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RESEARCH PAPER

The cognition-enhancing activity of E1R, a novel positive allosteric modulator of sigma-1 receptors

L Zvejniece[1], E Vavers[1,2], B Svalbe[1,3], R Vilskersts[1,2], I Domracheva[1], M Vorona[1], G Veinberg[1], I Misane[4], I Stonans[4], I Kalvinsh[1] and M Dambrova[1,2]

[1]*Latvian Institute of Organic Synthesis, Riga, Latvia,* [2]*Riga Stradins University, Riga, Latvia,* [3]*Faculty of Medicine, University of Latvia, Riga, Latvia, and* [4]*JSC Grindeks, Riga, Latvia*

Correspondence
L Zvejniece, Latvian Institute of Organic Synthesis, 21 Aizkraukles Street, Riga, LV-1006, Latvia.
E-mail: liga@biomed.lu.lv

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BACKGROUND AND PURPOSE
Here, we describe the *in vitro* and *in vivo* effects of (4R,5S)-2-(5-methyl-2-oxo-4-phenyl-pyrrolidin-1-yl)-acetamide (E1R), a novel positive allosteric modulator of sigma-1 receptors.

EXPERIMENTAL APPROACH
E1R was tested for sigma receptor binding activity in a [^{3}H](+)-pentazocine assay, in bradykinin (BK)-induced intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) assays and in an electrically stimulated rat vas deferens model. E1R's effects on cognitive function were tested using passive avoidance (PA) and Y-maze tests in mice. A selective sigma-1 receptor antagonist (NE-100), was used to study the involvement of the sigma-1 receptor in the effects of E1R. The open-field test was used to detect the effects of E1R on locomotion.

KEY RESULTS
Pretreatment with E1R enhanced the selective sigma-1 receptor agonist PRE-084's stimulating effect during a model study employing electrically stimulated rat vasa deferentia and an assay measuring the BK-induced $[Ca^{2+}]_i$ increase. Pretreatment with E1R facilitated PA retention in a dose-related manner. Furthermore, E1R alleviated the scopolamine-induced cognitive impairment during the PA and Y-maze tests in mice. The *in vivo* and *in vitro* effects of E1R were blocked by treatment with the selective sigma-1 receptor antagonist NE-100. E1R did not affect locomotor activity.

CONCLUSION AND IMPLICATIONS
E1R is a novel 4,5-disubstituted derivative of piracetam that enhances cognition and demonstrates efficacy against scopolamine-induced cholinergic dysfunction in mice. These effects are attributed to its positive modulatory action on the sigma-1 receptor and this activity may be relevant when developing new drugs for treating cognitive symptoms related to neurodegenerative diseases.

Abbreviations
BK, bradykinin; $[Ca^{2+}]i$, intracellular calcium ion concentration; E1R, (4R,5S)-2-(5-methyl-2-oxo-4-phenyl-pyrrolidin-1-yl)-acetamide; NE-100, 4-methoxy-3-(2-phenylethoxy)-N,N-dipropylbenzeneethanamine hydrochloride; PA, passive avoidance; PB-28, 1-cyclohexyl-4-[3-(1,2,3,4-tetrahydro-5-methoxy-1-naphthalenyl)propyl]piperazine dihydrochloride; PRE-084, 2-(4-morpholinethyl) 1-phenylcyclohexanecarboxylate hydrochloride

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Introduction

The sigma receptor was first identified as an opiate receptor subtype (Martin *et al.*, 1976). More than two decades ago, this separate receptor class was found to be expressed in both the periphery and CNS (Su *et al.*, 1988; Quirion *et al.*, 1992; Hanner *et al.*, 1996). Later, the sigma receptor was found to consist of two pharmacologically distinct subtypes, namely the sigma-1 and sigma-2 receptors (Hellewell *et al.*, 1994). Although sigma-1 receptors are localized in peripheral organs, they are expressed most abundantly in the CNS, where they participate in various cellular functions, including inositol 1,4,5-trisphosphate receptor-mediated Ca^{2+} signalling, ion channel firing, protein kinase localization and activation, cellular redox homeostasis, neurotransmitter release, inflammation, cellular differentiation, neuronal survival and synaptogenesis (Matsuno *et al.*, 1993; Hayashi and Su, 2007; Su *et al.*, 2010; Hayashi *et al.*, 2011). Accumulating evidence suggests that the sigma-1 receptor plays an important role in the pathophysiology of many neurological and psychiatric disorders, such as Alzheimer's disease, amnesia, pain, depression, schizophrenia, stroke and addiction (Maurice and Su, 2009; Hayashi *et al.*, 2011; Niitsu *et al.*, 2012). These findings indicate that the sigma-1 receptor may be an emerging CNS drug target.

Activity at sigma-1 receptors has been identified in several established CNS drugs and newly synthesized compounds (Cobos *et al.*, 2008; Su *et al.*, 2010). Both agonists and antagonists of sigma-1 receptors have been studied in an attempt to elucidate their possible pharmacological applications, which mainly involve learning and memory processes, depression and anxiety, schizophrenia, analgesia and some effects caused by certain drugs of abuse (Maurice and Lockhart, 1997; Monnet and Maurice, 2006; Cobos *et al.*, 2008; Banister and Kassiou, 2012). For example, the antidepressant fluvoxamine is a selective 5-HT reuptake inhibitor that possesses a high affinity for sigma-1 receptors (Narita *et al.*, 1996). In addition, donepezil is the most widely prescribed drug for Alzheimer's disease; it also binds to sigma receptors in the brain and occupies numerous sigma-1 receptors in the human brain at therapeutic doses (Ishikawa *et al.*, 2009). Sigma-1 ligands have demonstrated anti-amnesic actions in many studies of cholinergic hypofunction induced by pharmacological cholinergic receptor blockade (Earley *et al.*, 1991; Matsuno *et al.*, 1994), centrally administered neurotoxic agents [including β_{25-35}-amyloid peptides (Maurice *et al.*, 1996; 1998), ibotenic acid (Senda *et al.*, 1998)] and intraventricularly injected 192IgG-saporin (a selective immunotoxin; Antonini *et al.*, 2009). In addition, sigma-1 receptor ligands dose-dependently increased the extracellular acetylcholine level in rats' frontal cortices and hippocampi while leaving the striatum unaffected. Such absence of increased striatal acetylcholine levels after administering sigma-1 receptor agonists might explain why these drugs do not display the undesired side effects that are frequently observed after administering acetylcholinesterase inhibitors (Matsuno *et al.*, 1992; 1993; van Waarde *et al.*, 2011). During clinical studies, some sigma-1 receptor agonists, including fluvoxamine, donepezil and neurosteroids, improved the cognitive impairment and clinical symptoms associated with neuropsychiatric diseases (Silver and Shmugliakov, 1998; Kunitachi *et al.*, 2009; Marx *et al.*, 2009; Niitsu *et al.*, 2012). Recently, we described a novel compound called E1R ((4R,5S)-2-(5-methyl-2-oxo-4-phenyl-pyrrolidin-1-yl)-acetamide; Figure 1), an enantiomer of 4,5-disubstituted piracetam (2-(5-methyl-2-oxo-4-phenyl-pyrrolidin-1-yl)-acetamide; Kalvins *et al.*, 2011; Veinberg *et al.*, 2013). Its nootropic activity prompted further research into its molecular mechanisms of action and E1R was screened against a commercially available panel of 77 radioligand-binding assays, which provided evidence for the sigma receptor modulatory activity of E1R.

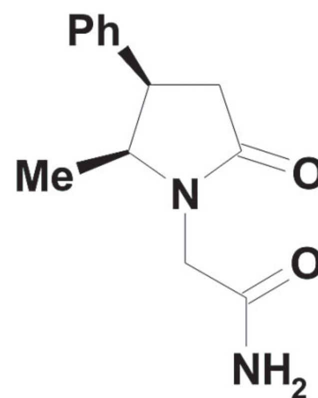

Figure 1
Structure of E1R.

In the present study, we characterized the mechanism of action of E1R on [^{3}H](+)-pentazocine binding, on the bradykinin (BK)-induced increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and on the function of peripheral sigma-1 and sigma-2 receptors, using the electrically stimulated rat vas deferens. The effects of E1R on cognition and locomotion were evaluated using passive avoidance (PA), Y-maze and open-field tests. Because sigma-1 receptor agonists potently modulate acetylcholine release (Matsuno *et al.*, 1994; van Waarde *et al.*, 2011), we used scopolamine-induced amnesia as an experimental model for the memory impairment caused by cholinergic dysfunction. The rota-rod, traction and cylinder tests were used to evaluate the influence of E1R on muscle strength and coordination. The results of these tests indicated that the cognition-enhancing properties of E1R were related to its modulatory activity at the sigma-1 receptor.

Methods

Animals

All animal care and experimental procedures complied with the guidelines reported in EU Directive 2010/63/EU and with local laws and policies; all of the procedures were approved by the Latvian Animal Protection Ethical Committee of Food and Veterinary Service in Riga, Latvia. Studies involving animals are reported in accordance with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). We used a total of 255 ICR male mice in the PA test, 40 in the open-field test, 25 in the muscle strength and coordination tests, 76

Balb/c male mice in the Y-maze test, 6 Wistar rats in the radioligand-binding assays and 5 Wistar rats in the isolated vas deferens mode. Male ICR and Balb/c mice weighed 23–25 g, while Wistar rats weighed 220–250 g (Laboratory Animal Breeding Facility, Riga Stradins University, Riga, Latvia); all animals were housed under standard conditions (21–23°C, 12 h light–dark cycle) with unlimited access to standard food (Lactamin AB, Mjölby, Sweden) and water.

Radioligand-binding assays

High-throughput profile. E1R was profiled in a commercially available panel of 77 radioligand-binding assays (CEREP, Poitiers, France). The molecular receptor nomenclature used throughout this paper conforms to the BJP's Concise Guide to Pharmacology (Alexander *et al*., 2013). A specific list of the assays performed with E1R is documented in the Results section of this paper, and further details regarding the methods used to conduct each assay are available from at http://www.cerep.fr/cerep/users/pages/catalog/profiles/DetailProfile.asp?profile=2118.

[^{3}H](+)-*pentazocine binding assay*. Binding experiments were carried out in the crude synaptosome fraction, obtained from Wistar rats, as described previously (Cobos *et al*., 2006). Membrane fraction aliquots were diluted with incubation buffer (50 mM Tris-HCl, pH 7.4) to reach a final protein concentration of 4–7 mg·mL^{-1}. E1R and PRE-084 were dissolved in saline, while haloperidol was dissolved in dimethyl sulfoxide at a concentration of 10 mM as stock solutions. Stock solutions were diluted with incubation buffer to the required concentrations (0.1 nM–100 μM). Dilutions at 1:1000 (v/v) from the stock [^{3}H](+)-pentazocine solution were prepared using deionized water. The binding assay buffer consisted of 60 μL of incubation buffer, 100 μL membrane aliquots, 20 μL of the tested drugs or incubation buffer for the control and 20 μL [^{3}H](+)-pentazocine. Before radioligand was added, membranes were incubated together with tested compounds for 10 min at room temperature. Non-specific binding was assessed by adding haloperidol (10 μM). The samples were incubated for 150 min at 30°C. The bound and free radioligands were separated by rapid filtration under a vacuum using Millipore GF/B filter paper (Merck Millipore, Billerica, MA, USA). The filters were washed three times with 0.25 mL of 10 mM Tris (pH 8.0, 4°C). The radioactivity in samples was measured with a liquid scintillation counter Wallac MicroBeta TriLux (PerkinElmer, Waltham, MA, USA) with a 60% efficiency. Each experiment was repeated at least three times and each assay was conducted in duplicate.

Measurement of the BK-induced increase in the $[Ca^{2+}]_i$

NG-108 cells were purchased from LGC Standards AB, Boras, Sweden. Cells were cultured and differentiated with the procedure described previously (Yamada *et al*., 2006). The changes in $[Ca^{2+}]_i$ were studied using a Fluo-4 NW Calcium Assay Kit (Invitrogen, Stockholm, Sweden) according to the manufacturer's instructions. The NG-108 cells were loaded with Fluo-4 NW for 45 min. The Fluo-4 NW-loaded cells were pre-incubated with 10 μM E1R, 2 μM PRE-084 or both in the dark at room temperature for 15 min. Pre-incubation with deionized water was used as a control. Subsequently, 1 μM BK was added to the wells to increase the $[Ca^{2+}]_i$. The changes in $[Ca^{2+}]_i$ were measured using the fluorescence emitted at 516 nm, which was generated by excitation at 494 nm, using the Fluoroskan Ascent Microplate Fluorometer (Thermo Labsystems, Helsinki, Finland). 40 μM NE-100 was used as the positive control and was pre-incubated with the cells for 20 min before the measurements were taken.

The selected compounds were diluted with deionized water. Cell survival was determined indirectly by measuring the total cellular protein via the Kenacid Blue R (KBR) dye-binding method (Clothier, 1995). The obtained relative fluorescence units (RFU) were standardized to the total cellular protein of each sample (RFUs = RFU $OD_{(KBR)}{}^{-1}$). The responses to the BK-induced $[Ca^{2+}]_i$ changes with or without pre-incubation with the test compounds were calculated as the per cent increase in the basal RFUs.

Sigma *receptor activity model of isolated vas deferens*

Wistar rats were decapitated. Both vasa deferentia were excised and immersed in an ice-cold Krebs–Henseleit buffer solution (content in mmol·L^{-1}: NaCl 118.0, KCl 4.75, $CaCl_2$ 2.52, $MgCl_2$ 1.64, $NaHCO_3$ 24.88, K_2HPO_4 1.18, glucose 10.0 and EDTA 0.05). Cleaned proximal portions of each vas deferens (~15 mm) were mounted in 50 mL organ baths and incubated in a Krebs–Henseleit buffer solution that was maintained at 32°C and bubbled with 95% CO_2 and 5% O_2 (Pubill *et al*., 1998). The passive tension was fixed at 1 g, and the buffer solution in the organ bath was changed every 15 min. After a 60 min adaptation period, the isolated vasa deferentia were stimulated with an electrical current (0.1 Hz, pulse duration of 1 ms 50 V). When the stimulation produced a stable contraction amplitude, cumulative doses (from 1 to 100 μM) of the sigma-1 receptor agonist PRE-084 were added. After reaching the plateau contraction amplitude, at the highest studied PRE-084 concentration (100 μM), the electrical stimulation was turned off, and each isolated vas deferens was washed several times with a Krebs–Henseleit buffer solution. After 30 min, electrical stimulation was resumed under the same parameters. When the electrical current induced a stable contraction amplitude, E1R was added to each isolated vas deferens at a concentration of 10 μM. After 10 min of electrical stimulation, cumulative doses of PRE-084 were added. To test for sigma-2 receptor activity, a selective sigma-2 receptor agonist PB-28; at concentrations ranging from 1 to 10 μM was used in a similar experimental set-up. Responses to selective sigma receptor agonists before and after the addition of the test compound were calculated as the percentage increase in the baseline contraction amplitude.

E1R dosing in vivo

In the PA test, the animals received an i.p. injection of E1R at doses of 0.1, 1 and 10 mg·kg^{-1} 60 min before training. The effect of E1R on scopolamine-induced cognitive deficits was assessed using the PA test, where E1R was administered i.p. at doses of 1, 5 and 10 mg·kg^{-1} 60 min before the training session and scopolamine was administered s.c. at a dose of 0.3 mg·kg^{-1} 20 min after the E1R injection. NE-100 was administered i.p. at a dose of 2 mg·kg^{-1} 20 min before E1R,

which was administered at a dose 5 mg·kg^{-1} 60 min before the acquisition trial in the scopolamine-induced cognitive deficit test. Prior to Y-maze test, the animals received an i.p. injection of E1R at a dose of 10 mg·kg^{-1} 60 min before the experiment, scopolamine was administered s.c. at a dose of 0.5 mg·kg^{-1} 20 min after E1R injection, and NE-100 was administered i.p. at a dose of 2 mg·kg^{-1} 20 min prior to E1R. In the open-field test, E1R was administered i.p. at doses of 1, 10 and 100 mg·kg^{-1} 30 min prior to experimentation. The control groups received an i.p. injection of saline. In the rota-rod, traction and chimney tests, measurements were made before i.p. administration of E1R at doses of 50, 100, 250, 500 and 630 mg·kg^{-1} and again at 30, 60, 120, 180 and 240 min after i.p. administration. All drugs were dissolved in 0.9% physiological saline (Fresenius Kabi, Warszawa, Poland) during the *in vivo* experiments.

Behavioural experiments

PA test. The PA test was performed as previously described (Zvejniece *et al.*, 2011). Briefly, on the training day, each mouse was individually placed in the light compartment of an apparatus with no access to the dark compartment and allowed to explore for 60 s (Ugo Basile, Comerio, Italy). After this time, the sliding door (4 × 4 cm) was automatically opened and the mouse was allowed to cross over into the dark compartment. Upon entering the dark compartment, the mouse received a shock of 0.1 mA for 3 s, the door was closed, and the mouse was returned to its home cage after 20 s. A retention test was performed on the next day (24 h later) without any shock. The time taken to enter the dark compartment was recorded as the retention latency. The maximum retention latency was set at 540 s.

Scopolamine-induced cognitive deficits in the PA test. The test was performed in essentially the same manner described in the PA test, with the exception that mice received a shock of 0.4 mA for 3 s.

Scopolamine-induced cognitive deficits in the Y-maze test. Working memory performance was assessed by recording spontaneous alternation behaviour in a Y-maze, as previously described (Yamada *et al.*, 1999). The experiment was conducted in a dim red-lit room. The mice were individually placed at the end of one arm in a symmetrical Y-shaped runway (arm length 35 cm, width 5 cm, height 21 cm) and allowed to explore the maze for 5 min. An alternation was defined as consecutive entries into all three arms. The total number and sequence of the arm entries were manually recorded, and the percentage of alternation was calculated (Yamada *et al.*, 1999).

Open-field test. To test the effects of E1R on locomotor activity, the open-field test was used. The test apparatus was a square arena (44 × 44 cm) with a black floor. The mice were gently placed in the centre of the field, and behavioural parameters were recorded using the EthoVision video tracking system (version 3.1., Noldus, Wageningen, The Netherlands). The distance moved (cm·4 min^{-1}) and velocity (cm·s^{-1}) were recorded. Testing consisted of five successive 4 min sessions that started 30, 60, 120, 180 and 240 min after compound administration.

Muscle strength and coordination. A rota-rod test was used to measure motor coordination (Model 7600, Ugo Basile). One day before the experiment, the animals were trained on the apparatus. On the day of the experiment, the animals were placed on a rota-rod (16 rpm), and the number of animals that fell off of the rota-rod within the 180 s session was recorded. The effect of drugs on motor performance was also tested using the chimney test (Dambrova *et al.*, 2008). In this test, mice had to climb backwards up a Pyrex glass tube (30 cm length, 3 cm inner diameter). Mice successfully reaching the 20 cm mark within 30 s were selected for further testing. The effect of drugs on muscle strength was examined using the traction test. Hence, the forepaws of a mouse were placed on a firmly fixed horizontal stick. The untreated mice grasped the stick with both forepaws and, when allowed to hang free, placed at least one hind foot on the stick within 5 s. Inability to perform this task was scored as a failure of traction.

Data analyses

The results are expressed as means ± SEM. The electrical current-induced contraction amplitudes of the isolated vasa deferentia were analysed using two-way repeated measures ANOVA followed by Bonferroni *post hoc* testing. Data for the BK-induced increase in $[Ca^{2+}]_i$ were analysed using one-way ANOVA followed by Tukey's test. For the PA and Y-maze experiments, data were analysed using one-way ANOVA followed by the Newman–Keuls test. For dose-related effects of E1R on the scopolamine-induced impairment of PA experiments, statistical analysis was performed using one-way ANOVA followed by the Mann–Whitney U-test. *P*-values less than 0.05 were considered statistically significant. The statistical calculations were performed using the GraphPad Prism 3.0 software package (GraphPad Software, Inc., La Jolla, CA, USA). The ED_{50} values were obtained by probit analysis.

Materials

E1R was prepared at the Latvian Institute of Organic Synthesis according to a previously published procedure (Kalvins *et al.*, 2011; Veinberg *et al.*, 2013). Haloperidol was purchased from Alfa Aesar, Karlsruhe, Germany. Bradykinin, NE-100, PRE-084 and PB-28 were purchased from Tocris Bioscience, Bristol, UK. (-)Scopolamine hydrochloride was obtained from Fluka (St. Louis, MO, USA). $[^3H]$(+)-pentazocine specific activity 33.9 Ci·mmol^{-1} was purchased from American Radiolabeled Chemicals, St. Louis, MO, USA.

Results

In vitro *selectivity profiling of E1R*

The pharmacological profiling of E1R against various possible targets was performed using a commercially available radioligand-binding assay screen that was performed by CEREP (see Methods). E1R at a 10 μM concentration had little or no activity in 77 radioligand displacement assays that included numerous ion channel, GPCR and CNS transporter targets (Supporting Information Table S1). The only target for E1R (inhibition or enhancement of radioligand binding exceeding 20%) was the sigma receptor. Here 10 μM E1R did

not displace the radioligand, but instead increased the specific binding of a non-selective radioligand ([^{3}H]1,3-di(2-tolyl)guanidine) for the sigma receptor by 38% in Jurkat cells (Supporting Information Table S1). In the same assay, the sigma receptor antagonist haloperidol inhibited the binding of the radioligand with an IC_{50} = 43 nM.

Action of E1R on [^{3}H](+)pentazocine binding

Unlike the selective sigma-1 receptor agonist PRE-084 (IC_{50} = 192 nM) or the non-selective sigma receptor antagonist [haloperidol (IC_{50} = 0.5 nM)], E1R did not displace [^{3}H](+)-pentazocine from the sigma-1 receptors (Figure 2). As seen in Figure 2, E1R did not modulate binding of [^{3}H](+)-pentazocine in this binding assay. It should be noted that we also failed to demonstrate sigma-1 receptor modulatory effect for phenytoin in this assay (data not shown).

Effects of E1R on the BK-induced increase of [Ca^{2+}]$_i$ in NG-108 cells

The selective sigma-1 receptor agonist PRE-084 at 2 μM enhanced the BK-induced [Ca^{2+}]$_i$ increase in NG-108 cells and E1R (10 μM) also enhanced the increase of [Ca^{2+}]$_i$ (Figure 3, $F_{7,75}$ = 94.15, P < 0.0001). Moreover, the effects of PRE-084 on the [Ca^{2+}]$_i$ changes were potentiated three times after pre-incubation with E1R (Figure 3, P < 0.001). The effects of PRE-084, E1R and their combination were antagonized by administering a selective sigma-1 receptor antagonist, NE-100, at 40 μM (Figure 3, $F_{7,75}$ = 94.15, P < 0.0001).

Effects of E1R on sigma-1 and sigma-2 receptors in the rat isolated vas deferens

The addition of cumulative doses of E1R did not influence the contractions of electrically stimulated rat vasa deferentia (Figure 4A) but these contractions were potentiated in the presence of the sigma-1 receptor agonist PRE-084 (100 μM) (Figure 4A,C; $F_{4,29}$ = 38.35, P < 0.0001). Pre-incubation of vasa deferentia with a 10 μM solution of E1R for 10 min prior to the addition of PRE-084 significantly increased the intensity of the contractions [Figure 4A,C; a two-way repeated ANOVA confirmed that the group ($F_{1,10}$ = 6.94, P < 0.05), dose ($F_{4,40}$ = 64.07, P < 0.0001) and dose-by-group interactions ($F_{4,40}$ = 3.18, P < 0.05) were the main effects]. The electrically stimulated contractions of rat vasa deferentia were also increased by the sigma-2 receptor agonist PB-28 (Figure 4B,D), but pretreatment with E1R did not affect this response to PB-28 [Figure 4B,D; in a two-way repeated ANOVA, dose ($F_{4,24}$ = 15.73, P < 0.0001) was a main effect, but there were no effects of the group (P > 0.05) or dose-by-group interactions (P > 0.05)].

Effects of E1R on cognition in the PA test

The PA test was used to examine the cognition-enhancing activity of E1R in mice. The retention latency, which was measured as response to a foot shock of 0.1 mA for 3 s, in control animals was 76 ± 16 s. Treatment with E1R significantly improved cognitive function in a dose-related manner ($F_{3,64}$ = 4.363, P < 0.01). As shown in Figure 5A, treatment with E1R at doses of 1 and 10 mg·kg^{-1} increased retention latency by 194 and 211%, respectively, compared with the control group. There were no differences in dark compartment entrance during training between the control and E1R-treated groups (data not shown).

The PA test was also used to detect the effects of E1R on scopolamine-induced memory impairment. Pretreatment with scopolamine markedly reduced the control (saline injection) retention latency (Figure 5B, P < 0.0001). E1R, at 5 and 10 mg·kg^{-1}, increased the retention latency of scopolamine-treated animals, by 237 and 209%, respectively ($F_{4,89}$ = 6.91, P < 0.0001). Treatment with the selective sigma-1 receptor

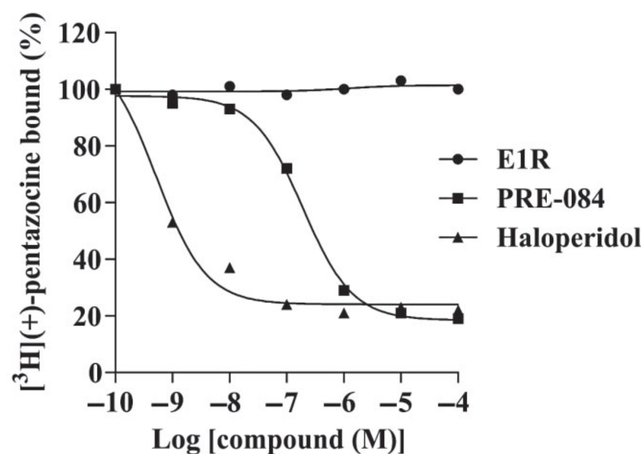

Figure 2

The effects of E1R and sigma receptor ligands on the binding of [^{3}H](+)-pentazocine to a sigma-1 receptor. Synaptosomes from rat brains were incubated with 1.5 nM [^{3}H](+)-pentazocine at 30°C for 150 min. Haloperidol (10 μM) was used to define non-specific binding. The data represent at least three experiments performed in duplicate.

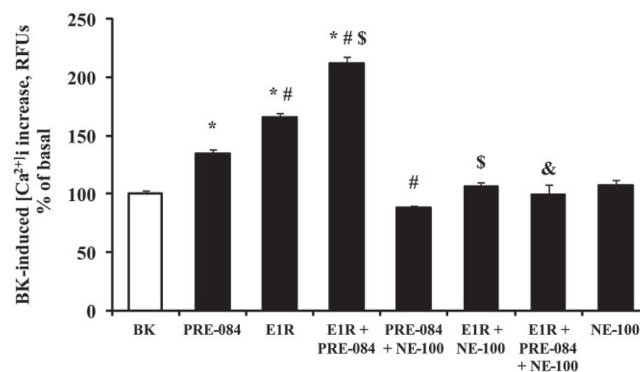

Figure 3

The effect of E1R, the selective sigma-1 receptor agonist PRE-084 and antagonist NE-100, as well as their combinations on 1 μM BK-induced [Ca^{2+}]$_i$ increase in NG-108 cells. The cells were pre-incubated with 10 μM E1R, 2 μM PRE-084 or both in the dark at room temperature for 15 min. 40 μM NE-100 was pre-incubated with the cells for 20 min before the measurements were taken. Changes in the [Ca^{2+}]$_i$ were calculated as the percentage increase of the basal RFUs. Each column represents the mean ± SEM. *P < 0.05 versus BK, #P < 0.05 versus PRE-084, $$P$ < 0.05 versus E1R, &P < 0.05 versus E1R and PRE-084 combination.

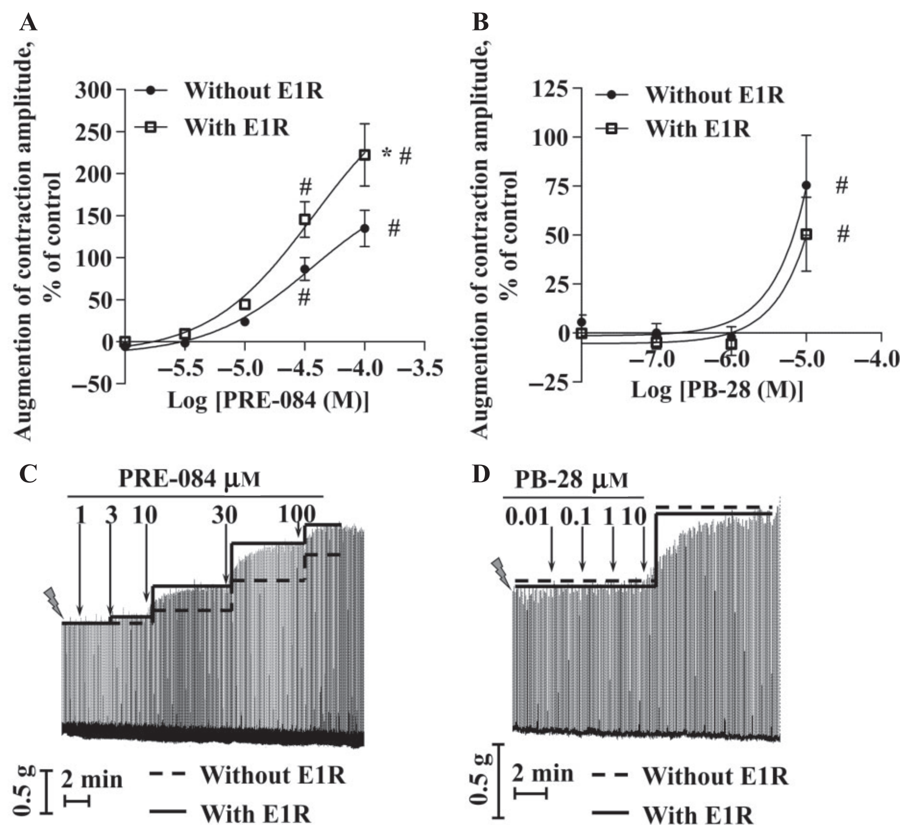

Figure 4

Sigma receptor activity assay in the electrically stimulated rat isolated vas deferens. (A,C) Effects of E1R (10 μM) on contractions potentiated by the selective sigma-1 receptor agonist (PRE-084). The results are expressed as the percentage of control contraction height and represent the means ± SEM; $n = 6$. (B,D) Effects of E1R (10 μM) on the selective sigma-2 receptor agonist (PB-28) in electrically stimulated rat vasa deferentia. The results are expressed as the percentage of control contraction height and represent the means ± SEM; $n = 4$. $^{*}P < 0.05$ versus PRE-084 treatment (as analysed using two-way repeated ANOVA followed by the Bonferroni *post hoc* test), $^{\#}P < 0.05$ versus the baseline concentration.

antagonist NE-100 inhibited the cognition-enhancing activity of E1R at a dose of 5 mg·kg^{-1} (Figure 5C, $F_{4,96} = 14.45$, $P < 0.0001$). The training latencies did not differ between the saline control, the scopolamine- and the E1R-treated groups (data not shown).

Effects of E1R on cognition in Y-maze test

The Y-maze test was used to detect effects of E1R on scopolamine-induced impairment of working memory (Figure 6). The spontaneous alternation behaviour in the control animals was reduced by pretreatment with scopolamine ($P < 0.001$). As shown in Figure 6, treatment with E1R (10 mg·kg^{-1}) increased the spontaneous alternation behaviour, compared with the scopolamine-treated group ($F_{4,71} = 6.19$, $P < 0.0002$). Treatment with the selective sigma-1 receptor antagonist NE-100 (2 mg·kg^{-1}) significantly inhibited the enhancement of working memory of E1R at a dose of 10 mg·kg^{-1} ($P < 0.05$).

Effects of E1R on locomotion

The open-field test was used to determine the influence of the compounds on locomotor activity. Doses of E1R up to 100 mg·kg^{-1} did not affect the distance moved, compared with the control animals [Table 1, two-way repeated ANOVA, main effect of group ($F_{3,28} = 6.86$, $P > 0.05$), time ($F_{4,112} = 49.99$, $P < 0.0001$) and time-by-group interaction ($P > 0.05$)].

Effects of E1R on muscle strength and coordination

In the rota-rod, chimney and traction tests, an inhibitory activity of E1R on muscle function was observed, with the following ED_{50} values (with ED_{16}–ED_{84}): 453 (398–516), 349 (199–611) and 595 (409–866) mg·kg^{-1} respectively.

Discussion

In the present study, we characterized a novel enantiomer of 4,5-disubstituted piracetam, E1R, as both a positive allosteric modulator of the sigma-1 receptor and a cognition enhancer. The compound had no effect on locomotor activity, muscle tone or coordination at doses up to 200 mg·kg^{-1}. Therefore, E1R was found to be free of potential motor side effects. The sigma receptor target site was the only site that E1R was discovered to target in the *in vitro* pharmacological profiling

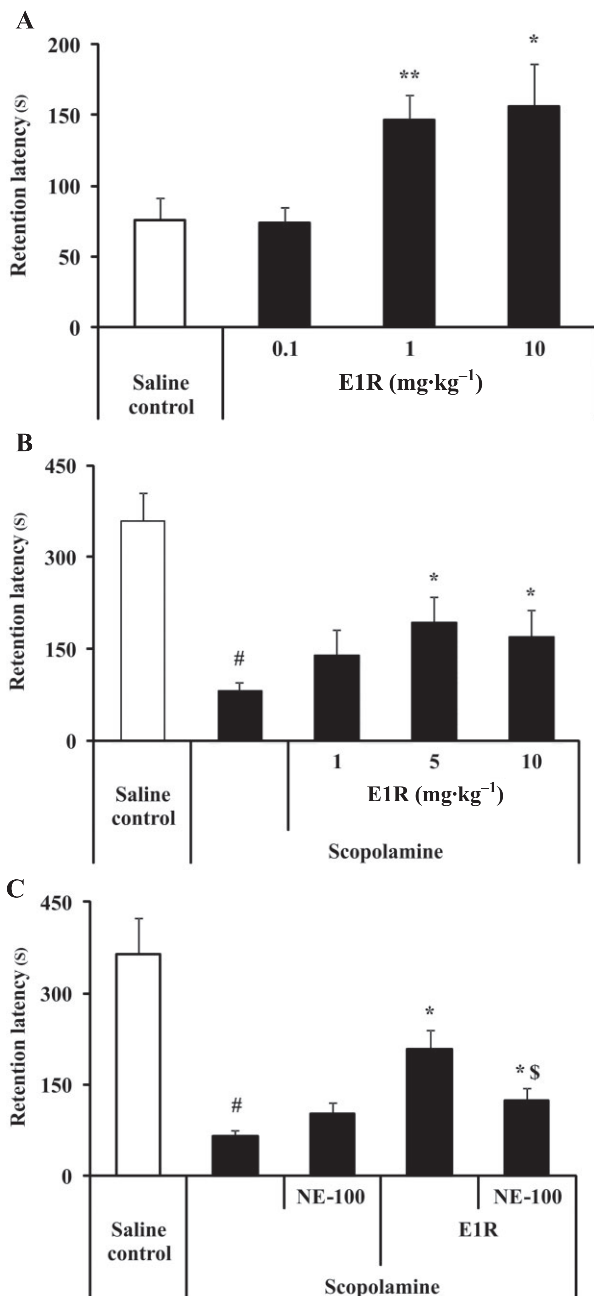

Figure 5

(A) Dose-related effects of E1R on PA retention in mice. E1R (0.1, 1 and 10 mg·kg^{-1}, i.p.) was administered 60 min before the training session. The retention test was performed 24 h later. The vertical bars represent the means ± SEM; n = 15–18. *P < 0.05 and **P < 0.01 versus the saline group. (B) Dose-related effects of E1R on the scopolamine-induced impairment of PA retention in mice. The mice were injected with E1R (1, 5 and 10 mg·kg^{-1}, i.p.) 60 min before the training session. Scopolamine (0.3 mg·kg^{-1}, s.c.) was administered 40 min prior to the training session. The vertical bars represent the means ± SEM; n = 17–20. (C) The effect of E1R (5 mg·kg^{-1}) was antagonized by the administration of the selective sigma-1 receptor antagonist NE-100 (2 mg·kg^{-1}) Each column represents the means ± SEM; n = 20–25. $^{\#}P$ < 0.05 of the scopolamine-treated group versus the saline control group, *P < 0.05 versus the scopolamine-treated group, and $^{\$}P$ < 0.05 versus the E1R-treated group.

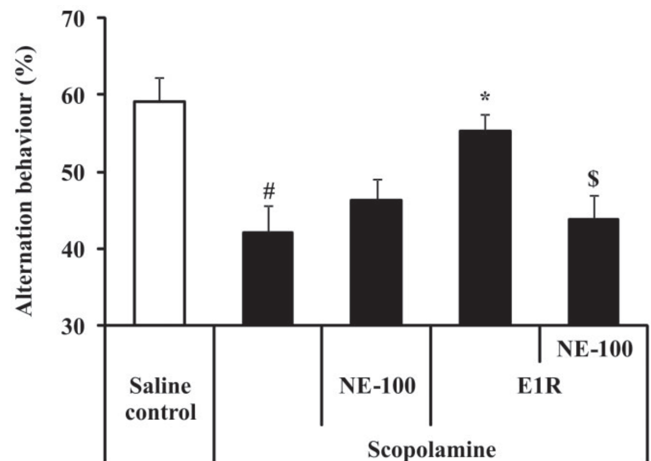

Figure 6

The effect of E1R on scopolamine-induced impairment of spontaneous alternation behaviour in the Y-maze test in mice. Mice were injected with E1R (10 mg·kg^{-1} i.p.) 60 min before the training session. Scopolamine (0.5 mg·kg^{-1}, s.c.) was administered 40 min before the training session. The effect of E1R was antagonized by the administration of the selective sigma-1 receptor antagonist NE-100 (2 mg·kg^{-1}) 20 min before E1R. The data are presented as the mean % of alternation behaviour ± SEM; n = 14–16. $^{\#}P$ < 0.05 of the scopolamine-treated group versus the saline group, *P < 0.05 versus the scopolamine-treated group, and $^{\$}P$ < 0.05 versus the E1R-treated group.

assays conducted, including a number of ion channels, GPCRs and CNS transporter targets. Our *in vitro* assays revealed that E1R did not bind directly to the sigma-1 receptors, but rather acted as a positive allosteric modulator. It should be noted that E1R enhanced the binding of an non-selective sigma receptor radioligand [^{3}H]1,3-di(2-tolyl) guanidine but we failed to demonstrate any modulatory effects of E1R on sigma-1 receptors, using the selective sigma-1 receptor radioligand, [^{3}H](+)-pentazocine binding assay. However, E1R potentiated the contractions of rat vasa deferentia in the presence of the sigma-1 receptor agonist PRE-084 and not in the presence of the sigma-2 receptor agonist PB-28 (Figure 4). In addition, E1R enhanced the effect of PRE-084 on the BK-induced $[Ca^{2+}]_i$ increase (Figure 3) thus confirming the positive allosteric modulation of sigma-1 receptors *in vitro*.

Several lines of evidence have suggested that activation of sigma-1 receptors ameliorates cognitive deficits in animal models of cholinergic dysfunction that mimic the cognitive symptoms of Alzheimer's disease (Earley *et al*., 1991; Matsuno *et al*., 1994; Maurice *et al*., 1998; Antonini *et al*., 2009; Maurice and Su, 2009; Hayashi *et al*., 2011). In addition, sigma-1 receptor agonists act as potent modulators of acetylcholine release (Matsuno *et al*., 1993; van Waarde *et al*., 2011). Because our *in vitro* studies identified E1R as a positive allosteric modulator of the sigma-1 receptor, we hypothesized that E1R might protect against scopolamine-induced cognitive deficits. E1R successfully alleviated the scopolamine-induced cognitive impairment assessed during the PA and Y-maze tests in mice. The effects of E1R were antagonized by the selective sigma-1 receptor antagonist NE-100, confirming

Table 1

The effects of E1R on moved distance in the open-field test

	Moved distance, cm (4 min)$^{-1}$				
Dose (mg kg^{-1} i.p.)	30 min	60 min	120 min	180 min	240 min
Saline	1124 ± 121	623 ± 136	578 ± 126	436 ± 125	398 ± 70
E1R 1	1402 ± 101	871 ± 111	688 ± 82	559 ± 84	626 ± 110
E1R 10	1026 ± 65	640 ± 73	472 ± 60	406 ± 62	359 ± 58
E1R 100	1076 ± 78	648 ± 71	470 ± 75	450 ± 87	458 ± 35

Data represent the means ± SEM; *n* = 10.

the sigma-1 receptor modulatory activity of E1R *in vivo*. Therefore, we propose that the cognition-enhancing effects of E1R may involve modulation of the activity of endogenous agonists of sigma-1 receptors. To date, memory enhancements have been observed only for endogenous sigma-1 receptor agonists including dehydroepiandrosterone and its sulphate (Roberts *et al*., 1987; Flood *et al*., 1988); exogenous sigma-1 receptor agonists induce memory enhancements only in amnesia models.

The neurosteroids are considered to be the most probable endogenous sigma-1 receptor ligands (Cobos *et al*., 2008; Niitsu *et al*., 2012). Neurosteroids such as pregnenolone and dehydroepiandrosterone are known to bind to sigma-1 receptors under physiological conditions, and sigma-1 receptors constitute one of the key targets in their trophic, neuromodulatory and behavioural effects (Su *et al*., 1988; Monnet and Maurice, 2006). Pregnenolone, dehydroepiandrosterone and other nonsteroidal sigma-1 receptor agonists, affect the learning and memory processes in cholinergic and NMDA receptor-dependent models of amnesia and aging (Maurice *et al*., 2001; Monnet and Maurice, 2006). A significant correlation between the levels of pregnenolone in the hippocampus of aged rats and memory performance has been observed (Robel *et al*., 1995). Interestingly, sigma-1 receptor density is frequently preserved during aging (van Waarde *et al*., 2011).

Apart from E1R, few positive allosteric modulators of sigma-1 receptors have been described (Cobos *et al*., 2008; Guo *et al*., 2013). Phenytoin has been reported to decrease motor activity in mice (Poncelet *et al*., 1984), reduce increases in extracellular K^+ concentrations (Nobile and Lagostena, 1998) and inhibit both Na^+ (Rush and Elliott, 1997) and T-type Ca^{2+} currents (Todorovic and Lingle, 1998). Unlike E1R, treatment with phenytoin triggered memory impairment during the PA task (Reeta *et al*., 2009). Consequently, we examined the effects of E1R on locomotor activity using the open-field test. Unlike phenytoin, E1R did not affect locomotion at doses up to 100 mg·kg^{-1} (Table 1) and did not influence Na^+ and K^+ channels in pharmacological profiling assays (Supporting Information Table 1). Therefore, E1R is the first reported positive allosteric modulator of sigma-1 receptors that enhances cognition without affecting locomotor activity.

Recently published reviews suggest a renewed interest in sigma-1 receptors (Maurice and Su, 2009; Kourrich *et al*., 2012) and a need for further research in this field (Abate, 2012). Therefore, because E1R has unique pharmacological and behavioural profiles as well as low toxicity, it may become a useful tool for detailed studies of sigma-1 receptors and an emerging drug target in CNS pharmacology. The pharmacological profile of E1R may be of particular relevance for the development of new therapies for the treatment of cognitive disorders, including those that are associated with neurodegenerative diseases. In support of this idea, some of the currently approved Alzheimer's disease medications, such as the cholinesterase inhibitor donepezil, are potent sigma-1 receptor ligands (Kato *et al*., 1999). The symptomatic and potential neuroprotective effects of donepezil in Alzheimer's disease (Francis *et al*., 2005) may arise from both direct and indirect cholinergic mechanisms, as well as an interaction with the sigma-1 receptor, as this receptor provides neuroprotection against glutamate and amyloid toxicities.

The memory-improving effects of E1R in both drug-naïve and scopolamine-treated mice in the PA test are of particular interest because of the piracetam-like structure of E1R. Many racetams share piracetam's nootropic properties in several mammalian species ranging from rodents to humans (Froestl and Maitre, 1989; Gouliaev *et al*., 1995; Malykh and Sadaie, 2010). Racetams enhance performance during various learning and memory tasks, particularly in the PA test in mice and rats (Mondadori *et al*., 1989; Krylova *et al*., 1991; Zvejniece *et al*., 2011). Piracetam and its derivatives are known to alleviate memory deficits caused by scopolamine and other amnesic drugs, as well as electroconvulsive shock and hypoxia (Malykh and Sadaie, 2010; Zvejniece *et al*., 2011). Recent studies have indicated that phenylpiracetam and its most active R-enantiomer (R-phenylpiracetam) possesses both memory-improving activity in the PA task and motor-stimulant properties in the open-field test (Tiurenkov *et al*., 2007; Zvejniece *et al*., 2011). E1R is a close structural analogue of these two compounds, which differ in structure by only one methyl group (Kalvins *et al*., 2011; Zvejniece *et al*., 2011; Veinberg *et al*., 2013), suggesting that E1R may exhibit similar behavioural effects. However, our present data show that although E1R exhibited cognition-enhancing activity, similar to that of R-phenylpiracetam, E1R did not affect performance in the open-field test at doses up to 100 mg·kg^{-1}. Therefore, even minor structural alterations may contribute to rather significant differences in the pharmacological activity of piracetam-like compounds.

Consequently, E1R is a unique racetam compound because it displays cognition enhancements linked to positive allosteric sigma-1 receptor modulation. To our knowledge, E1R is the first piracetam derivative reported to modulate sigma-1 receptors, prompting further studies to elucidate the exact molecular mechanisms and possible structure-activity relationships underlying its memory-improving effects.

In conclusion, E1R is a novel 4,5-disubstituted piracetam derivative that enhanced cognition and alleviated scopolamine-induced cholinergic dysfunction, without affecting locomotor activity in mice. These effects are related to the positive allosteric modulation of sigma-1 receptors by E1R. Therefore, E1R may be interesting as both a novel tool for studying sigma-1 receptor pharmacology and a novel drug candidate for treating cognitive disorders.

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Conflicts of interest

I Misane and I Stonans are employees of JSC Grindeks. There are no other conflicts to declare.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

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Table S1 The screening profile of E1R *in vitro* binding assays.