydrochloride; RS-102221, *N*-{5-[5-(2,4-dioxo-1,3,8-triazaspiro[4.5]dec-8-yl)pentanoyl]-2,4-dimethoxyphenyl}-4-(trifluoromethyl)benzenesulfonamide; S 24795, 2-[2-(4-bromophenyl)-2-oxoethyl]-1-methyl pyridinium; SSR180711, 1,4-diazabicyclo(3.2.2)nonane-4-carboxylic acid, 4-bromophenyl ester; T1, trial 1; T2, trial 2; TC-5619, *N*-[2-(pyridin-3-ylmethyl)-1-azabicyclo[2.2.2]oct-3-yl]-1-benzofuran-2-carboxamide; T_{max}, time of maximal concentration.

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of EVP-6124 with acetylcholine on α7 nAChRs. The concentrations of EVP-6124 that resulted in physiological potentiation were consistent with the free drug concentrations in brain that improved memory performance in the ORT. These data suggest that the selective partial agonist EVP-6124 improves memory performance by potentiating the acetylcholine response of α7 nAChRs and support new therapeutic strategies for the treatment of cognitive impairment.

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1. Introduction

Nicotine has been shown to improve attention, learning, and memory through interaction with neuronal nAChRs (Levin et al., 2006). Several subtypes of nAChRs are expressed in the mammalian brain, each of them displaying distinct physiological and pharmacological properties. Functional nAChRs are assembled from five subunits around an axis of pseudosymmetry and can be composed of identical subunits (homopentamers) or different subunits (heteropentamers) (Dani and Bertrand, 2007). The α4 and β2 subunits are thought to form high affinity brain nAChRs, whereas the homopentameric α7 nAChR, which is expressed throughout the entire central nervous system, is less sensitive to ACh and nicotine (Albuquerque et al., 2009; Dani and Bertrand, 2007).

The α7 nAChRs are highly expressed in the hippocampus, a brain region that is very important for the formation of several types of memory. Hippocampal LTP, the sustained increase in the efficiency of synaptic transmission that is induced by multiple high frequency trains of electrical stimulation, is a potential cellular mechanism for learning and memory. In rat and mouse hippocampal slices, the partial α7 nAChR agonists GTS-21, SSR180711, and S 24795 improved LTP (Biton et al., 2007; Hunter et al., 1994; Lagostena et al., 2008). These effects were blocked by co-application of the α7 nAChR antagonist MLA and were absent in α7 nAChR knock-out mice (Lagostena et al., 2008). Furthermore, activation of the MAPK signaling pathway, with phosphorylation of ERK and CREB, is linked to the establishment of LTP and the formation of long-term memory. The α7 nAChR agonists A-582941 and ABT-107 increased phosphorylation of ERK and CREB in the mouse brain (Bitner et al., 2007, 2010).

Agonists of α7 nAChRs improved performance in learning and memory tasks (for review, see Kem, 2000). GTS-21 improved inhibitory avoidance responding, one-way active avoidance, as well as performance in the Lashley III maze and the 17-arm radial maze in rats (Arendash et al., 1995; Meyer et al., 1994). In addition, GTS-21 facilitated performance in a delayed-matching-to-sample test in monkeys (Briggs et al., 1997). In clinical trials with healthy volunteers, GTS-21 improved attention, working memory, and episodic memory (Kitagawa et al., 2003). In a randomized double-blind crossover trial in nonsmoking subjects with schizophrenia, stably treated with antipsychotics, GTS-21 caused significant cognitive improvement on the Repeatable Battery for the Assessment of Neuropsychological Status total scale (Olincy et al., 2006). GTS-21 is a weak partial agonist of human α7 nAChRs and inhibits α4β2 nAChRs and 5-HT_3 receptors (Briggs et al., 1997). The more selective α7 nAChR agonist, AR-R17779, improved long-term win-shift acquisition in the eight-arm radial maze and social recognition memory in rats (Levin et al., 1999; van Kampen et al., 2004). Improvements in social recognition, object recognition, and water maze performance were observed with several quinuclidine amide α7 nAChR agonists (Boess et al., 2007; Hauser et al., 2009; Wallace et al., 2011; Wishka et al., 2006), as well as with a number of other α7 nAChR agonists, including other types of quinuclidines (Bitner et al., 2010; Feuerbach et al., 2009; Pichat et al., 2007; Sydserff et al., 2009) and novel, structurally unrelated compounds (Bitner et al., 2007; Briggs et al., 2009; Roncarati et al., 2009). These cognitive enhancing effects were blocked by MLA (Boess et al., 2007; Pichat et al., 2007; van Kampen et al., 2004; Wallace et al., 2011), but were maintained by the co-infusion of donepezil, an AChEI (Bitner et al., 2010).

In this work, we examined the physiological and pharmacological properties of a novel and selective quinuclidine amide α7 nAChR agonist, EVP-6124, and its effects on memory in the ORT. Memory loss was either pharmacologically induced (i.e. disruption of the cholinergic system by administration of the muscarinic antagonist scopolamine) or was natural (i.e. a 24 h retention interval). Furthermore, EVP-6124 was co-administered with MLA (i.p. or i.c.v.) to investigate whether the pro-cognitive effects of EVP-6124 could be antagonized. Since α7 nAChR agonists have been suggested for the treatment of AD, and AD patients are often treated with an AChEI, the potential beneficial interaction between AChEIs and EVP-6124 was investigated in the present study at the behavioral level in rodents and at the electrophysiological level in oocytes expressing human α7 nAChRs.

2. Material and methods

2.1. Reagents

EVP-6124 was synthesized by Bayer Healthcare AG (Wuppertal-Elberfeld, Germany) and Ricerca Biosciences (Concord, OH). MLA and scopolamine hydrobromide were obtained from Research Biochemicals International/Sigma-Aldrich (Deisenhofen, Germany). Donepezil was a generous gift from Solvay Pharmaceuticals (Weesp, The Netherlands). Reagents for binding and electrophysiological studies were from Sigma-Aldrich. Reagents used in pharmacokinetic studies were from VWR International, Ltd. (Poole, Dorset, UK).

2.2. Animals

All animal experiments were approved by local ethical committees, followed the principles of laboratory animal care, and were in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC), or the Guide for the Care and Use of Laboratory Animals from the U.S. Department of the Health and Human Services. All efforts were made to minimize animal suffering, to reduce the number of animals used, and to use alternatives to *in vivo* methods where possible.

2.3. Selectivity profiling

Binding or activity of EVP-6124 was measured at 10 μM in a selectivity panel according to standard validated protocols under conditions defined by the contractor (MDS Pharma Services, Taipei, Taiwan; http://www.mdsps.com). Reference standards were run as an integral part of each assay to ensure the validity of the results.

For the 5-HT_{2A} receptor binding assay, membranes were prepared from HEK293 cells expressing the human recombinant 5-HT_{2A} receptor. For 5-HT_{2B} and 5-HT_{2C} receptor binding assays, membranes were prepared from CHO cells expressing the human recombinant 5-HT_{2B} or 5-HT_{2C} receptor. Affinity was determined by incubating different concentrations of EVP-6124 in binding buffer for 1 h. For 5-HT_{2A} binding, the incubation was at 22 °C in the presence of 0.5 nM [^{3}H]-ketanserin; for 5-HT_{2B}, at 22 °C in the presence of 2 nM [^{3}H]-mesulergine; and for 5-HT_{2C}, at 37 °C in the presence of 1 nM [^{3}H]-mesulergine. Nonspecific binding was determined in the presence of 1 μM ketanserin, 10 μM mesulergine, or 10 μM RS-102221 for 5-HT_{2A}, 5-HT_{2B}, or 5-HT_{2C}, respectively. All measurements were performed in triplicate. EVP-6124 was also tested in the 5-HT_{2B} rat gastric fundus tissue response assay according to standard protocols under conditions defined by the contractor (MDS Pharma Services). Briefly, inhibition of α-methyl serotonin-induced contraction was isometrically measured. All measurements were performed in duplicate.

2.4. Binding assays

Brains from male Sprague-Dawley rats were rapidly removed and placed in ice cold homogenization buffer (10% w/v 0.32 M sucrose, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, and 0.01% w/v NaN_3, pH 7.4, 4 °C). Brains were homogenized at 600 rpm in a Potter glass Teflon® homogenizer. The resulting suspension was centrifuged (1000 × g, 4 °C, 10 min) and the supernatant was collected. The pellet was resuspended in homogenization buffer (20% w/v) and the suspension was recentrifuged (1000 × g, 4 °C, 10 min). Both supernatants were combined and centrifuged (15,000 × g, 4 °C, 30 min). The resulting pellet (P2 fraction) was resuspended in binding buffer (50 mM Tris–HCl, 1 mM $MgCl_2$, 120 mM NaCl, 5 mM KCl, and 2 mM $CaCl_2$, pH 7.4) and centrifuged (15,000 × g, 4 °C, 30 min). The resuspension and centrifugation were repeated once.

For the α7 nAChR assay, the final pellet was resuspended in binding buffer and incubated in a final volume of 250 μl (0.2 mg membrane protein per assay) in the presence of 2 nM [^{3}H]-MLA, 0.1% w/v BSA and different concentrations of the test substance for 2 hat 25 °C. Nonspecific binding was determined in the presence of 10 μM MLA. The incubation was terminated by the addition of 4 ml PBS (20 mM Na_2HPO_4, 5 mM KH_2PO_4, 150 mM NaCl, pH 7.4, 4 °C) and rapid filtration using a cell harvester (Brandel, Inc., Gaithersburg, MD) and type A/E glass fiber filters (Gelman Sciences, Inc., Ann Arbor, MI), pretreated for 3 h with 0.3% v/v polyethylenimine. Filters were washed twice with 4 ml PBS (4 °C) and bound [^{3}H]-MLA was determined by scintillation counting. All measurements were performed in triplicate.

To determine the affinity of the compound at rat brain α4β2 nAChRs, different concentrations of EVP-6124 were incubated in a final volume of 250 μl binding buffer for 2 h at 4 °C in the presence of 2 nM [^{3}H]-cytisine. Nonspecific binding was determined in the presence of 10 μM nicotine. All measurements were performed in triplicate.

The 5-HT_3 receptor is a homolog of the α7 nAChR and is often potently antagonized by α7 nAChR agonists (Bodnar et al., 2005; Briggs et al., 1997; Sydserff et al., 2009; Wallace et al., 2011; Wishka et al., 2006). For 5-HT_3 receptor binding assays, membranes from HEK293 cells expressing human recombinant 5-HT_3 receptor (RB-HS3, Receptor Biology, Inc., Beltsville, MD) were diluted according to manufacturer's instructions in incubation buffer (50 mM Tris-base, pH 7.4, 5 mM $MgCl_2$, 0.5 mM EDTA, 0.1% ascorbic acid, 10 μM pargyline) and incubated in a volume of 200 μl (membrane protein concentration: 3 μg per assay) for 60 min at 21 °C in the presence of 0.5 nM of the selective 5-HT_3 receptor radioligand [^{3}H]-GR65630 and different concentrations of EVP-6124. Nonspecific binding was determined in the presence of 100 μM 5-HT. The incubation was terminated by filtration through type A/E glass fiber filters (Gelman Sciences, Inc.) or GF/B filters (Whatman, Maidstone, UK) that were pretreated for at least 1 h with 0.3% v/v polyethylenimine. Filters were washed three times with 3 ml buffer (50 mM Tris–HCl, pH 7.4; 4 °C) and bound radioactivity was determined by scintillation counting. All measurements were performed in triplicate.

The IC_{50} values were determined from plots of binding activity versus log compound concentration using a sigmoidal curve fit (Prism, v. 2.0, GraphPad Software Inc., San Diego, CA). The dissociation constants K_i of test compounds were determined from their IC_{50} values, the dissociation constants K_D, and the concentrations L of [^{3}H]-MLA, [^{3}H]-cytisine or [^{3}H]-GR65630 as appropriate, using the equation $K_i = IC_{50}/(1 + L/K_D)$.

2.5. Electrophysiological recordings

Experiments were carried out with human α7 nAChRs expressed in *Xenopus laevis* oocytes. Oocytes were prepared, injected with cDNA encoding α7 nAChR subunits, and recorded using standard procedures (Hogg et al., 2008). Additional studies were carried out with rat α3β4, α4β2, and muscle α1β1γδ nAChRs expressed in oocytes. Briefly, ovaries were harvested from *X. laevis* females that were deeply anesthetized by cooling at 4 °C and with tricaine mesylate (3-aminobenzoic acid ethyl ester, methane sulfonate salt, 150 mg/l). Small pieces of ovary were isolated in sterile Barth solution (88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 10 mM HEPES, 0.82 mM $MgSO_4 \cdot 7H_2O$, 0.33 mM $Ca(NO_3)_2 \cdot 4H_2O$, and 0.41 mM $CaCl_2 \cdot 6H_2O$, pH 7.4) and supplemented with 20 μg/ml kanamycin, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Injections of cDNAs encoding for the receptors were performed in at least one hundred oocytes using an automated injection device (Roboinject, Multi Channel Systems, Reutlingen, Germany); and receptor expression was examined at least two days later. Oocytes were impaled with two electrodes filled with 3 M KCl, and their membrane potentials were maintained at −80 mV throughout the experiment. All recordings were performed at 18 °C and cells were superfused with OR2 medium (82.5 mM NaCl, 2.5 mM KCl, 5 mM HEPES, 1.8 mM $CaCl_2 \cdot 2H_2O$, and 1 mM $MgCl_2 \cdot 6H_2O$, pH 7.4). Currents were recorded using an automated process equipped with standard two-electrode voltage-clamp configuration (HiClamp, Multi Channel Systems). Data were captured and analyzed using Matlab (Mathworks, Inc., Natick, MA) or Excel (Microsoft, Redmond, WA) software. ACh and EVP-6124 were prepared as concentrated stock solutions in water and then diluted in the recording medium to obtain the desired test concentrations. All experiments were carried out using three or more cells.

2.6. Object recognition task

2.6.1. Animals

In the dose–response experiment assessing the effect of EVP-6124 on a scopolamine-induced deficit, twenty-four 2.5-month-old male Wistar rats (Harlan Laboratories, Inc., Horst, The Netherlands; average body weight: 329 g) were used. Each rat was tested with 4 treatments (including the control conditions). In the ORT combination study of EVP-6124 and donepezil, another cohort of twenty-four 5-month-old male Wistar rats was used (Harlan; average body weight: 465 g), of which 23 animals were included in the final analysis due to the continuous escaping of one rat from the apparatus. All 23 rats received each treatment, i.e. were tested 5 times. For the experiments using natural forgetting (i.e. a 24 h retention interval) and MLA, twenty-four 2-month-old male Wistar rats were obtained from Charles River Laboratories International, Inc. (Sulzfeld, Germany). In the dose–response experiment, the rats were 2.5 months old (average body weight: 319 g). For the co-administration of EVP-6124 and MLA (i.p.), the rats were 3 months old (average body weight: 357 g). For the co-administration of EVP-6124 and MLA (i.c.v.), the rats were 4 months old (average body weight: 407 g). Across the 3 experiments, rats were tested a total of 5–10 times. The effects of EVP-6124 on memory consolidation in particular were investigated in the natural forgetting test in the ORT, using twenty-four 3-month-old Wistar rats (Harlan Laboratories, Inc.; average body weight: 357 g). All 24 rats received each treatment, i.e. each rat was tested 7 times in two experiments. All animals were housed individually, which improves ORT performance (Beck and Luine, 2002), in standard type III Makrolon cages on sawdust bedding. The animals were on a reversed 12/12-h light/dark cycle (lights on from 19:00 to 7:00 h); and food and water were given *ad libitum*. The rats were housed and tested in the same room. A radio, playing softly provided background noise to mask noises in the room. All testing was performed between 9:00 and 18:00 h under low illumination (20 lux).

2.6.2. Object recognition memory task

The ORT was performed as described elsewhere (Ennaceur and Delacour, 1988; Prickaerts et al., 1997). The apparatus and objects are identical to those described previously (Rutten et al., 2007). The same four objects were used during adaptation and in the studies. For two weeks, the animals were handled daily and adapted to the test procedures. For two days, the rats were allowed to explore the apparatus with no objects present, twice for 3 min each day. Afterward, animals were adapted to the testing sessions and treatments with saline injections (i.p., p.o., and i.c.v.), until stable discrimination performance was achieved at the 1 and 24 h retention intervals.

A testing session consisted of two trials (T1 and T2), each with a duration of 3 min. The times spent exploring each object during T1 and T2 were recorded manually with a personal computer. Object exploration was defined as directing the nose to the object at a distance of not more than 2 cm or touching the object with the nose. During T1, the apparatus contained two identical objects. After T1, the animal was returned to its home cage. After a retention interval of 1 h (scopolamine-induced short-term memory deficit) or 24 h (natural forgetting), the animal was returned to the apparatus for T2. In T2, one of the two familiar objects was replaced by a new object. All objects and locations were used in a balanced manner to exclude possible object and/or location preferences. To avoid olfactory cues, the objects were thoroughly cleaned with 70% ethanol after each trial. The testing order of treatments was determined randomly and several treatment groups were tested each day ($n = 8$ per treatment group per testing day). The experimenter was blind to the treatments. Because rats were retested with different compound doses, test sessions were scheduled to allow at least a two day washout period.

2.6.3. Drug administration

EVP-6124 was prepared in deionized water at an injection volume of 2 ml/kg. Scopolamine was prepared in saline at an injection volume of 1 ml/kg, with doses based on the weight of the salt. Donepezil was dissolved in saline at an injection volume of 2 ml/kg. For i.p. administration, MLA was dissolved in deionized water at an injection volume of 1 ml/kg. MLA was dissolved in saline for i.c.v. administration.

2.6.4. ORT study designs

2.6.4.1. Dose–response effect of EVP-6124 on a scopolamine-induced deficit. Before testing EVP-6124, the effects of scopolamine alone at 0.03, 0.1, or 0.3 mg/kg, i.p. in the ORT were determined ($n = 8$ per treatment). Scopolamine (0.1 mg/kg, i.p.) injected 30 min before T1 resulted in a robust deficit at T2 when a 1 h interval was used (data not shown). The d2 index was not significantly different from the chance level of performance; and there were no changes in exploratory behavior for 0.1 mg/kg, i.p. of scopolamine compared with saline. Subsequently, the ability of EVP-6124 to reverse the memory impairment induced by 0.1 mg/kg of scopolamine was tested. First, scopolamine and then EVP-6124 (0.03, 0.1, 0.3, and 1.0 mg/kg, p.o.) were administered 30 min before T1. For the control treatments, animals received either deionized water (p.o.) plus saline (i.p.) or deionized water (p.o.) plus 0.1 mg/kg scopolamine (i.p.).

2.6.4.2. Effect of EVP-6124 and donepezil in combination on a scopolamine-induced deficit. The AChEI, donepezil, was used to elevate ACh levels and was tested in

combination with EVP-6124. Sub-efficacious doses of EVP-6124 (0.03 mg/kg) and donepezil were administered p.o. A 1 h retention interval between T1 and T2 was used with the scopolamine-induced deficit (0.1 mg/kg, i.p.). In a previous study we found that 0.1 mg/kg of donepezil was the highest dose with no effect on a scopolamine-induced memory deficit (data not shown), and hence was considered to be the sub-efficacious dose for this compound. The order of compound administration was scopolamine, EVP-6124, and donepezil, 30 min before T1.

2.6.4.3. Dose–response effect of EVP-6124 on natural forgetting. Untreated Wistar rats show no significant object memory after 24 h (Rutten et al., 2007). The effect of EVP-6124 on long-term memory was investigated using a 24 h interval between T1 and T2. EVP-6124 was tested at 0.1, 0.3, and 1.0 mg/kg, p.o., administered 30 min before T1.

2.6.4.4. Effect of EVP-6124 and an α7 nAChR antagonist on natural forgetting. In order to determine whether the pro-cognitive effects of EVP-6124 (0.3 mg/kg, p.o.) on natural forgetting could be antagonized, MLA was co-administered. MLA was administered 1 h before T1 and EVP-6124, 30 min before T1. The optimal dose of EVP-6124 in the 24 h retention interval study was 0.3 mg/kg, and was used in combination with 0.1, 0.3, and 1.0 mg/kg, i.p. of MLA.

An additional MLA experiment was conducted via i.c.v. administration in 21 cannulated rats to confirm that the action of EVP-6124 was mediated through centrally located α7 nAChRs. Rats were fully anesthetized with isoflurane (induction: 5% and maintenance: 2%), were fixed in a stereotaxic frame, and a cannula was placed above the left lateral ventricle at the following coordinates with reference to bregma: 0.8 mm posterior, 1.55 mm lateral and 3.8 mm ventral (Paxinos and Watson, 1996). The tip of the cannula ended 1.0 mm above the lateral ventricle. The cannula was affixed to the skull using acrylic dental cement and two small screws. Animals were allowed to recuperate for two weeks before the adaptation and drug testing procedures started.

An injection needle, that was 1.0 mm longer than the guide cannula, was inserted into the cannula to deliver MLA (3 or 10 μg, i.c.v.) into the lateral ventricle in an injection volume of 2 μl (1 μl/min), 4 min before T1. The needle was left in place for an additional minute to prevent the reflux of infused MLA. EVP-6124 (0.3 mg/kg, p.o.) was administrated 30 min before T1.

At the conclusion of testing, verification of the correct cannula placement was performed in two randomly selected animals. After injection of methylene blue, the animals were decapitated and the brains were rapidly removed and sliced with a razor blade. The presence of dye in the ventricular system verified the correct placement of the cannula in both animals. No methylene blue was observed in the surrounding tissue.

2.6.4.5. Effect of EVP-6124 on memory consolidation in the natural forgetting ORT. In order to assess effects on memory consolidation in particular since administration of a drug before T1 can affect both acquisition and consolidation processes, EVP-6124 was tested using a 24 h interval between T1 and T2 in the natural forgetting ORT. EVP-6124 was administered immediately after T1 (0.3, 1.0, and 3.0 mg/kg, p.o.) to assess memory consolidation and was compared to administration of EVP-6124 2 h before T1 (0.1, 0.3, 1.0, and 3.0 mg/kg, p.o.).

2.6.5. Statistical analyses

The ORT provides measures for exploration time and discrimination (Prickaerts et al., 1997). The relative discrimination (d2) index, which is independent of exploratory activity, was calculated as $d2 = (b - a)/(a + b)$, where the times spent exploring the familiar and new objects during T2 were represented as '*a*' and '*b*', respectively. The d2 index ranged from −1 to 1, with −1 or 1 indicating complete preference for the familiar or novel objects, respectively and 0 signifying no preference for either object.

One-sample *t*-tests were performed for each treatment to assess whether the d2 index significantly differed from zero, the chance level of performance. Effects between the treatments were assessed by either one-way or repeated measures ANOVAs. In the combination study of EVP-6124 and donepezil, all 23 animals received each treatment (within-subjects design), and a repeated measures ANOVA was used. In the other studies, different subsets of animals were used for each treatment and between-subjects, one-way ANOVAs were performed. When the overall ANOVA was significant, *post hoc* Bonferroni *t*-tests (all pairwise comparisons) were used. An α level of 0.05 was considered significant. Of note, overall the total object exploration times in T1 and T2 did not differ after treatment with EVP-6124, MLA, scopolamine, or donepezil in any of the tests (data not shown), indicating that the compounds did not affect activity and/or exploratory behavior per se. In the consolidation study, in which rats were treated after T1, there was an incidental finding of differences between the groups for exploration times during T1 (data not shown).

2.7. Pharmacokinetics and determination of plasma protein binding

2.7.1. Pharmacokinetics

Male Wistar rats (Charles River Labs, Wilmington, MA; approximately 270 g, $n = 60$) were treated with EVP-6124. EVP-6124 was prepared in 0.25% aqueous methylcellulose (Sigma-Aldrich, St. Louis, MO) at a volume of 1 ml/kg for p.o. administration. Animals were sacrificed with CO_2 at 1, 2, 4, or 8 h after dosing; blood was collected via cardiac puncture and brains were removed and frozen. Blood was collected in lithium heparin tubes and centrifuged; and plasma was removed and stored frozen until analysis. Brains were homogenized in PBS (0.1 M, pH 7.4), centrifuged at 15,000 rpm for 10 min at 4 °C, and the supernatant collected and stored frozen until analysis.

2.7.2. Plasma protein binding

The rat plasma protein binding assays were performed using an equilibrium dialysis method adapted from Ponganis and Stanski (1985). Each dialysis cell (Dianorm, Munich, Germany) consisted of two compartments separated by a cellulose semipermeable membrane with a molecular weight cut off of 5000 Da. The membrane was rehydrated before use in deionized water followed by the dialysis buffer. Plasma (Harlan Sera-Lab Limited, Loughborough, UK) was heated to 37 °C and adjusted to pH 7.4 before use. Five micromolar EVP-6124 solutions were prepared in isotonic phosphate buffer and species-specific plasma (final dimethyl sulfoxide concentration of 0.5%). The plasma was introduced to one side of the membrane, and dialysis buffer to the plasma-free, other side. Incubations were performed for 2 h in duplicate. The dialysis cells were mounted and rotated in a drive unit to ensure that a uniform equilibrium was obtained. The equilibrium was temperature controlled by immersing the drive unit in a water bath at 37 °C. At the end of the equilibration time the cells were emptied.

2.7.3. Bioanalytical methods

Following protein precipitation by the addition of methanol containing an internal standard, the samples were centrifuged and analyzed by LC-MS/MS. The LC system consisted of an Agilent HP 1100 binary LC pump (Agilent Technologies, UK, Ltd., Stockport, Cheshire, UK) and CTC Analytics HTS autosampler (Presearch Ltd, Hitchin, Herts, UK). This system used an Atlantis C18 3 μm column (10 × 2.1 mm) (Waters Ltd, Elstree, Herts, UK) maintained at 40 °C running a solvent gradient of 10 mM ammonium formate in deionized water containing 0.1% formic acid (Eluent A) and 10 mM ammonium formate in methanol containing 0.1% formic acid (Eluent B). Solvent composition was maintained at 100% A for 0.1 min following injection of each sample. A linear LC gradient was then employed reaching 5% Eluent A at 1.5 min (held for 0.3 min), and 100% Eluent A from 1.85 min until the end of the run. The flow rate was 0.5 ml/min. Triple quadrupole MS/MS analyses were performed using a Quattro Micro (Waters Ltd). Analyte detection used parent and daughter ion masses identified by an automated optimization process using Masslynx software with the Quanlynx application manager (Waters Ltd). The plasma and brain samples from rats were quantified using standard curves prepared in plasma or brain homogenate, respectively. The dialysis cell samples from the protein-containing compartment were quantified using calibration standards prepared in plasma and the protein-free compartments were quantified using calibration standards prepared in dialysis buffer. The fraction unbound was determined using the formula: $fu = 1 - ((PC - PF)/PC)$, where PC = sample concentration in protein-containing side, PF = sample concentration in protein-free side.

3. Results

3.1. Selectivity profile of EVP-6124

EVP-6124 is a novel synthetic molecule (Fig. 1) that was found to bind with high affinity to α7 nAChRs in rat brain membranes and to displace radioactive ligands such as the snake toxin α-bungarotoxin and MLA, two ligands that are specific for α7 nAChRs. EVP-6124 displaced [^{3}H]-MLA (K_i = 9.98 nM, pIC_{50} = 7.65 ± 0.06, n = 3; Fig. 2A) and [^{125}I]-α-bungarotoxin (K_i = 4.33 nM, pIC_{50} = 8.07 ± 0.04, n = 3; data not shown). EVP-6124 was approximately 300 fold more potent than the natural agonist ACh (K_i = 3 μM), measured in binding assays using [^{3}H]-MLA (data not shown).

The specificity of EVP-6124 for α7 nAChRs was confirmed by the absence of displacement of [^{3}H]-cytisine from α4β2 nAChRs by 10 μM EVP-6124, suggesting that the affinity of EVP-6124 for these heteromeric receptors was at least 1000 fold lower than the affinity

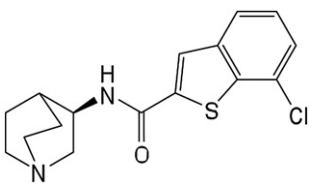

Fig. 1. Representation of the chemical structure of EVP-6124, (*R*)-7-chloro-*N*-(quinuclidin-3-yl)benzo[b]thiophene-2-carboxamide.

of nicotine ($K_i = 8$ nM) for the α4β2 nAChRs labeled by [^{3}H]-cytisine. The selectivity of EVP-6124 was further examined using a panel of more than 60 molecular targets, including peptide and non-peptide receptors, ion channels, and amine transporters (Table 1). No appreciable interaction was found with any of the examined targets in this panel, other than at the 5-HT$_3$ receptor subtype. EVP-6124 inhibited the 5-HT$_3$ receptor by 51% at 10 nM, the lowest concentration tested (data not shown). Evaluation of the human 5-HT$_{2B}$ receptor expressed in CHO cells demonstrated displacement of [^{3}H]-mesulergine ($K_i = 14$ nM) and only antagonist activity in the rat gastric fundus assay at an IC$_{50}$ of 16 μM.

3.2. Determination of the activity of EVP-6124

In electrophysiological studies on *Xenopus* oocytes expressing human α7 nAChRs, brief pulses of EVP-6124 produced strong

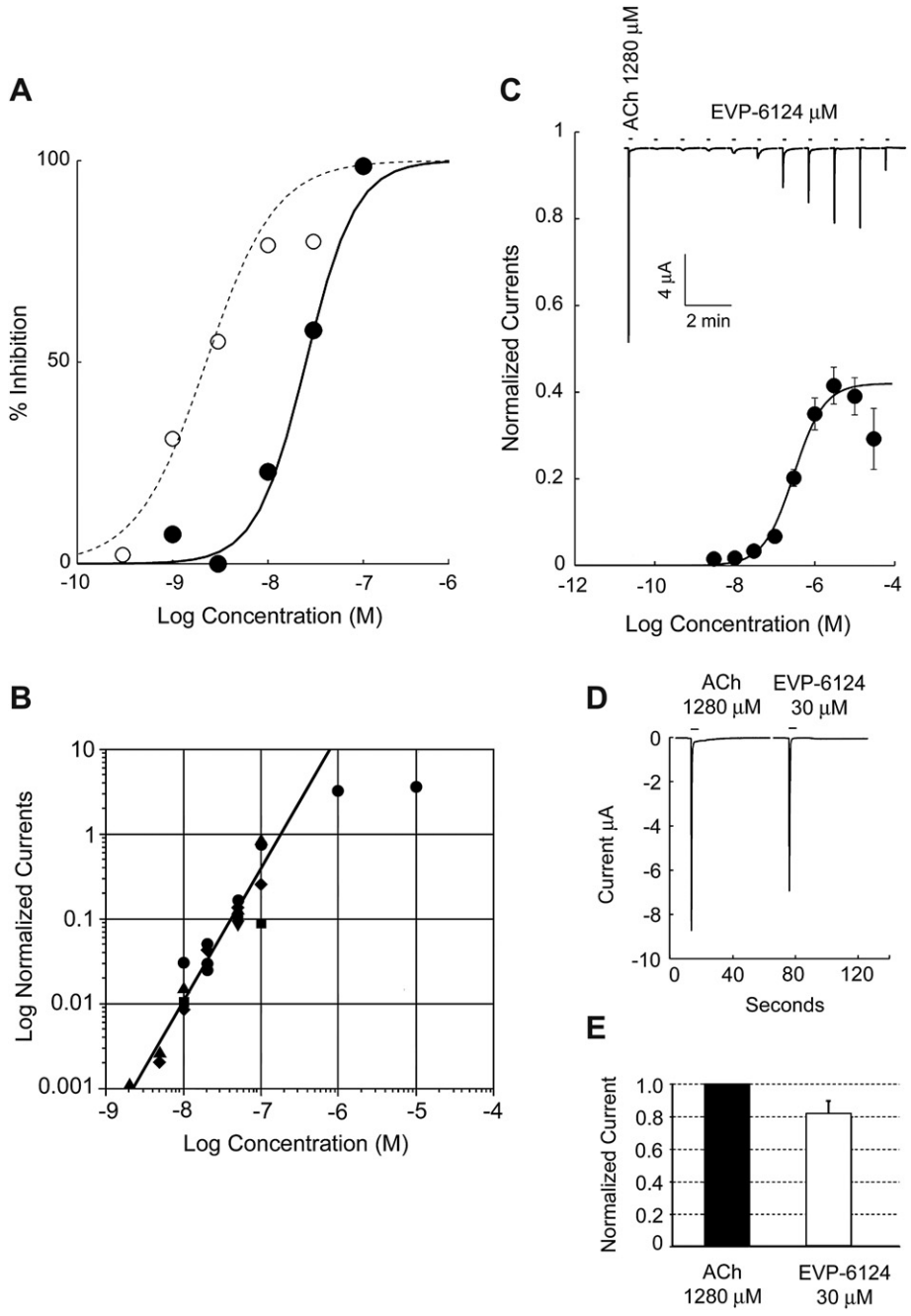

Fig. 2. A, EVP-6124 efficiently displaced [^{3}H]-MLA binding in rat brain homogenates, yielding a K_i of 9.98 nM (filled symbols and continuous line). As a positive control, open symbols and dashed line indicate the displacement of [^{3}H]-MLA caused by α-bungarotoxin with a K_i of 1.14 nM. B, EVP-6124 acted as an agonist of human α7 nAChRs expressed in *Xenopus* oocytes. The dose–response curve for peak current was plotted in double-logarithmic format, normalized to the current evoked by 50 μM ACh. The EC$_{50}$ was 0.16 μM and the slope was 1.6. C, EVP-6124 acted as a partial agonist at human α7 nAChRs expressed in *Xenopus* oocytes. Inset, typical currents evoked by a brief pulse of ACh (1280 μM), a pulse with no compounds, and a series of pulses of increasing concentrations of EVP-6124 from 0.003 to 30 μM are shown. Detectable responses were seen at concentrations greater than 0.03 μM. The continuous line on the concentration activation curve is the best fit obtained with the Hill equation, an EC$_{50}$ of 0.39 ± 0.07 μM, and a Hill coefficient of 1.45 ± 0.11. Bars indicate SEM ($n = 8$). D, EVP-6124 acted as a potent partial agonist at human α7 nAChRs. Typical currents recorded in an oocyte expressing human α7 nAChRs in response to 1280 μM ACh and to a single pulse of 30 μM EVP-6124. Bars above the traces indicate the timing of the drug application. E, Histogram of the currents normalized versus the ACh response for experiments as described in D. $N = 8$, bars indicate SEM. A comparison of C with D and E shows that repeated application of EVP-6124 desensitized human α7 nAChRs.

Table 1
In vitro binding or activity of EVP-6124.

Target	Percent inhibition (10 μM)	Target	Percent inhibition (10 μM)
Adenosine A_1 (*h*)	−7	Neuropeptide Y Y_1 (*h*)	−4
Adenosine A_{2A} (*h*)	12	Neuropeptide Y Y_2 (*h*)	−3
Adenosine A_3 (*h*)	10	Nicotinic ACh (*h*)	34
Adrenergic α_{1A} (*r*)	14	Nicotinic ACh, α7 (*r*)	99
Adrenergic α_{1B} (*r*)	46	Nicotinic ACh bungarotoxin-sensitive neuromuscular (*h*)	15
Adrenergic α_{1D} (*h*)	39	Opiate δ (*h*)	0
Adrenergic α_{2A} (*h*)	46	Opiate κ (*h*)	11
Adrenergic β_1 (*h*)	24	Opiate μ (*h*)	33
Adrenergic β_2 (*h*)	6	Platelet activating factor	−2
Bradykinin B_1 (*h*)	10	Prostanoid EP_4 (*h*)	−4
Bradykinin B_2 (*h*)	12	Purinergic P_{2x} (*rb*)	−5
Dopamine D_1 (*h*)	37	Purinergic P_{2Y} (*r*)	5
Dopamine D_{2S} (*h*)	36	5-HT_{1A} (*h*)	31
Dopamine D_3 (*h*)	16	5-HT_3 (*h*)	99
Dopamine $D_{4.2}$ (*h*)	14	Sigma σ_1 (*h*)	62
Endothelin ET_A (*h*)	4	Sigma σ_2 (*r*)	54
Endothelin ET_B (*h*)	−11	Tachykinin NK_1 (*h*)	−24
Epidermal growth factor (EGF) (*h*)	6	Testosterone (*r*)	2
Estrogen ERα (*h*)	4	Thyroid hormone (*r*)	−16
$GABA_A$, agonist site (*r*)	14	Calcium channel L-type, benzothiazepine (*r*)	13
$GABA_A$, central benzodiazepine (*r*)	1	Calcium channel L-type, dihydropyridine (*r*)	24
$GABA_{B1A}$ (*h*)	−18	Calcium channel N-type (*r*)	4
Glucocorticoid (*h*)	0	Potassium channel [K_{ATP}] (*h*)	−1
Glutamate, kainate (*r*)	−8	Sodium channel, site 2 (*r*)	59
Glutamate, NMDA, agonism (*r*)	5	Dopamine transporter (*h*)	29
Glutamate, NMDA, glycine (*r*)	−6	GABA transporter (*r*)	18
Glutamate, NMDA, phencyclidine (*r*)	0	Norepinephrine transporter (*h*)	53
Histamine H_1 (*h*)	24	5-HT transporter (*h*)	2
Histamine H_2 (*h*)	15	Phorbol ester (*m*)	−7
Histamine H_3 (*h*)	−6	Rolipram (*r*)	2
Imidazoline I_2, central (*r*)	21	CYP450 1A2 (*h*)	7
Interleukin IL-1 (*m*)	−16	CYP450 2C19 (*h*)	5
Leukotriene $CysLT_1$ (*h*)	4	CYP450 2C9 (*h*)	3
Melotonin MT_1 (*h*)	5	CYP450 2D6 (*h*)	−20
Muscarinic M_1 (*h*)	19	CYP450 3A4 (*h*)	8
Muscarinic M_2 (*h*)	5		
Muscarinic M_3 (*h*)	7		

h, human; *m*, mouse; *r*, rat; *rb*, rabbit.

inward currents relative to 50 μM ACh (Fig. 2B). In double-logarithmic format, the straight-line fit of the dose–response curve yielded an EC_{50} of 0.16 μM and a slope of 1.6 for EVP-6124. Relative to 1280 μM ACh, EVP-6124 acted as a partial agonist, with maximum current amplitude of 42 ± 3% (Fig. 2C). Typical EVP-6124-evoked currents (Fig. 2C, inset), and the corresponding concentration activation curve (Fig. 2C) were generated by pulsing EVP-6124 every minute at increasing concentrations up to 30 μM. These data were readily fitted with a single Hill equation with an EC_{50} of 0.39 ± 0.07 μM and a Hill coefficient of 1.45 ± 0.11 ($n = 8$). The reduction in the amplitude of the inward current at 10 and 30 μM, after increasing concentrations of EVP-6124 suggested that the compound desensitized α7 nAChRs. To better assess the agonist activity of EVP-6124 without desensitization, cells expressing α7 nAChRs were first challenged with an ACh test pulse (1280 μM) and then with a single pulse of 30 μM EVP-6124 (Fig. 2D). Currents evoked by 30 μM reached 82 ± 7% of the current evoked by ACh (Fig. 2E), which was considerably larger than the 42% observed in Fig. 2C. Additionally, single pulses of 30 μM EVP-6124 demonstrated that the compound acted as a partial but potent agonist at α7 nAChRs (Fig. 2D and E).

Selectivity of EVP-6124 was further confirmed using electrophysiological characterization of the rat α3β4, α4β2, and muscle α1β1γδ receptors. There was no detectable agonist activity at concentrations up to 100 μM. Antagonist activity was, however, observed at the rat α3β4 receptor with an IC_{50} of 16 μM (data not shown).

3.3. Effects of EVP-6124 on memory in the object recognition task

3.3.1. EVP-6124 reverses a scopolamine-induced deficit

Comparisons between treatments indicated differences for the d2 indices ($F(5,90) = 15.60$, $p < 0.001$). *Post hoc* analysis showed that the d2 indices were significantly higher after treatment with saline and water vehicles (without a scopolamine deficit) and in the presence of 0.3 and 1.0 mg/kg of EVP-6124 (with a scopolamine deficit) than after treatment with scopolamine alone (Fig. 3A). One-sample *t*-tests showed significant differences from chance performance for the d2 indices at 0.1, 0.3, and 1.0 mg/kg of EVP-6124 and 0.1 mg/kg of scopolamine, indicating recognition of the familiar object and reversal of the scopolamine-induced deficit (Fig. 3A). After treatment with both vehicles, the d2 index was also significantly different from chance performance.

3.3.2. Sub-efficacious doses of EVP-6124 and an AChEI (donepezil) reverse a scopolamine-induced deficit

The repeated measures ANOVA revealed differences between the treatments for the d2 indices ($F(4, 88) = 8.18$, $p < 0.001$). *Post hoc* analysis revealed that the d2 indices were significantly higher for the vehicle and the EVP-6124 and donepezil combined treatments, when compared with scopolamine alone (Fig. 3B). One-sample *t*-tests showed significant differences from chance performance for the d2 indices for the vehicle and for the combination of EVP-6124 and donepezil (Fig. 3B). This was in contrast to the other treatments, which showed no significant differences from chance performance.

3.3.3. EVP-6124 prevents natural forgetting

Comparison between treatments indicated differences for the d2 indices ($F(3,60) = 3.62$, $p < 0.05$). *Post hoc* analysis showed that the d2 index was significantly higher for the 0.3 mg/kg EVP-6124 treatment when compared with the vehicle (Fig. 4A). One-sample *t*-tests showed significant differences from chance performance for the d2 indices at EVP-6124 doses of 0.1, 0.3, and 1.0 mg/kg, indicating recognition of the familiar object and prevention of natural forgetting (Fig. 4A).

3.3.4. MLA antagonism of the memory enhancing effects of EVP-6124 on natural forgetting

For the i.p. MLA study, comparison between treatments indicated differences for the d2 indices ($F(4,75) = 16.94$, $p < 0.001$). *Post hoc* analysis showed that the d2 indices were significantly lower after treatment with only the vehicles and with 0.3 and 1.0 mg/kg of MLA in combination with 0.3 mg/kg of EVP-6124 than after treatment with 0.3 mg/kg of EVP-6124 alone (Fig. 4B). One-sample *t*-tests showed significant differences from chance performance for the d2 indices for the combination of 0.3 mg/kg of EVP-6124 with vehicle or 0.1 mg/kg of MLA, indicating prevention of natural forgetting (Fig. 4B).

For the i.c.v. MLA study, comparison between treatments indicated differences for the d2 indices ($F(3,60) = 10.17$, $p < 0.001$). *Post hoc* analysis showed that the d2 indices were significantly lower after treatment with only the vehicles and with 10 μg of MLA in combination with 0.3 mg/kg of EVP-6124 than after treatment with

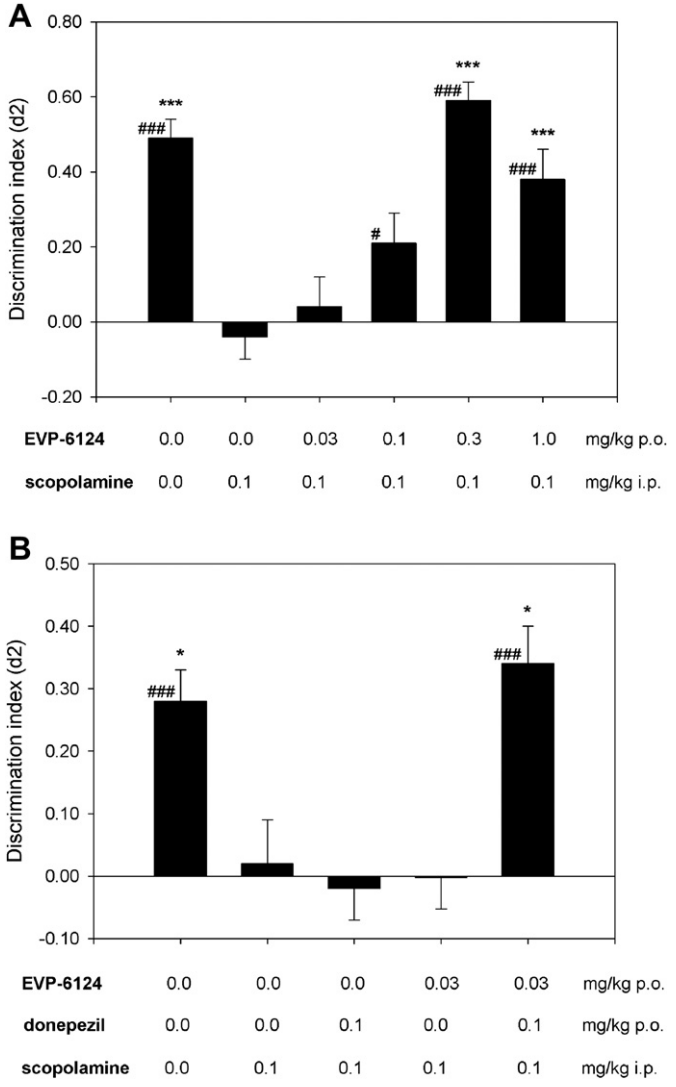

Fig. 3. Scopolamine-induced short-term memory deficit in an object recognition task. All drugs were administered 30 min before T1. A, Effects of EVP-6124 (p.o.) on the discrimination index (d2) using scopolamine treatment (0.1 mg/kg, i.p.) to impair memory and a 1 h interval in 2.5-month-old male Wistar rats (mean + SEM). When compared to scopolamine alone, EVP-6124 (0.3 and 1.0 mg/kg) reversed the short-term memory impairment induced by treatment with scopolamine. Differences from scopolamine alone: $^{***}p < 0.001$. Differences from zero (chance performance): $^{\#}p < 0.05$; $^{\#\#\#}p < 0.001$. $N = 24$ per treatment for vehicle and scopolamine alone, and 12 per treatment with EVP-6124. B, The effects of donepezil (p.o.) and EVP-6124 (p.o.) on the d2 index in an ORT using a 1 h interval, after scopolamine treatment (i.p.) in 5-month-old male Wistar rats (mean + SEM). When compared to scopolamine alone, EVP-6124 and donepezil combined reversed the scopolamine-induced memory deficit. Differences from scopolamine alone: $^{*}p < 0.05$. Differences from chance performance: $^{\#\#\#}p < 0.001$. $N = 23$ per treatment.

0.3 mg/kg of EVP-6124 alone (Fig. 4C). One-sample *t*-tests showed significant differences from chance performance for the d2 indices for the combination of 0.3 mg/kg of EVP-6124 with vehicle or 3 μg of MLA, indicating prevention of natural forgetting (Fig. 4C).

3.3.5. Effects of EVP-6124 on consolidation in the natural forgetting ORT

A vehicle treatment was not used in these studies, since in the previous experiments vehicle administered before T1 produced natural forgetting. Thus, no ANOVAs were performed. When EVP-6124 was administered before T1, the one-sample *t*-tests showed significant differences from chance performance for the d2 indices for 0.3, 1, and 3 mg/kg of EVP-6124 (Fig. 5A). When EVP-6124 was administered immediately after T1, to selectively assess the effect on memory consolidation, the one-sample *t*-tests showed significant differences from chance performance for the d2 indices for EVP-6124 at 1 and 3 mg/kg (Fig. 5B).

3.4. Pharmacokinetics and brain penetration of EVP-6124 in rats

EVP-6124 was found to bind moderately to rat plasma proteins with a mean fu of 0.11 ± 0.01 (mean ± SD) or 11%. Over a range of 0.1–30 mg/kg, p.o., EVP-6124 demonstrated proportional dose escalation. Table 2 shows the plasma and brain concentrations and B:P ratios of EVP-6124 after a single dose of 0.3 mg/kg, p.o., an efficacious dose in the ORT. T_{max} was at 4 h in plasma and 2 h brain, although the brain concentrations remained similar between 2 and 8 h. The B:P ratios were 1.7–5.1 between 1 and 8 h. Assuming that the free drug fraction in the brain was equal to or at least similar to the free drug fraction in the plasma, the corresponding brain free drug concentrations were between 0.14 and 0.24 nM up to 8 h after treatment with EVP-6124. The unbound concentration of EVP-6124 was assumed to reflect the concentration available for binding to brain α7 nAChRs.

3.5. Low concentrations of EVP-6124 potentiate ACh-evoked currents

Estimation of the brain concentration of EVP-6124 attained during positive behavioral tests indicated that this compound was present in the sub- to low nanomolar range. Importantly, however, these concentrations were insufficient to activate or desensitize α7 nAChRs in electrophysiological studies (Fig. 2). To understand the possible mechanisms underlying the positive effects of EVP-6124 on cognition and memory, additional functional investigations were carried out.

The α7 nAChR desensitization profile caused by sustained application of EVP-6124 was determined by exposing oocytes expressing human α7 nAChRs to a succession of increasing EVP-6124 concentrations and measuring the response to fixed, low concentration ACh (40 μM) test pulses. Plotting the amplitude of the current evoked by ACh test pulses as a function of the logarithm of the concentration of EVP-6124 yielded a concentration inhibition curve with a sharp decline and an IC_{50} of 3 nM (Fig. 6A). This IC_{50} was in good agreement with the EVP-6124 K_i of 9.98 nM for displacement of [^{3}H]-MLA binding (Fig. 2A) and of 4.33 nM for displacement of [^{125}I]-α-bungarotoxin. At lower concentrations, the concentration inhibition curve for EVP-6124 showed an increase in the response to a pulse of ACh in the range of 0.3–1 nM. To assess the effect of EVP-6124 at potentiating (0.3 nM) and desensitizing (>1 nM) concentrations on ACh-evoked responses over time, oocytes were exposed to a sustained concentration of EVP-6124 and their responses to 40 μM ACh test pulses were monitored at 2-min intervals (Fig. 6B). A marked and sustained increase (potentiation) in the ACh-evoked α7 nAChR response was observed at 0.3 nM of EVP-6124 (Fig. 6B, upper panel). At 3 nM of EVP-6124, an increase in the first ACh-evoked current was observed, followed by a rapid decline in the ACh-evoked α7 nAChR response, attributable to α7 nAChR desensitization (Fig. 6B, lower panel).

The effect of sustained application of EVP-6124 was then tested with ACh (40 μM) pulsed with a long time delay between pulses (Fig. 6C and D). Oocytes expressing α7 nAChRs were first tested with brief ACh pulses (40 μM, 5 s) during a stabilization period (8 min) and then exposed for a prolonged period (20 min) to EVP-6124 at 0.3 nM. During the initial co-exposure to ACh and EVP-6124, the response amplitudes were larger than the response amplitudes for ACh alone, confirming the result in Fig. 6B, upper panel. Exposure to 0.3 nM EVP-6124, without pulses of ACh, did not

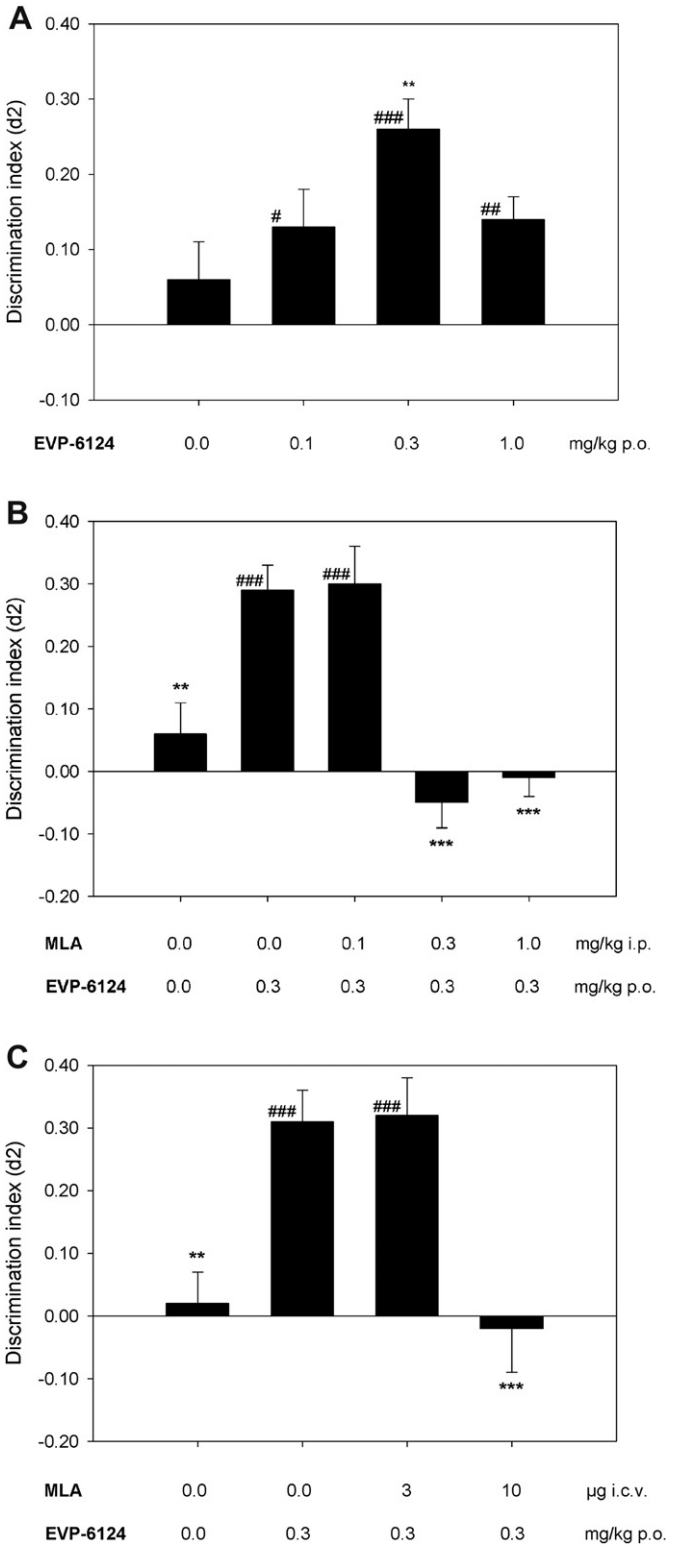

Fig. 4. Natural forgetting in an object recognition task. EVP-6124 was always administered 30 min before T1. A, Effects of EVP-6124 (p.o.) on the d2 index using a 24 h retention interval in 2.5-month-old male Wistar rats (mean + SEM). When compared to vehicle, EVP-6124 (0.3 mg/kg) prevented loss of long-term memory. Differences from vehicle: $^{**}p < 0.01$. Differences from chance performance: $^{\#}p < 0.05$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$. $N = 16$ per treatment. B, The effects of co-administration of 0.1–1 mg/kg of MLA (i.p.) 1 h before T1 and 0.3 mg/kg of EVP-6124 (p.o.) on the d2 index using a 24 h retention interval in 3-month-old male Wistar rats (mean + SEM). When compared to the administration of 0.3 mg/kg of EVP-6124 alone, 0.3 and 1.0 mg/kg of MLA significantly antagonized the pro-cognitive effect of 0.3 mg/kg of EVP-6124. Differences from 0.3 mg/kg EVP-6124 alone: $^{**}p < 0.01$; $^{***}p < 0.001$. Differences from chance performance: $^{\#\#\#}p < 0.001$. $N = 16$ per treatment. C, The effects of co-administration of 3 and 10 µg of MLA (i.c.v.) 4 min before T1 and 0.3 mg/kg of EVP-6124 (p.o.) on the d2 index using a 24 h retention interval in 4-month-old male Wistar rats (mean + SEM). When compared to the administration of 0.3 mg/kg of EVP-6124 alone, 10 µg of MLA significantly blocked the pro-cognitive effect of 0.3 mg/kg of EVP-6124. Differences from 0.3 mg/kg EVP-6124 alone: $^{**}p < 0.01$; $^{***}p < 0.001$. Differences from chance performance: $^{\#\#\#}p < 0.001$. $N = 16$ per treatment.

evoke a sizeable current. The periodic test pulses of ACh, in the presence of 0.3 nM EVP-6124 in the medium, produced increased response amplitudes, larger than the amplitudes during the initial sustained co-application of ACh and EVP-6124. The minimal decline of the ACh-evoked current observed during washout of EVP-6124 suggested a slow off-rate of EVP-6124 from the receptors, which was consistent with the high affinity binding observed by the displacement of [^{3}H]-MLA and [^{125}I]-α-bungarotoxin from α7 nAChRs (Fig. 2A).

4. Discussion

The identification of a high expression level of neuronal nAChRs in many brain areas, including the hippocampus and cortex, and more specifically of those containing homopentameric α7 subunits suggested that these receptors may play an important role in cognitive functions (Dani and Bertrand, 2007). This hypothesis was confirmed by the finding that specific agonists of α7 nAChRs improved memory performance in animals, as shown initially by the effects of molecules such as GTS-21 (reviewed in Kem, 2000) and more recently by more selective and potent agonists.

Recently described α7 nAChRs agonists have demonstrated marked improvements in binding displacement of MLA or α-bungarotoxin, with K_i values in the range of 1–8.8 nM (Hauser et al., 2009; Malysz et al., 2010; Sydserff et al., 2009; Wallace et al., 2011; Wishka et al., 2006). EVP-6124 similarly displaced binding to the rat brain α7 nAChR with a K_i in the low nanomolar range (i.e. 9.98 nM for the displacement of [^{3}H]-MLA and 4.33 nM for [^{125}I]-α-bungarotoxin). Like EVP-6124, all of these potent agonists (TC-5619, ABT-107, AZD0328, RG3487, and PHA-543613) are quinuclidines. Earlier quinuclidines (e.g. PNU-282987, ABBF, and SSR180711), demonstrated less potency (K_i values of 22–62 nM) (Biton et al., 2007; Bodnar et al., 2005; Boess et al., 2007).

The selectivity of EVP-6124 for α7 nAChRs was demonstrated by the lack of effect on cytisine binding at α4β2 nAChRs. In *Xenopus* oocytes, EVP-6124 also did not activate heteromeric nAChRs, such as the α3β4, α4β2, and the embryonic muscle α1β1γδ subtypes. The selectivity for α7 nAChRs versus other nAChRs is characteristic of the other recently described compounds (Hauser et al., 2009; Malysz et al., 2010; Sydserff et al., 2009; Wallace et al., 2011; Wishka et al., 2006). EVP-6124 at a 10 µM concentration also lacked appreciable interactions with more than 60 molecular targets in a selectivity screening panel that included receptors, ion channels, and amine transporters. The antagonist activity of EVP-6124 at 5-HT$_{3A}$ receptors, homologs of α7 nAChRs, is a common feature of some of the recently characterized compounds, such as AZD0328 and RG3487 (Sydserff et al., 2009; Wallace et al., 2011), and could prove beneficial in reducing the potential emetic effects of nicotinic agonists. In contrast, some of the quinuclidines achieved greater separation between the activities at α7 nAChRs and 5-HT$_{3A}$ receptors (Hauser et al., 2009; Malysz et al., 2010; Wishka et al., 2006). Given the sub- to low nanomolar brain concentrations required for activation of α7 nAChRs, this greater separation may provide no therapeutic benefit in the clinic.

Functional assessment of EVP-6124 at α7 nAChRs revealed that the compound acted as a partial agonist and evoked up to 80% of the ACh-evoked current for single pulses of 30 µM. With

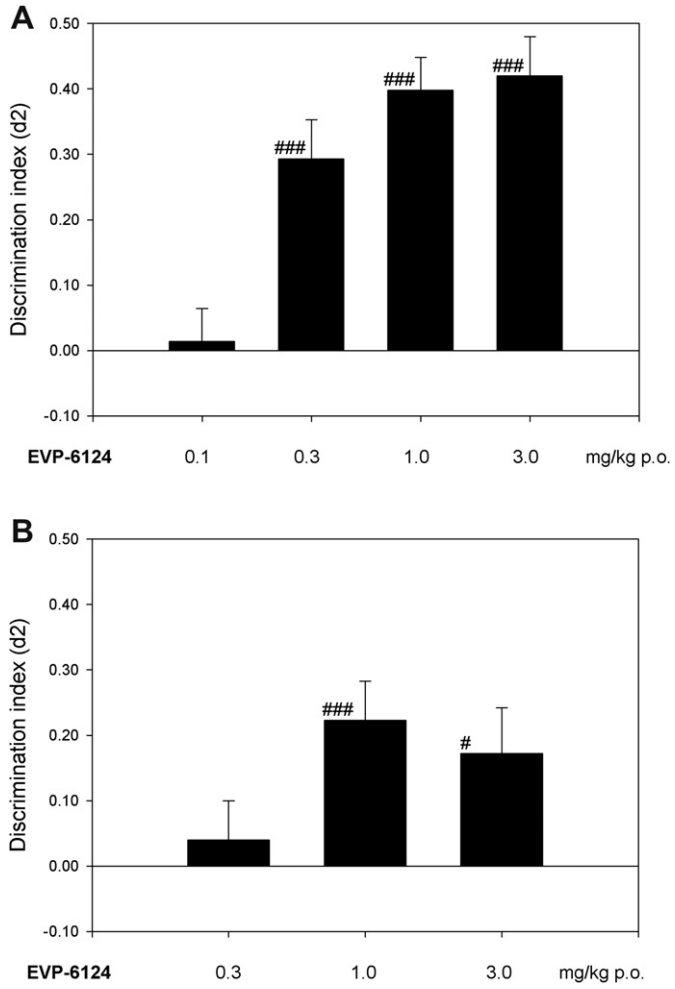

Fig. 5. Effect of EVP-6124 on memory consolidation in the natural forgetting ORT A, Effects of EVP-6124 (p.o.) on the d2 index using a 24 h retention interval in 3-month-old male Wistar rats (mean + SEM). When compared to chance level, EVP-6124 (0.3–3 mg/kg, p.o.) administered 2 h before T1 improved memory. Differences from chance performance: $^{###}p < 0.001$. $N = 24$ per treatment. B, Effects of EVP-6124 (p.o.) on the d2 index using a 24 h retention interval in 3-month-old male Wistar rats (mean + SEM). When compared to chance performance, EVP-6124 (1 and 3 mg/kg) administered immediately after T1 improved memory consolidation. Differences from chance performance: $^{#}p < 0.05$; $^{###}p < 0.001$. $N = 24$ per treatment.

application of ascending concentrations of EVP-6124, the maximum activity was 42% of ACh. Under similar experimental conditions, AZD0328, RG3487, and ABT-107 acted as partial agonists, demonstrating 58, 63, 79%, respectively, of the activity of ACh (Malysz et al., 2010; Sydserff et al., 2009; Wallace et al., 2011). TC-5619 was a full agonist (Hauser et al., 2009). The high affinity of EVP-6124 for α7 nAChRs, with an EC_{50} in the range of 0.16–0.39 μM, was similarly observed in other recently described α7 nAChR agonists. In studies reporting peak current, as reported in this study, EC_{50} values ranged between 0.28 and 1.04 μM (Malysz et al., 2010; Sydserff et al., 2009; Wallace et al., 2011). The EC_{50} for TC-5619 of 0.033 μM was reported as net charge, a method that produced a 3–6 fold lower value than that produced by peak current analysis, when both analyses were performed in the same studies (Malysz et al., 2010; Sydserff et al., 2009; Wallace et al., 2011). In summary, the EC_{50} values for EVP-6124, as well as the other recently described quinuclidines, suggested that therapeutic concentrations should be expected in the high nanomolar or low micromolar range, if the compounds were to act solely as receptor agonists that required near to full receptor occupancy.

Table 2
Total concentrations of EVP-6124 and (estimated free concentrations of EVP-6124) after a single dose of 0.3 mg/kg, p.o.

Time (h)	Mean ± SEM		
	Plasma concentration (nM)	Brain concentration (nM)	Brain to plasma ratio
1	0.71 ± 0.05 (0.078 ± 0.006)[a]	1.3 ± 0.2 (0.14 ± 0.02)	1.8
2	0.84 ± 0.13 (0.092 ± 0.014)	2.2 ± 0.2 (0.24 ± 0.03)	2.6
4	1.1 ± 0.1 (0.12 ± 0.01)	1.8 ± 0.5 (0.20 ± 0.05)	1.7
8	0.42 ± 0.11 (0.046 ± 0.012)	2.1 ± 0.5 (0.24 ± 0.05)	5.1

[a] Estimated free drug concentration = total drug concentration × fu, where fu = 0.11. $N = 5$ per group.

EVP-6124 demonstrated good brain penetration after oral administration, with B:P ratios of approximately 2 between 1 and 4 h and 5 at 8 h. Recently described agonists have varied widely in B:P ratios. RG3487 had the lowest B:P ratio of 0.13, followed by ABT-107 and PHA-543613 (1 and 1.5, respectively) (Bitner et al., 2010; Wallace et al., 2011; Wishka et al., 2006). Of the quinuclidines, PNU-282987 had a high B:P ratio of 5 (Bodnar et al., 2005). The B:P ratio of about 2 for EVP-6124 supported further study of the compound in cognitive tests.

The memory enhancing effects of EVP-6124 *in vivo* were demonstrated in the ORT in a test of short-term memory impairment caused by scopolamine and in a test of natural forgetting, with a 24 h retention interval between the two trials. These data clearly illustrated that EVP-6124 improved memory in a dose-dependent manner, reaching a peak of activity at brain concentrations in the low nanomolar range. Efficacy in cognitive tests at low nanomolar concentrations have similarly been reported for ABT-107 and RG3487 (Bitner et al., 2010; Malysz et al., 2010; Wallace et al., 2011).

Since EVP-6124 (0.3 mg/kg, p.o.) was administered before T1 in the ORT and therefore could have exerted an effect on memory consolidation, as well as on acquisition, additional studies were performed in which the effect of EVP-6124 on memory consolidation could be investigated. EVP-6124 improved memory consolidation when administered immediately after T1 in an ORT, as was found for RG3487 (Wallace et al., 2011).

In addition, the effect of 0.3 mg/kg of EVP-6124 in the natural forgetting test was blocked by administration of the selective α7 nAChR antagonist MLA (0.3 mg/kg, i.p. or 10 μg, i.c.v.). These studies indicated that the pro-cognitive effect of EVP-6124 was specifically mediated via brain α7 nAChRs. Cognitive improvements by other α7 nAChR agonists were similarly blocked by MLA administered either centrally or peripherally at comparable concentrations (Boess et al., 2007; Pichat et al., 2007; van Kampen et al., 2004; Wallace et al., 2011). Some of the α7 nAChR agonists, including EVP-6124, are also 5-HT_3 receptor antagonists (Boess et al., 2007; Sydserff et al., 2009; Wallace et al., 2011), and may therefore enhance cognition via this latter mechanism (for review, Walstab et al., 2010). However, the selective 5-HT_3 receptor antagonist ondansetron (0.1–10 mg/kg, p.o.) did not prevent natural forgetting in an ORT, while the α7 nAChR agonist RG3487 was effective (Wallace et al., 2011). Additionally, the α7 nAChR agonist AZD0328, with a 25 nM K_i at rat 5-HT_3 receptors, was not effective in an ORT in mice lacking α7 nAChRs (Sydserff et al., 2009). Furthermore, the brain concentrations of EVP-6124 and other α7 nAChR agonists required for efficacy in cognitive tests (sub- to low nanomolar) are below the concentrations required for antagonist activity at 5-HT_3 receptors where near to full receptor occupancy would likely be required.

Since treatment with α7 nAChR agonists may benefit AD patients, and they are often treated with an AChEI, we investigated the potential beneficial interaction between AChEIs and EVP-6124. Co-infusion studies of ABT-107 with donepezil demonstrated that

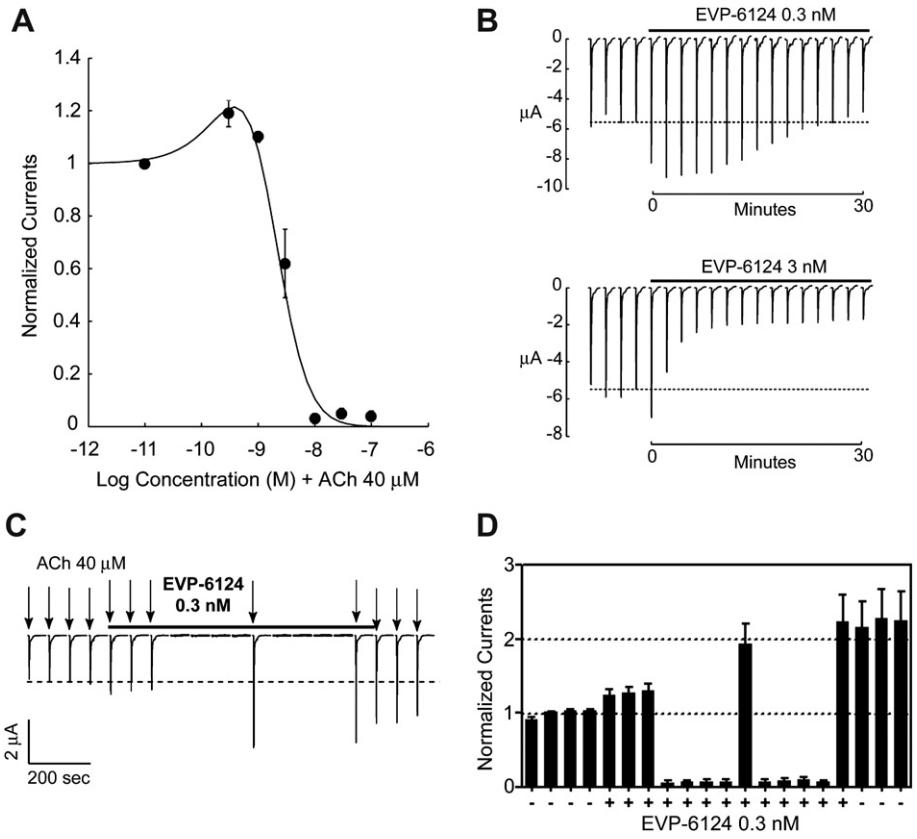

Fig. 6. Low concentrations of EVP-6124 potentiate ACh-evoked currents. A, The response to a test pulse of ACh (40 μM) was measured during sustained exposure to EVP-6124 at the indicated logarithmic concentrations. The concentration inhibition curve for EVP-6124 showed an increase in the response to a pulse of ACh in the range of 0.3–1 nM. Higher concentrations of EVP-6124 yielded a concentration inhibition curve with a sharp decline and an IC_{50} of 3 nM. Filled circles are the mean of the normalized response ($n \geq 3$). Error bars are SEM. The curve is the best fit obtained with the dual binding site model (Cachelin and Rust, 1994; Zwart et al., 2000). B, Currents evoked by 40 μM ACh for 5 s were measured at regular intervals of every 2 min, before and during a sustained exposure to EVP-6124 (0.3 and 3 nM). Bars above the traces indicate the timing of the sustained application of EVP-6124. EVP-6124 in the sub-nanomolar range potentiated ACh-evoked currents (upper panel), while EVP-6124 in the low nanomolar range reduced ACh-evoked currents (lower panel). C, α7 nAChRs were assessed by exposing the oocytes to brief ACh test pulses (arrows, 40 μM, 5 s), first during a stabilization period of 8 min and then for a period of 20 min to EVP-6124 at 0.3 nM (indicated by the horizontal bar above the traces). Exposure to 0.3 nM EVP-6124 increased the ACh-evoked current without desensitizing the receptors. The magnitude of the increase in the ACh-evoked current was greater when the pulse was preceded by a rest period. D, Histogram of the currents (mean + SEM) normalized versus the initial responses to ACh alone for a series of experiments ($n = 7$) utilizing the protocol described in C.

the combination of an α7 nAChR agonist and an AChEI was not deleterious to cognition (Bitner et al., 2010). Since the doses of both ABT-107 and donepezil were efficacious alone, a ceiling effect may have precluded the observation of an additive effect of the combination. Accordingly, we tested the combination of previously established sub-efficacious doses of EVP-6124 (0.03 mg/kg, p.o.) and donepezil (0.1 mg/kg, p.o.) and found that the combined treatment completely reversed the scopolamine-induced deficit in the ORT. The results of this combination study further suggested that the pro-cognitive effects of AChEIs, which are dose limited by adverse effects, primarily gastrointestinal (Birks and Flicker, 2006), could be enhanced if AChEIs are combined in low doses with an α7 nAChR agonist in AD patients.

Based on the pharmacokinetic data, estimation of the maximum effective brain concentration in the ORT after a dose of 0.3 mg/kg, p.o. was about 2 nM (total concentration of EVP-6124) and less than 1 nM free drug. Thus, in spite of the high affinity of EVP-6124 for α7 nAChRs, a concentration lower than 3 nM was clearly insufficient to activate the receptor in *in vitro* studies, suggesting an alternative mechanism of action. Moreover, in the animal studies, the EVP-6124 concentration slowly built up and remained stable before and while the behavioral tests were performed. Therefore, these conditions cannot be compared to a brief pulse exposure to EVP-6124 used for the determination of the concentration activation curve *in vitro*.

Since AChEIs inhibit the cleavage of ACh and thereby increase the level of this neurotransmitter, we further examined the interaction of EVP-6124 and ACh at the cellular level in sustained exposure experiments that were aimed at mimicking the conditions of an animal treated with EVP-6124 (Fig. 6A and B). These data clearly illustrated that sustained exposure to a concentration of EVP-6124 below 1 nM potentiated the ACh-evoked current. RG3487 potentiated the ACh-evoked current at 3–10 nM (Wallace et al., 2011). Increasing the EVP-6124 concentration to 3 nM or above, caused a marked reduction of the ACh-evoked current that was attributable to receptor desensitization. Under similar experimental conditions of paired application of an α7 nAChR agonist and ACh, α7 nAChR desensitization was observed in the low nanomolar range with ABT-107 and RG3487 at 10 and 30 nM, respectively (Malysz et al., 2010; Wallace et al., 2011). The pro-cognitive effects after EVP-6124, ABT-107, and RG3487 administration appear to have occurred at brain concentrations below those that caused desensitization. Reducing the frequency of stimulation diminished the desensitization caused by successive agonist exposures and revealed a larger degree of potentiation (Fig. 6C and D). These data illustrated that the presence of a low concentration of EVP-6124, in the sub-nanomolar range increased the ACh-evoked current by a factor of two or more.

Although different mechanisms can be postulated to account for the observed potentiation, the simplest and most probable model

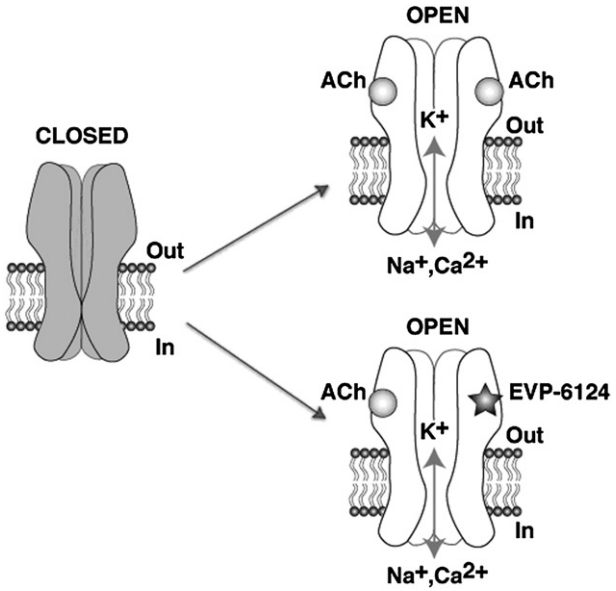

Fig. 7. Model of EVP-6124 and ACh binding. In the absence of ligand the receptor is closed (left panel). ACh applications allowing two (or more) molecules to bind and activate the receptor (upper right panel). Exposure to a low concentration of EVP-6124 causes binding of one EVP-6124 molecule on the receptor that is insufficient to activate the receptor. Brisk activation of the receptor will, however, occur upon exposure to ACh and a single molecule might be sufficient to trigger the opening of the channel (lower right panel).

considers the co-agonistic behavior of EVP-6124 and ACh at α7 nAChRs. In light of our findings in oocytes treated with EVP-6124 and ACh in sustained and intermittent exposure experiments, we were directed to a model of co-agonist activity described for tubocurarine and ACh at α3β4 nAChRs (Cachelin and Rust, 1994). Following this initial description, additional evidence for co-agonist activities was reported for other nAChRs subtypes (Papke et al., 2011; Smulders et al., 2005; Wallace et al., 2011; Zwart et al., 2000). In this model, a single receptor displays at least two pockets where the ligand can bind. Similarly, two molecules of ACh must bind to the α7 nAChR to activate it. Exposure to a low concentration of a high affinity ligand, such as EVP-6124, will increase the probability that a single molecule of this ligand occupies the receptor. As occupancy of the receptor by a single molecule is assumed to be insufficient to activate the receptor, exposure to such a low concentration of ligand is not expected to cause channel opening. Brief and intermittent exposure to another ligand with lower affinity, such as ACh, will then trigger channel opening and an inward current. These steps, which correspond to the model formulated by Cachelin and Rust (1994), are summarized in Fig. 7.

Altogether, these data provide evidence that the novel synthetic compound, EVP-6124, acted as a potent partial and selective agonist at α7 nAChRs. Exposure of EVP-6124 to α7 nAChRs at a sub- to low nanomolar range, which corresponded to the active doses in behavioral tests, caused a potentiation of the ACh-evoked current that can account for the pro-cognitive effects observed in animals. These data suggested that a novel mechanism of action at low agonist concentrations of EVP-6124 was responsible for its pro-cognitive effects, a notion that needs to be investigated further in controlled experiments.

The finding of a novel mechanism of action of a partial agonist acting at a concentration in the sub-nanomolar range through a co-agonism mechanism may lead to a more desirable side-effect profile than more classical approaches which dictate that the drug be dosed to full agonist concentrations. Activation of α7 nAChRs by exposure to a low agonist concentration, of a drug such as EVP-6124 utilizing this co-agonist mechanism, is expected to increase the drug safety margin, to minimize undesired interactions with other receptors, and to open new and promising therapeutic avenues in combination with classical AChEIs at lower than typically prescribed doses.

Conflict of interest

EnVivo Pharmaceuticals, Inc. and Bayer have a financial interest in EVP-6124. This research was funded by EnVivo Pharmaceuticals, Inc. RC, GS, DGF, DH, MG, and GK received compensation as employees from EnVivo Pharmaceuticals, Inc. and participated in study design, development of reagents, conduct of experiments, data analysis, and report preparation. During the initial phases of the discovery of EVP-6124, FGB and CM were employed by Bayer and participated in study design, conduct of experiments, data analysis, and report preparation. JP, OAHR, NPG, DB, and SB received compensation from EnVivo Pharmaceuticals, Inc. for conducting some of the studies described in this report and participated in study design, conduct of experiments, data analysis, and report preparation.

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