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DISCOVERY OF E1R: A NOVEL POSITIVE ALLOSTERIC MODULATOR OF SIGMA-1 RECEPTOR

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ANNOTATION

Sigma-1 receptor (Sig1R) is a new drug target. Notably, the International Union of Basic and Clinical Pharmacology included Sig1R in its list of receptors only in 2013. It is believed that Sig1R ligands are potential novel drug candidates for the treatment of central and peripheral nervous system diseases. The aim of this thesis was to evaluate the pharmacological activity of novel Sig1R ligands and to search for their possible clinical applications.

Methylphenylpiracetam (2-(5-methyl-2-oxo-4-phenyl-pyrrolidin-1-yl)-acetamide) was synthesised in the Latvian Institute of Organic Synthesis. There are two chiral centres in the molecular structure of methylphenylpiracetam; therefore, it is possible to isolate four individual stereoisomers, which are denoted E1R, T1R, E1S and T1S. The activity of E1R was profiled using commercially available screening assays, which showed that the compound selectively modulates Sig1R activity but does not affect the other investigated neuronal receptors and ion channels. The following detailed *in vitro* studies, in which more selective Sig1R ligands were used, provided solid evidence that E1R is a positive allosteric modulator of Sig1R. It was found that the enantiomers with the R-configuration at the C-4 chiral centre in the 2-pyrrolidone ring (E1R and T1R) are more effective positive allosteric modulators of Sig1R than their optical antipodes.

Since Sig1R agonists demonstrate neuroprotective properties and are effective in the treatment of cognitive disorders of various origins, the activity of E1R was tested in experimental models *in vivo*. The obtained results showed that E1R markedly improved memory function by activating Sig1R because the effects of E1R were blocked by a selective Sig1R antagonist. In addition, E1R demonstrated a significant Sig1R-dependent anti-seizure effect.

The obtained results provide evidence that E1R is a unique compound since among the six other positive allosteric Sig1R modulators identified to date, E1R is the only one that demonstrates neuroprotective activity and improves memory.

The thesis is presented as a unifying material of peer-reviewed publications, and it summarizes the study materials and results of the evaluations of the pharmacological activities of E1R. The obtained results are important for the global understanding of Sig1R physiological function and are expected to hasten the possible use of Sig1R ligands in clinical practice.

ANOTĀCIJA

Promocijas darba tēma: **E1R: jauns pozitīvs allostēriskais sigma-1 receptora modulators**

Sigma-1 receptors (Sig1R) ir jauns zāļu mērķis, kurš starptautiskajā receptoru klasifikatorā ir iekļauts tikai 2013. gadā. Tiek uzskatīts, ka Sig1R darbību regulējošie ligandi varētu būt potenciāli zāļu līdzekļi centrālās un perifērās nervu sistēmas saslimšanu ārstēšanai. Promocijas darba mērķis bija pētīt jaunu Sig1R receptora ligandu farmakoloģisko aktivitāti un meklēt praktiskos pielietojumus potenciālai izmantošanai klīnikā.

Latvijas Organiskās sintēzes institūtā tika sintezēts jauns savienojums, piracetāma atvasinājums metilfenilpiracetāms. Savienojuma molekulā ir divi hirālie centri, līdz ar to šim savienojumam ir iespējami četri dažādi stereoizomēri: E1R, T1R, E1S, T1S. Veiktais metilfenilpiracetāma aktivitātes skrīnings parādīja, ka savienojums selektīvi modulē Sig1R aktivitāti, bet neietekmē citus pētītos neironālos receptorus un jonu kanālus. Izmantojot selektīvus Sig1R ligandus un kalcija jonu transporta pētījumus šūnu kultūrās, tika pierādīts, ka visi metilfenilpiracetāma enantiomēri spēj pastiprināt selektīvo Sig1R agonistu aktivitāti, tādējādi apliecinot, ka savienojumi darbojas kā pozitīvi allostēriski Sig1R modulatori. Enantiomēri ar R konfigurāciju pie C-4 hirālā centra (E1R, T1R) uzrādīja augstāku bioloģisko aktivitāti par to optiskajiem izomēriem.

Ņemot vērā to, ka zināmajiem Sig1R agonistiem piemīt neiroprotektīvas īpašības un tie ir efektīvi dažādas izcelsmes kognitīvo traucējumu ārstēšanā, E1R darbība tika pārbaudīta eksperimentālajos modeļos *in vivo*. Iegūtie rezultāti liecina, ka E1R spēj ievērojami uzlabot atmiņas procesus, un šo procesu regulācijā ir iesaistīta Sig1R aktivācija, jo selektīvs Sig1R antagonists novērsa E1R efektus. Papildus tam E1R pārsteidzoši uzrādīja arī anti-konvulsīvu efektu, kas ir ievērojami izteiktāks nekā zināmajiem Sig1R agonistiem.

Promocijas darba rezultāti ir nozīmīgi, jo līdz šim bez E1R ir zināmi tikai 6 savienojumi, kuri darbojas kā pozitīvi allostēriski Sig1R modulatori, bet E1R ir vienīgais šāda veida savienojums, kuram ir izteikta atmiņas procesus uzlabojoša darbība un neiroprotektīvas īpašības.

Promocijas darbs veidots kā rakstu kopa, kas apkopo pētījumu materiālus. Promocijas darbā iegūtie rezultāti ir starptautiski nozīmīgi gan Sig1R receptoru fizioloģiskās lomas pētījumos, gan Sig1R ligandu tālākai virzībai izmantošanai klīniskajā praksē.

TABLE OF CONTENTS

INTRODUCTION....7
Aim of the study....7
Objectives of the study....8
Hypothesis of the study....8
Scientific novelty of the study....8
1. LITERATURE....9
1.1. Sigma-1 receptor (Sig1R)....9
1.2. Ligands of the Sig1R....11
1.3. Allosteric Sig1R modulators....13
1.4. The pharmacological activity of allosteric Sig1R modulators....16
1.5. Racetams: a compound class with unrevealed therapeutic potential....17
2. METHODS....19
2.1. Animals....19
2.2. Chemicals....19
2.3. *In vitro* methods....20
2.3.1. Radioligand binding assays....20
2.3.2. Measurement of the bradikynin (BDK)-induced increase in the $[Ca^{2+}]_i$....20
2.3.3. SigR activity model of isolated rat *vas deferens*....21
2.4. *In vivo* methods: behavioural experiments....21
2.4.1. Passive avoidance (PA) test....21
2.4.2. Scopolamine-induced cognitive deficits in the Y-maze test....22
2.4.3. Open-field test....22
2.4.4. Muscle strength and coordination....22
2.4.5. Chemoconvulsant-induced seizures....23
2.4.6. NE-100-induced seizures....23
2.4.7. E1R dosing *in vivo*....24
2.5. Data and statistical analyses....24
3. RESULTS....26
3.1. The synthesis of methylphenylpiracetam and separation of the individual stereoisomers....26
3.2. *In vitro* selectivity profiling of E1R....26
3.3. Activity of E1R on [^{3}H]-(+)-pentazocine binding....29
3.4. Effects of E1R on the BDK-induced increase of $[Ca^{2+}]_i$ in NG108-15 cells....29
3.5. Effects of E1R on Sig1R and Sig2R in an experimental model of isolated *vas deferens*....30
3.6. Effects of E1R on cognition in the PA test....31
3.7. Effects of E1R on cognition in Y-maze test....33
3.8. Effects of E1R on locomotion, muscle strength and coordination....34
3.9. Activity of E1R in the PTZ- and BIC-induced seizure models....35
3.10. Anti-seizure effects of E1R are Sig1R dependent....37
3.11. The activity of E1R on NE-100-induced seizures....38
4. DISCUSSION....41
4.1. Allosteric modulators of Sig1R....41
4.2. Modulation of Sig1R and regulation of memory and cognition....45
4.3. Sig1R ligands and seizures....47
4.4. Sig1R allosteric modulators: possible molecular mechanisms....49
5. CONCLUSIONS....53
6. APPROBATION OF THE STUDY – PUBLICATIONS AND THESIS....54
ACKNOWLEDGEMENTS....57
REFERENCES....58
SUPPLEMENTS....65

ABBREVIATIONS

ANOVA – analysis of variance

Ala – alanine

AMPA – α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

ARRIVE – Animal Research: Reporting of In Vivo Experiments

AUC – area under curve

BDK – bradykinin

BIC – (+)-bicuculline

$[Ca^{2+}]_i$ – intracellular Ca^{2+} concentration

CCK - cholecystokinin

CGRP – calcitonin gene-related peptide

CNS – central nervous system

$D_{1–5}$ – dopamine receptors

DM – dextromethorphan

DMSO – dimethyl sulfoxide

DHEA – dehydroepiandrosterone

DTG – 1,3-di(2-tolyl)guanidine

ED_{50} – median effective dose

E1R – 2-(5S-methyl-2-oxo-4R-phenyl-pyrrolidin-1-yl)-acetamide

E1S – 2-(5S-methyl-2-oxo-4S-phenyl-pyrrolidin-1-yl)-acetamide

GABA – gamma-aminobutyric acid

Gln – glutamine

Gly – glycine

HEK293 – human embryonic kidney cell line

5−HT – 5-hydroxytryptamine (serotonin)

IC_{50} – half maximal inhibitory concentration

IL−1β – interleukin-1 beta

i.p. – intraperitoneal

IP_3 – inositol 1,4,5-triphosphate receptor

i.v. – intravenous

K_i – equilibrium dissociation constant

Leu – leucine

LPS – lipopolysaccharide

$M_{1–5}$ – muscarinic receptors

NE−100 – 4-Methoxy-3-(2-phenylethoxy)-N,N-dipropylbenzeneethanamine hydrochloride

NG108−15 – neuroblastoma-glioma hybrid cell line

NMDA – N-methyl-D-aspartic acid receptor

NPY – neuropeptide Y

OD – optical density

PA – passive avoidance

PAM – positive allosteric modulator

PB−28 – 1-Cyclohexyl-4-[3-(1,2,3,4-tetrahydro-5-methoxy-1-naphthalenyl)propyl]piperazine dihydrochloride

PDGF – platelet-derived growth factor

PRE−084 – 2-(4-Morpholinethyl)-1-phenylcyclo-hexanecarboxylate hydrochloride

PTZ – pentylenetetrazole

RFU – relative fluorescence units

s.c. – subcutaneous

S.E.M. – standard error of the mean

SigR – Sigma receptor

Sig1R – Sigma-1 receptor

Sig2R – Sigma-2 receptor

SKF−10,047 – N-allylnormetazocine

SV2A – synaptic vesicle glycoprotein 2A

Thr – threonine

TNFα – tumor necrosis factor alpha

T1R – 2-(5R-methyl-2-oxo-4R-phenyl-pyrrolidin-1-yl)-acetamide

T1S – 2-(5R-methyl-2-oxo-4S-phenyl-pyrrolidin-1-yl)-acetamide

TRIS – 2-Amino-2-(hydroxymethyl)-1,3-propanediol

US – United States

VIP – vasoactive intestinal polypeptide

INTRODUCTION

Sigma-1 receptor (Sig1R) is a unique protein that regulates cellular protein functions, G-protein-coupled receptors and cell signalling molecules (Chu and Ruoho, 2016). Sig1R has become increasingly studied as a target for developing drugs for neurological disorders. This molecular chaperone protein can be regulated by several ligands. For example, several established central nervous system (CNS) drugs and newly synthesized compounds have shown Sig1R activity (Cobos *et al*., 2008; Su *et al*., 2010). Both agonists and antagonists of Sig1R have been studied in an attempt to elucidate their possible pharmacological applications, which mainly involve the improvement of learning and memory processes and treatment of depression, anxiety, schizophrenia, analgesia, seizures and some effects caused by certain drugs of abuse (Banister and Kassiou, 2012; Cobos *et al*., 2008; Maurice and Lockhart, 1997; Monnet and Maurice, 2006). Positive allosteric modulators (PAMs) of Sig1R have also been described, but, compared to other known compounds, PAMs are least studied.

There has been significant interest in the investigation of structure-activity relationships aimed at searching for novel nootropic compounds for the treatment of cognitive disorders. Since the 1960s, drugs from the so-called racetam family, which differ from each other in the structure of the substituents around the pyrrolidin-2-one heterocycle, have maintained a well-deserved reputation as leading therapeutic agents for the improvement of cognitive functions, attention abilities, information storage and retrieval, and mental conditions associated with head traumas, stroke, age, and age-related pathologies (Gouliaev and Senning, 1994; Gualtieri *et al*., 2002; Malykh and Sadaie, 2010), as well as seizures (Arroyo and Crawford, 2003; Klitgaard *et al*., 2016). Discovery of drugs with better efficacy based on the pyrrolidin-2-one pharmacophore can lead to new pharmacological applications of piracetam-like compounds. E1R was synthesized in the Latvian Institute of Organic Synthesis.

Aim of the study

To evaluate the molecular mechanisms and pharmacological activity of E1R in experimental models *in vitro* and *in vivo*.

Objectives of the study

1. To compare the activity of the stereoisomers of methylphenylpiracetam E1R, T1R, E1S and T1S.
2. To determine the binding activity of E1R to Sig1R using radioligand binding assays.
3. To determine the effects of E1R *in vitro* and *ex vivo* using selective Sig1R ligands.
4. To determine the effect of E1R on memory function and cognition *in vivo*.
5. To determine the activity of E1R in chemoconvulsant-induced seizure models *in vivo*.

Hypothesis of the study

E1R is a relevant drug for neuropharmacological applications.

Scientific novelty of the study

The International Union of Basic and Clinical Pharmacology included Sig1R in its list of receptors only in 2013. It was classified as a non-opioid intracellular receptor with no evidence for coupling through conventional signalling pathways. The molecular mechanisms and physiological functions of ligand-regulated Sig1R have not been fully understood thus far. It has been shown that the clinical use of allosteric modulators is associated with a reduction of side effects and an increase of activity of conventional drugs. Therefore, allosteric modulation of Sig1R is an emerging and important target for designing novel drugs. In addition to E1R, there are only a few compounds that act as allosteric Sig1R modulators. The obtained results provide key principles for the pharmacological activity of allosteric Sig1R modulators and increase the global understanding of the physiological function of Sig1R. E1R demonstrates a novel mechanism for the improvement of memory and cognition. In addition, the effects of E1R indicate that it might be a promising novel anti-seizure drug with none of the negative influences on memory typically encountered with many anti-epileptic drugs. The activity and pharmacological profile of E1R presented in this thesis can help generate strategies for the design of drugs for allosteric Sig1R sites, which offers new opportunities for the development of novel and highly selective therapeutic agents.

1. LITERATURE

1.1. Sigma-1 receptor (Sig1R)

The name “Sigma” for sigma receptor (SigR) was derived from the first letter of SKF−10,047 (N-allylnormetazocine), a compound from the benzomorphan family that was initially used to study opioid receptors. Based on the pharmacological activity of SKF−10,047, SigR was identified first as an opioid receptor subtype (Martin *et al*., 1976). In later studies, when the isomers of benzomorphans were evaluated separately, it was found that the (+)- and (−)-isomers of the compounds have different pharmacological activities (Martin *et al*., 1984). In the case of SKF−10,047, it was shown that the (−)-isomer accounted for the vast majority of the opioid-mediated effects (Aceto and May, 1983; Khazan *et al*., 1985; Zukin, 1982), while the activity of (+)-SKF−10,047 was not blocked by the opioid receptor antagonists naltrexone and naloxone (Harris, 1980; Vaupel, 1983). In classic radioligand binding studies, it was demonstrated that SigR displayed high affinity for several (+)-benzomorphans, including (+)-pentazocine, (+)-cyclazocine, dextrallorphan and dextrorphan, while a number of established opiates and opioid peptides failed to display significant affinities for these sites (Su, 1982).

SigR was found to consist of two pharmacologically distinct subtypes, namely, sigma-1 receptor (Sig1R) and sigma-2 receptor (Sig2R) (Hellewell *et al*., 1994; Hellewell and Bowen, 1990; Quirion *et al*., 1992). Sig1R was identified as being expressed in both the periphery and CNS (Quirion *et al*., 1992; Su *et al*., 1988b). Sig1R is widely distributed in the brain, and it especially concentrates in specific areas involved in memory, emotion and sensory and motor functions (Cobos *et al*., 2008). Sig1R was first cloned from guinea pig liver in 1996 (Hanner *et al*., 1996). Shortly afterwards, the Sig1R protein was obtained from rat and mouse brains, rat kidneys, and a human choriocarcinoma cell line, and it was shown that the receptor contains 90% identical and 95% similar amino acid sequences across species (Kekuda *et al*., 1996; Seth *et al*., 1998).

Sig1R is a unique 223 amino acid integral membrane-bound protein that is found not only in the plasma membrane but also in intracellular membranes such as the endoplasmic reticulum and mitochondria-associated membrane and the nuclear membrane (Mavlyutov *et al*., 2015; Mori *et al*., 2013; Su *et al*., 2010). Unlike many transmembrane receptors that belong to large, extensively studied families, such as G-protein-coupled receptors or ligand-gated ion channels, Sig1R is an evolutionarily isolated receptor class with no sequence homology with any known mammalian proteins (Schmidt *et al*., 2016). It was found that the

receptor shares 30% identity and 67% similarity with a yeast sterol C8−C7 isomerase, but it does not display any sterol isomerase activity (Moebius *et al*., 1997). It seems that Sig1R might represent a repurposed enzyme in which the catalytic site has been formed as a ligand-binding site (Schmidt *et al*., 2016). Different hypotheses and models have been proposed for the Sig1R structure and localization in the membranes, including a single membrane-spanning domain from alanine (Ala)92 to glycine (Gly)112 and two transmembrane domains from Ala10 to leucine (Leu)30 and from glutamine (Gln)80 to Leu100, as well as an additional third (putatively membrane flanking) hydrophobic region from Gly176 to threonine (Thr)203 (Brune *et al*., 2013). In 2016, a crystal structure of human Sig1R was published, showing that the overall structure of Sig1R features a trimeric organization with only a single transmembrane domain for each protomer (Schmidt *et al*., 2016). However, the validity of this Sig1R structural model remains to be demonstrated.

For Sig1R, the evidence for coupling through conventional signalling pathways is lacking (IUPHAR/BPS Guide to Pharmacology). However, it is known that Sig1R can amplify or reduce the signalling initiated when interacting with target proteins (Zamanillo *et al*., 2012). Therefore, from a functional perspective, Sig1R has been identified as a unique ligand-regulated molecular chaperone protein (Su *et al*., 2010; Zamanillo *et al*., 2012). Sig1R participates in the regulation and modulation of voltage-regulated and ligand-gated ion channels, including Ca^{2+}, K^{+}, Na^{+}, Cl^{-}, and small conductance calcium-activated potassium channels, and N-methyl-D-aspartate (NMDA) and inositol 1,4,5-triphosphate (IP_3) receptors (Maurice and Su, 2009). Sig1R regulates various cellular functions, including IP_3 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 (Hayashi *et al*., 2011; Hayashi and Su, 2007; Matsuno *et al*., 1993; Su *et al*., 2010). The modulation of the activity of other proteins by Sig1R is derived from the monomeric and oligomeric states of Sig1R (Gromek *et al*., 2014). The monomeric form of Sig1R has been reported to interact with target proteins such as the voltage-gated Na^{+} channel (Balasuriya *et al*., 2012), acid sensing channels (Carnally *et al*., 2010), and dopamine receptor-1 (D_1) (Navarro *et al*., 2010) and is associated with Sig1R chaperone activity (Gromek *et al*., 2014). Sig1R ligands may regulate the activity of the receptor interaction with client proteins by altering the oligomeric/monomeric receptor ratio and favouring the oligomeric states (Mishra *et al*., 2015). Several previous experiments support the conclusion that in ligand binding, Sig1R functions as an oligomer (Chu *et al*., 2013; Gromek *et al*., 2014; Schuster *et al*., 1995). Therefore, oligomerization is a key

functional property of the Sig1R that may be linked to ligand efficacy (Schmidt *et al*., 2016) and possibly could explain how ligands can regulate the activity of Sig1R.

1.2. Ligands of the Sig1R

An endogenous ligand for Sig1R has not been discovered thus far. However, compounds such as dehydroepiandrosterone (DHEA) and its sulphate, pregnenolone and its sulphate, sphingosines, progesterone, neuropeptide Y (NPY) and N,N-dimethyltryptamine have attracted interest as endogenous compounds (Zamanillo *et al*., 2012) that can bind to Sig1R. (+)-SKF−10,047 and (+)-pentazocine are prototypical Sig1R ligands. [^{3}H]-(+)-Pentazocine is still widely used to evaluate the binding affinities of novel synthesised Sig1R compounds in radioligand binding experiments. Currently, several compounds with different therapeutic and pharmacological applications, such as antipsychotics (e.g., haloperidol, chlorpromazine, promethazine, and pimozide); antidepressants (e.g., fluvoxamine, sertraline, clorgyline, fluoxetine, imipramine, and citalopram); anxiolytics (e.g., opipramol and diazepam); antitussives (e.g., carbetapentane, dextromethorphan (DM), and dimemorfan); antihistamines (e.g., pyrilamine, promethazine, and chlorpheniramine); antifungals (e.g., fenpropimorph and tridemorph); Ca^{2+} channel blockers (e.g., verapamil and emopamil); drugs for the treatment of neurodegenerative disorders such as Parkinson disease (e.g., amantadine) or Alzheimer disease (e.g., memantine and donepezil); and psychostimulants and drugs of abuse (e.g., phencyclidine, cocaine, methamphetamine, amphetamine, lysergic acid diethylamide, and ecstasy) are known to be able to bind to Sig1R with moderate to high affinity (Cobos *et al*., 2008; Matsumoto *et al*., 2007; Zamanillo *et al*., 2012). These ligands are diverse in chemical structure, sharing few common features, and based on the chemical structures of these compounds, several pharmacophore models have been proposed for Sig1R (Matsumoto *et al*., 2007). The central pharmacophore model for Sig1R is the Ar−X_5−N pharmacophore (Glennon *et al*., 1994); Figure 1.1.), which demonstrates that the chemical structures of Sig1R ligands include an aryl (or some other hydrophobic) group separated from an amine, usually by a five-membered (could be shorter or longer) chain. The spacer group “X” can be linear or branched (including unsaturated and cyclic structures) and can contain functionalities such as a ketone, amino, or ester group. The terminal amine “N” can be secondary, tertiary, or quaternary. Active searching for novel Sig1R ligands has resulted in discovery of very potent and selective compounds (Figure 1.1.) that are now extensively used to study the mechanisms of Sig1R.

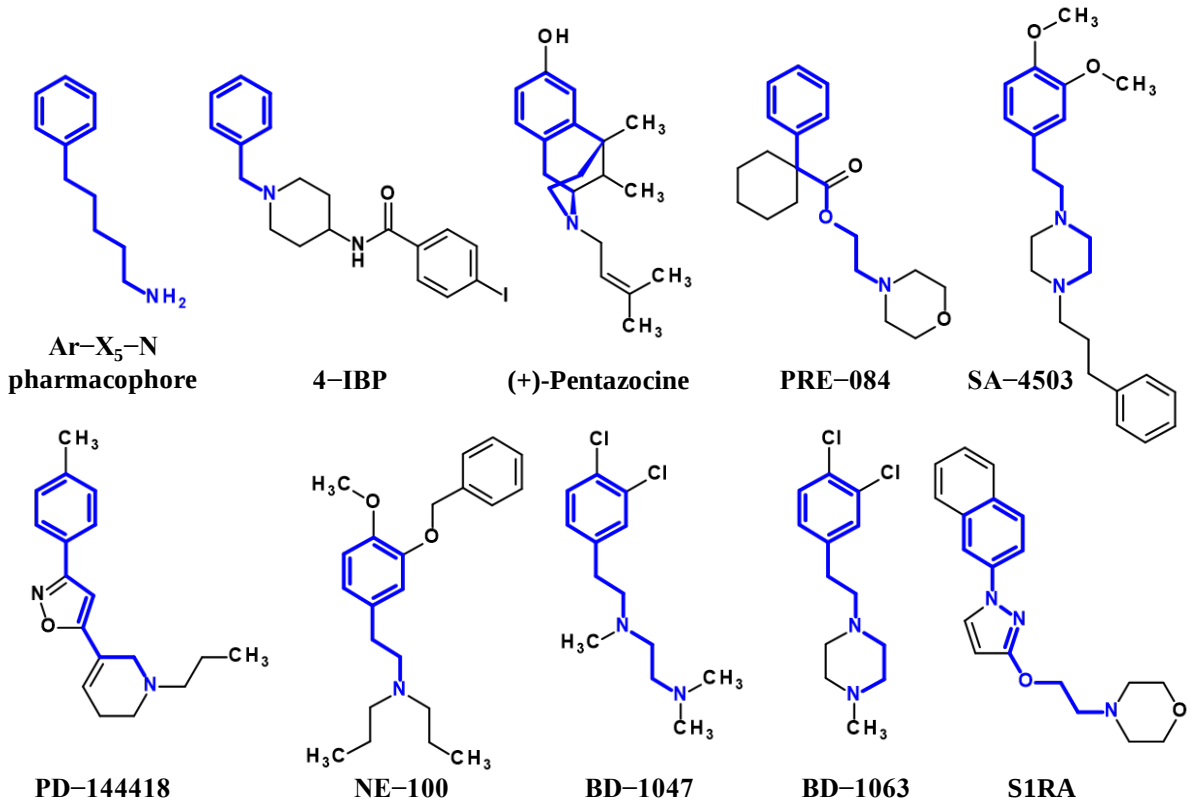


Figure 1.1. **Selective Sig1R ligands**
Prototypic agonist: (+)-pentazocine (K_i = 17 nM). Agonists: 4−IBP (K_i = 1.7 nM), PRE−084 (K_i = 44 nM), SA−4503 (K_i = 17 nM). Antagonists: NE−100 (K_i = 1.5 nM), BD−1047 (K_i = 0.9 nM), BD−1063 (K_i = 9 nM), S1RA (K_i = 17 nM). Putative antagonist: PD−144418 (K_i = 0.08 nM).

Which ligands possess agonist activity and which possess antagonist activity at Sig1R are still actively researched questions. Traditionally, antagonists are compounds that can attenuate or block the effects of Sig1R agonists. Growing evidence suggests that antagonists of Sig1R may function as inverse agonists (Chu and Ruoho, 2016). The classification of Sig1R ligands is difficult and is based on *in vivo* studies, which are necessary to fully describe and understand the pharmacological activity of Sig1R ligands. In addition, different tests and assays, including molecular modelling, radioligand binding, *in vivo* imaging, cultured cells or isolated tissue systems for functionality assays, are used.

It has been shown that Sig1R plays an important role in the pathophysiology of many neurological and psychiatric disorders, such as Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, amnesia, pain, depression, schizophrenia, stroke, seizures and addiction (Cai *et al*., 2017; Hayashi *et al*., 2011; Maurice and Goguadze, 2017; Maurice and Su, 2009; Mavlyutov *et al*., 2017; Niitsu *et al*., 2012). Therefore, Sig1R ligands could be used for the treatment of all abovementioned neurological disorders, which affect millions of people; however, for some of these disorders, effective treatments still have not been found. Some selective Sig1R ligands have entered clinical trials, which in near future

will probably give an answer regarding the importance of Sig1R as a molecular target for clinical applications.

1.3. Allosteric Sig1R modulators

Allosteric regulation is the regulation of a protein by binding an effector molecule at a site other than the orthosteric or active site of a protein (Figure 1.2.). The binding of allosteric modulators to a target protein induces a conformational change in the protein structure (Figure 1.2.). Allosteric modulators can be positive or negative allosteric modulators (PAMs or NAMs, respectively). PAMs increase the activity of the ligand, while NAMs block it (Figure 1.2.). To date, only a few compounds are known to act as allosteric Sig1R modulators (Figure 1.3.).

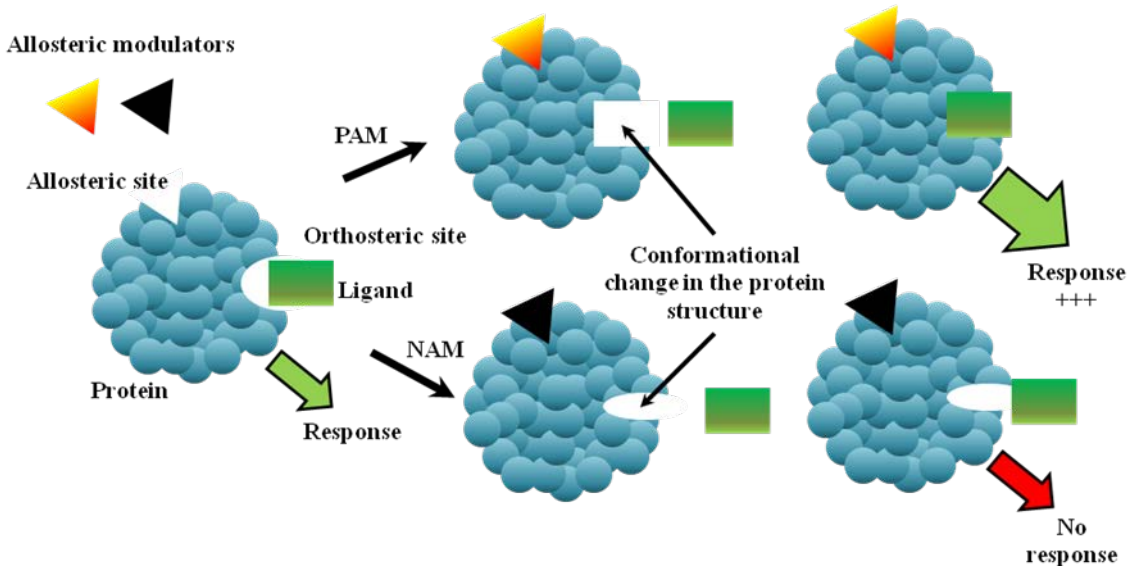


Figure 1.2. **Classic model for allosteric regulation of protein**
PAM – positive allosteric modulation; NAM – negative allosteric modulation.

The discovery of allosteric Sig1R modulators is linked to the results obtained from radioligand binding studies using [^{3}H]-DM in the 1980s. Although the cough suppressant DM is a drug from the morphinan class, it was shown that similar to (+)-SKF−10,047, the DM binding sites in the brain were distinct from the opiate receptors and were not associated with the receptor binding sites for several putative central neurotransmitters (Craviso and Musacchio, 1983). Later, sigma ligands were shown to inhibit [^{3}H]-DM high affinity binding with a rank order of potency similar to that for sites labelled with high affinity sigma ligands, namely, [^{3}H]-(+)-3-(3-hydroxyphenyl)-N-(1-propyl) piperidine hydrochloride ([^{3}H]-(+)-3-PPP) or [^{3}H]-(+)-SKF−10,047 (Musacchio *et al*., 1989a). Through the use of more

selective ligands, DM has been confirmed to bind to Sig1R with significant affinity (Shin *et al*., 2007), and a number of studies indicate that DM acts as an agonist at Sig1R (Taylor *et al*., 2016). Over the course of the competition binding studies, it was found that the anti-epileptic drug phenytoin (diphenylhydantoin, Figure 1.3.) increased the binding of [^{3}H]-DM in the guinea pig brain (Craviso and Musacchio, 1983). In addition, phenytoin was found to increase the binding of [^{3}H]-(+)-3-PPP in the same fashion as it increased that of [^{3}H]-DM (Musacchio *et al*., 1989b). Ropizine (SC−13504), an anti-convulsant benzhydryl piperazine (Figure 1.3.), also induced a marked concentration-dependent increase in the binding of [^{3}H]-DM (Musacchio *et al*., 1988) and [^{3}H]-(+)-3-PPP (Musacchio *et al*., 1989b) and is one of the first allosteric Sig1R modulators identified. The allosteric modulation of sigma recognition sites by phenytoin has been demonstrated by the ability of phenytoin to stimulate the binding of various tritiated Sig1R agonists (Chaki *et al*., 1996; Cobos *et al*., 2005), to slow dissociation from sigma sites and to shift sigma sites from a low-affinity state to a high-affinity state (DeHaven-Hudkins *et al*., 1993). A detailed comparison of the effects of phenytoin on the binding of [^{3}H]-(+)-pentazocine and [^{3}H]-NE−100 using saturation and kinetics assays showed that phenytoin acts as PAM and can negatively modulate the binding of [^{3}H]-NE−100 by decreasing the specific binding and increasing dissociation rate from Sig1R (Cobos *et al*., 2006).

Interestingly, it was shown that compound SR−31747A ((Z)-N-cyclohexyl-N-ethyl-3-(3-chloro-4-cyclohexylphenyl)propen-2-ylamine hydrochloride) could be an allosteric modulator of peripheral sigma binding sites (Paul *et al*., 1994). Although SR−31747A modulated the activity of Sig1R ligands *in vivo* and *in vitro*, the use of radiolabeled [^{3}H]-SR−31747A demonstrated that SR−31747A binds specifically, saturably and reversibly to rat spleen membranes and human lymphocytes at a single class of high affinity sites, which were clearly different from the [^{3}H]-(+)-pentazocine and [^{3}H]-(+)-3-PPP binding sites (Paul *et al*., 1994). A few years later, the purified amino acid sequence of the [^{3}H]-SR−31747A binding site was found to be a nuclear membrane protein related to a fungal C8−C7 sterol isomerase; additionally, this protein, which was called SR−31747A-binding protein, was encoded by the ERG2 gene (Jbilo *et al*., 1997). The exact molecular mechanism of SR−31747A is not fully described so far, but SR−31747A is currently not considered an allosteric Sig1R modulator.

Recently, a group of structurally similar compounds have been shown to act as allosteric Sig1R modulators (Guo *et al*., 2013). These benzazepine derivatives include the 1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine analogues SKF−83959, SKF−38393, SCH−23390 and SOMCL−668 (Figure 1.3.).

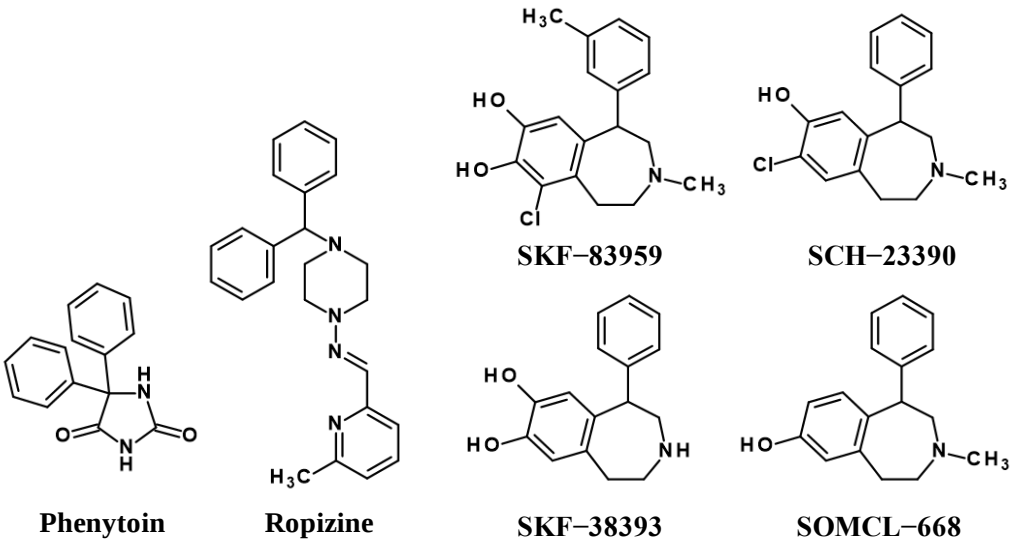


Figure 1.3. **Positive allosteric modulators of Sig1R**

SKF−83959 is an atypical D_1 agonist (Deveney and Waddington, 1995; Downes and Waddington, 1993), while SKF−38393 is selective D_1 agonist (Molloy and Waddington, 1984) and SCH−23390 acts as a D_1 antagonist (Hyttel, 1983). Previously, it was shown that SKF−83959 exerts many D_1 receptor-independent pharmacological effects. For example, SKF−83959 suppresses excitatory synaptic transmission and voltage-activated Na^+ current in rat hippocampus (Chu *et al*., 2011), inhibits the delayed rectifier K^+ channel in primary culture neurons (Chen *et al*., 2009), and promotes the spontaneous release of glutamate in the rat somatosensory cortical neurons (Chu *et al*., 2010). Based on the pharmacological activity of SKF−83959 and similarities of the pharmacophore with some other Sig1R ligands, the activity of SKF−83959 and its analogues was related to Sig1R. It was shown that these compounds can enhance the binding activity of [^{3}H]-(+)-pentazocine in brain and liver tissues, shift the saturation curve towards the left, and decrease the dissociation rate in binding kinetic analysis (Guo *et al*., 2013). To exclude the potential involvement of other receptors and to confirm that the activity of these compounds is related to Sig1R modulation, several chemical derivatives of SKF−83959 were synthesized in order to find a selective Sig1R allosteric modulator. One of these newly synthesized compounds, called SOMCL−668 (Figure 1.3.), did not exhibit affinity for human D_1, D_2, D_3, serotonin $(5-HT)_{1A}$, or $5-HT_{2A}$ receptors (Zhang *et al*., 2014) but did show potent allosteric modulating activity at Sig1R (Guo *et al*., 2015). Therefore, SOMCL−668 has been proposed as a selective allosteric Sig1R modulator.

1.4. The pharmacological activity of allosteric Sig1R modulators

It has been shown that Sig1R modulators act as anti-convulsive, anti-inflammatory and anti-depressant drugs.

Allosteric modulators of Sig1R possess anti-convulsive activity. Phenytoin has been used in the clinic against various types of epileptiform seizures for more than 70 years (Santulli *et al*., 2016). However, the Sig1R antagonist BD-1047 did not block the anti-seizure effects of phenytoin (Guo *et al*., 2015), suggesting that the mechanism by which phenytoin exerts its anti-seizure activity is primarily related to the inhibition of voltage-gated sodium channels (Tunnicliff, 1996). The allosteric Sig1R modulator ropizine possess limited anti-convulsant activity against chemically induced seizures (Edmonds *et al*., 1979, 1978), while it is similar in efficacy in maximal electroshock-induced seizures to phenytoin (Novack *et al*., 1979). The anti-convulsive effects of SKF−83959 at doses of 20 and 40 mg/kg and SOMCL−668 at a dose of 40 mg/kg in models of pentylenetetrazol (PTZ)- and kainic acid-induced seizures were demonstrated to be mediated by modulating Sig1R (Guo *et al*., 2015). The anti-convulsive effects of SKF−83959 and SOMCL−668 were blocked by the selective Sig1R antagonist BD−1047 at a dose of 1 mg/kg.

SKF−83959 has been shown to inhibit the generation of intracellular reactive oxygen species and the expression of tumour necrosis factor α (TNFα), interleukin-1 beta (IL−1β), and cytokine-inducible nitric oxide synthase in lipopolysaccharide (LPS)-stimulated mice brain microglial BV2 cells (Wu *et al*., 2015). The effects of SKF−83959 were blocked by BD−1047 or BD−1063 (Wu *et al*., 2015). In the same study, it was shown that in a [^{3}H]-(+)-pentazocine binding assay, SKF−83959 enhanced the binding activity of DHEA by shifting the DHEA binding curve to the left. In addition, SKF−83959 enhanced the anti-inflammatory effect of exogenous DHEA in a synergistic manner, which was dependent on Sig1R (Wu *et al*., 2015), thus showing that the anti-inflammatory effects of SKF−83959 are due to positive allosteric Sig1R modulator activity in cells.

Since Sig1R may serve as a novel target for anti-depressant development (Fishback *et al*., 2010), the selective Sig1R allosteric modulator SOMCL−668 has been tested for its potential antidepressant activity (Wang *et al*., 2016). SOMCL−668 at doses of 10 and 20 mg/kg significantly decreased the immobility time of mice in the forced-swimming and tail suspension tests, and this effect was blocked by the BD−1047 (Wang *et al*., 2016). In addition, the daily administration of SOMCL−668 at a dose of 10 mg/kg for one week significantly reversed the decrease in the sucrose preference in the chronic mild stress model in mice (Wang *et al*., 2016).

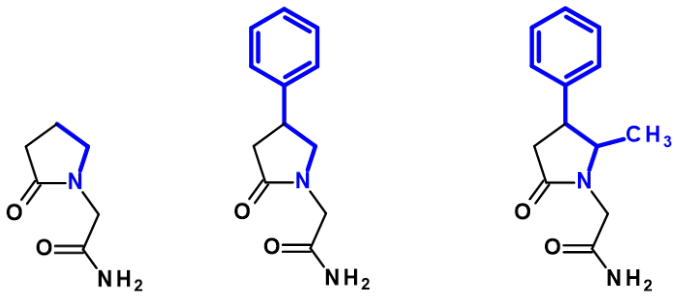
1.5. Racetams: a compound class with unrevealed therapeutic potential

Racetams are a group of compounds that are derived from piracetam (2-oxo-1-pyrrolidine acetamide) originally and possess nootropic activity. The term nootropic, from the Greek *noos* (mind) and *tropos* (to bend or turn), was coined by Giurgea in 1964 to describe the discovered properties of the racetams (Robbins, 2009), which included the enhancement of learning and memory, improvement of resistance against chemical and physical injuries, and absence of the usual psychological and general cardiovascular pharmacological activity of psychopharmaceuticals (Gouliaev and Senning, 1994).

Piracetam and its derivatives, such as aniracetam, pramiracetam, nefiracetam, oxiracetam and phenylpiracetam, are the drugs of choice for the specific therapy of cognition/memory disorders (Gouliaev and Senning, 1994). Although racetam-like compounds have been used in clinical practice since the early seventies, in the United States (US), a number of racetams, including piracetam, still are not approved for medical use by the US Food and Drug Administration. The reason for this could be that the racetams have poorly understood mechanisms of action. Structurally, piracetam is a cyclic analogue of the mammalian CNS inhibitory neurotransmitter gamma-aminobutyric acid (GABA, Figure 1.4.). However, the activity of piracetam is not related to GABA receptors (Gouliaev and Senning, 1994). Piracetam was shown to act as a PAM of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (Ahmed and Oswald, 2010). Piracetam is well-tolerated drug with documented clinical benefit for the treatment of several conditions, including age-related cognitive disorders, vertigo and dyslexia (Winblad, 2005), and has shown anti-convulsive activity (Schmidt, 1990). In addition, a piracetam derivative, levetiracetam, is an anti-seizure drug that binds to synaptic vesicle glycoprotein 2A (SV2A) in synaptic and endocrine vesicles (Rogawski, 2016). The discovery of the increased activity of the (S)-enantiomer levetiracetam was possible after the stereoselective resolution of racemic etiracetam (Gower *et al*., 1992).

Another optically active derivative of piracetam, phenylpiracetam (Figure 1.4.), was originally designed as a nootropic drug for the sustenance and improvement of the physical condition and cognition abilities of Soviet space crews (Malykh and Sadaie, 2010). Later, phenylpiracetam was introduced into general clinical practice in Russia and in some Eastern European countries. Because of its use as a doping drug for the stimulation of physical activity, such as sports, phenylpiracetam was included in the list of banned substances issued by the World Anti-Doping Agency (Georgakopoulos *et al*., 1999; The World Anti-Doping Code, 2013). The possible target receptors and mechanisms for the acute activity of this drug

remained unclear until it was found that (R)-phenylpiracetam (MRZ−9547) is a selective dopamine transporter inhibitor that moderately stimulates striatal dopamine release (Sommer *et al*., 2014). According to previous *in vitro* studies, racemic phenylpiracetam binds with low micromolar affinity to nicotinic acetylcholine receptors but has no affinity for D_1, D_2 and D_3 or $5{-}HT_2$ receptors (Firstova *et al*., 2011). Phenylpiracetam, especially (R)-phenylpiracetam, possesses antidepressant and stimulatory activity and enhances memory processes, as shown in the passive avoidance response test (Tiurenkov *et al*., 2007; Zvejniece *et al*., 2011).



Piracetam **Phenylpiracetam** **Methylphenylpiracetam***

Figure 1.4. **Chemical structures of piracetam and its derivatives**
*Methylphenylpiracetam was synthesized in the Latvian Institute of Organic Synthesis (Kalvins *et al*., 2011).

A unifying hypothesis for the mechanism of action of racetam compounds is still required. Interestingly, it has been reported that piracetam can affect the fluidity of membranes (Winblad, 2005). Membrane fluidity is believed to be important for the structural organization of proteins and other molecules within the membrane and thus can influence membrane transport, enzyme activity, receptor binding and activation (Winblad, 2005). However, this so-called “membrane hypothesis” has not been attributed to all racetam compounds. The search for the possible mechanism of action of racetams can reveal new therapeutic opportunities.

2. METHODS

2.1. Animals

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. Animals were adapted for two weeks prior to the experiments. All studies involving animals were conducted in accordance with ARRIVE guidelines (Kilkenny *et al*., 2010; McGrath *et al*., 2010). Experimental procedures were performed in accordance with the guidelines reported in the EU Directive 2010/63/EU and in accordance with local laws and policies. All procedures were approved by the Latvian Animal Protection Ethical Committee of Food and Veterinary Service in Riga, Latvia.

2.2. Chemicals

E1R ((4R,5S)-2-(5-methyl-2-oxo-4-phenyl-pyrrolidin-1-yl)-acetamide), T1R ((4R,5R)-2-(5-methyl-2-oxo-4-phenyl-pyrrolidin-1-yl)-acetamide), T1S ((4S,5R)-2-(5-methyl-2-oxo-4-phenyl-pyrrolidin-1-yl)-acetamide) and E1S ((4S,5S)-2-(5-methyl-2-oxo-4-phenyl-pyrrolidin-1-yl)-acetamide) were prepared at the Latvian Institute of Organic Synthesis (Kalvins *et al*., 2011; Publication I). [^{3}H]-(+)-pentazocine ([ring-1,3-^{3}H](1R,9R,13R)-1,13-dimethyl-10-(3-methylbut-2-en-1-yl)-10-azatricyclo[7.3.1.0^{2,7}]trideca-2,4,6-trien-4-ol; specific activity 33.9 Ci/mmol) was purchased from American Radiolabeled Chemicals, St. Louis, Missouri, USA. Bradykinin (2-[[2-[[1-[2-[[2-[[2-[[1-[1-[2-amino-5-(diaminomethylideneamino)pentanoyl]pyrrolidine-2-carbonyl]pyrrolidine-2-carbonyl]amino]acetyl]amino]-3-phenylpropanoyl]amino]-3-hydroxypropanoyl]pyrrolidine-2-carbonyl]amino]-3-phenylpropanoyl]amino]-5-(diaminomethylideneamino)pentanoic acid; BDK), NE−100 (4-Methoxy-3-(2-phenylethoxy)-N,N-dipropylbenzeneethanamine hydrochloride), PRE−084 (2-(4-Morpholinethyl)-1-phenylcyclo-hexanecarboxylate hydrochloride) and PB−28 (1-Cyclohexyl-4-[3-(1,2,3,4-tetrahydro-5-methoxy-1-naphthalenyl)propyl]piperazine dihydrochloride) were purchased from Tocris Bioscience, Bristol, UK. Haloperidol (4-[4-(4-Chlorophenyl)-4-hydroxy-1-piperidyl]-1-(4-fluorophenyl)-butan-1-one) was purchased from Alfa Aesar, Karlsruhe, Germany. (−)-Scopolamine hydrochloride was obtained from Fluka, St. Louis, Missouri, USA. Pentylenetetrazole (PTZ) and (+)-bicuculline (BIC) were procured from Sigma-Aldrich Co. (St. Louis, MO, USA). 0.9% physiological saline was purchased from Fresenius Kabi (Warszawa, Poland).

For the competitive binding experiments E1R and PRE−084 were dissolved in 0.9% physiological saline, while haloperidol was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 mM as stock solutions. Stock solutions were diluted with 50 mM TRIS-HCl 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. PTZ was weighed and dissolved in 0.9% physiological saline to make a 1% PTZ solution. BIC was dissolved in DMSO to prepare a 1% stock solution, which was then diluted with saline to make a 0.01% BIC solution. PTZ and BIC solutions were freshly prepared before each experiment. All other compounds were dissolved in deionized water or 0.9% physiological saline before use in *in vitro* or *in vivo* experiments, respectively.

2.3. *In vitro* methods

2.3.1. Radioligand binding assays

E1R was profiled in a commercially available panel of 77 radioligand binding assays (CEREP, Poitiers, France). A specific list of the assays performed with E1R is documented in the Results section, and further details regarding the methods are available at http://www.cerep.fr/cerep/users/pages/catalog/profiles/DetailProfile.asp?profile=2118.

The method for [^{3}H]-(+)-pentazocine binding assay is described in Publication II. Briefly, binding experiments were carried out in the crude synaptosome fraction which was obtained from Wistar rats. The binding assay buffer consisted of 60 μL of incubation buffer (50 mM TRIS-HCl, pH 7.4), 100 μL membrane aliquots, 20 μL of the tested drugs or incubation buffer for the control, and 20 μL [^{3}H]-(+)-pentazocine. Nonspecific 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, Massachusetts, USA). The filters were washed three times with 0.25 ml of 10 mM TRIS (pH 8.0, 4 °C). The samples radioactivity was measured with a liquid scintillation counter Wallac MicroBeta TriLux (PerkinElmer, Waltham, Massachusetts, USA). Each experiment was repeated at least three times, and each assay was conducted in duplicate.

2.3.2. Measurement of the bradikynin (BDK)-induced increase in the $[Ca^{2+}]_i$

The method is described in Publication II. Briefly, 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 Fluo-4 NW-loaded NG108−15 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 deionised water was used as a control. Subsequently, 1 μM BDK 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.

2.3.3. SigR activity model of isolated rat *vas deferens*

The method is described in Publication I and Publication II. 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 ºC 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 electrical current induced a stable contraction amplitude, cumulative doses (from 1 to 100 μM) of the Sig1R 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 Sig2R activity, a selective Sig2R agonist PB−28 (at concentrations ranging from 1 to 10 μM) was used in a similar experimental set-up.

2.4. *In vivo* methods: behavioural experiments

2.4.1. Passive avoidance (PA) test

The method is described in Publication II. 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 (UgoBasile, Comerio, Italy). When 60 s had expired, the sliding door (4 x 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 hours 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.

The PA test to evaluate the effects of E1R on scopolamine-induced cognitive deficits was performed in essentially the same manner described above, with the exception that mice received a shock of 0.4 mA for 3 s. Scopolamine was administered s.c. at a dose of 0.3 mg/kg.

2.4.2. Scopolamine-induced cognitive deficits in the Y-maze test

Working memory performance was assessed by recording spontaneous alternation behaviour in a Y-maze. 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. Scopolamine was administered s.c. at a dose of 0.5 mg/kg (Publication II).

2.4.3. Open-field test

To test the effects of E1R on locomotor activity, the open-field test was used. 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, Netherland). The distance moved (cm/4min) and velocity (cm/s) were recorded. Testing consisted of five successive 4 min sessions that started 30, 60, 120, 180 and 240 min after compound administration.

2.4.4. Muscle strength and coordination

A rota-rod test was used to measure motor coordination (Model 7600, Ugo Basile, Comerio, Italy). One day prior to 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. In the rota-rod, traction and chimney tests, measurements were made prior or 30, 60, 120, 180 and 240 min after intraperitoneal (i.p.) administration of E1R.

2.4.5. Chemoconvulsant-induced seizures

Chemoconvulsant-induced clonic and tonic seizures were initiated by inserting a 27-gauge needle into the tail veins and infusing 1% PTZ (Mandhane *et al*., 2007; Zvejniece *et al*., 2010) or 0.01% BIC (Meldrum, 1975) at a constant rate of 20 µl/2 s to restrained animals. The infusion was halted when forelimb clonus followed by tonic seizures of the full body were observed. Minimal doses of PTZ or BIC (mg/kg of mouse weight) necessary to induce clonic and tonic seizures were considered as indices of seizure threshold.

The activity of Sig1R ligands on clonic and tonic seizure thresholds was studied in PTZ- and BIC-induced seizure models and each experimental set included a respective control group. The minimal dose of PTZ and BIC to induce clonic and tonic seizures in each experimental group is expressed as percentage from control, where 100% represents the seizure threshold for control group. Animals received i.p. injection of saline for control or Sig1R ligand 60 min before PTZ or BIC intravenous (i.v.) infusion. Each animal received a single dose of Sig1R ligand.

2.4.6. NE−100-induced seizures

The NE−100-induced seizures were studied at a dose of 75 mg/kg, which induced seizures in 100% of the animals. The method is described in Publication IV. Briefly, compounds or saline were administered 30 min prior to NE−100. Mice were then placed immediately in observation chambers (40×25×15 cm) and video recorded for 25 min using a digital HD video camera recorder (Handycam HDR-CX11E, Sony Corporation, Tokyo, Japan). Scoring scale for observed behavioural responses of animals was adapted from previously published seizure rating scale (Lüttjohann *et al*., 2009). Behavioural responses of animals were scored from the video files. Latency time until the first occurrence of seizures induced by NE−100 was also determined from the video files.

2.4.7. 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 60 min prior to 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 60 min prior to the training session. Prior to Y-maze test, the animals received an i.p. injection of E1R at a dose of 10 mg/kg 60 min prior to experiment. In the open-field test, E1R was administered i.p. at doses of 1, 10 and 100 mg/kg 30 min prior to experimentation. In the rota-rod, traction and chimney tests, measurements were made after i.p. administration of E1R at doses of 50, 100, 250, 500 and 630 mg/kg. In the chemoconvulsant-induced seizure models animals received i.p. injection of E1R at doses of 10 and 50 mg/kg 60 min before administration of chemoconvulsant. To test the activity of E1R on NE−100-induced seizures, the animals received i.p. administration of E1R at a dose of 75 mg/kg 30 min before the i.p. administration of NE−100.

2.5. Data and statistical analyses

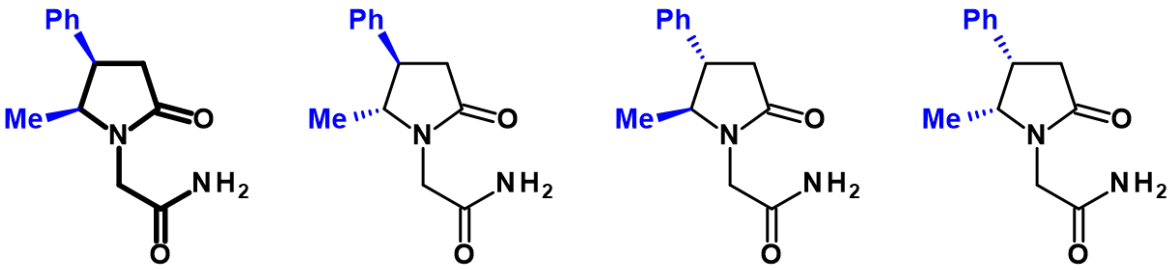
The results are expressed as the mean ± standard error of the mean (S.E.M.). Non-linear regression analysis was used to determine IC_{50} values of the tested compounds in competitive radioligand binding assay. In the SigR activity model of isolated rat *vas deferens* responses to selective SigR agonists before and after the addition of the test compound were calculated as the percentage increase in the baseline contraction amplitude. The electrical current-induced contraction amplitudes of the isolated *vasa deferentia* were analysed using a two-way repeated measures ANOVA followed by Bonferroni post-hoc testing. The responses to the BDK-induced $[Ca^{2+}]_i$ changes with or without pre-incubation with the test compounds were calculated as the percent increase in the basal relative fluorescence units (RFUs). The data for the BDK-induced increase in $[Ca^{2+}]_i$ were analysed using a one-way ANOVA followed by Tukey's test. The total number and sequence of the arm entries in the Y-maze test were manually recorded, and the percentage of alternation was calculated. For the PA and Y-maze experiments, the data were analysed using a one-way ANOVA followed by the Newman-Keuls multiple comparison test. For E1R's dose-related effect on the scopolamine-induced impairment of passive avoidance experiments, statistical analysis was performed using a one-way ANOVA followed by the Mann-Whitney U-test. To evaluate the effects of E1R on muscle strength and coordination the ED_{50} values were obtained by probit analysis. The data for the chemoconvulsant-induced seizures were analysed using Student's t-test and

one-way ANOVA followed by Newman-Keuls multiple comparison 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, California, USA).

3. RESULTS

3.1. The synthesis of methylphenylpiracetam and separation of the individual stereoisomers

The synthesis, isolation and purification of all four stereoisomers (Figure 3.1.) of methylphenylpiracetam were performed by Prof. G. Veinbergs, Dr. chem. M. Vorona, Dr. chem. H. Kazoka, Dr. phys. S. Belyakov, Dr. phys. A. Mishnev, J. Kuznecovs, S. Vikainis, N. Orlova, Dr. chem. A. Lebedev, Y. Ponomaryov and Dr. habil. chem. E. Liepinsh. Details are described in Publication I and Publication III.



E1R **T1R** **T1S** **E1S**

Figure 3.1. **Individual stereoisomers of methylphenylpiracetam**

3.2. *In vitro* selectivity profiling of E1R (Publication II)

The pharmacological profiling of E1R against various possible targets was performed using a commercially available radioligand binding assay screen that was performed by CEREP. E1R at a 10 μM concentration had little to no activity in 76 radioligand displacement assays that included numerous ion channel, G protein-coupled receptor and central nervous system transporter targets (Table 3.1.). The only target for E1R (inhibition or enhancement of radioligand binding exceeding 20%) was the SigR; 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 (DTG) by 38% in Jurkat cells (Table 3.1.). In the same assay, the SigR antagonist haloperidol inhibited the binding of the radioligand with an IC_{50} = 43 nM.

Table 3.1.

The screening profile of E1R in *in vitro* binding assays (CEREP)

Receptor/ Ion Channel/Enzyme	Inhibition by 10 μM E1R, %	Reference Compound	IC_{50}, nM
Adenosine A_1	2	Dipropylcyclopentylxanthine	0.87
Adenosine A_{2A}	−16	5'-N-ethylcarboxamidoadenosine	10
Adrenergic α_1	−5	prazosin	0.17
Adrenergic α_2	−4	yohimbine	36
Adrenergic α_1	−5	atenolol	230
Adrenergic α_2	−2	ICI−118551	0.5
Angiotensin AT_1	−14	saralasin	0.6
Angiotensin AT_2	−5	angiotensin	0.13
Benzodiazepine (central)	−18	diazepam	110
Benzodiazepine (peripheral)	3	PK−11195	1.1
Bombesin BB	−5	bombesin	0.22
Bradykinin B_2	3	NPC−567	10
Calcitonin gene-related peptide (CGRP)	1	Human CGRP	0.04
Cannabinoid CB_1	−3	CP−55940	0.21
Cholecystokinin $(CCK)_1$	9	CCK-8s	0.2
Cholecystokinin CCK_2	8	CCK-8s	0.26
Dopamine D_1	−6	SCH−23390	0.22
Dopamine D_{2S}	5	(+)-butaclamol	1.4
Dopamine D_3	7	(+)-butaclamol	1.2
Dopamine D_4	−3	clozapine	32
Dopamine D_5	−3	SCH−23390	0.15
Endothelin ET_A	−2	endothelin	0.11
Endothelin ET_B	2	endothelin	0.04
GABA (nonselective)	13	GABA	73
Galanin GAL_1	−2	galanin	0.16
Galanin GAL_2	−4	galanin	0.8
Platelet-derived growth factor (PDGF)	−6	glycoprotein PDGF-BB	0.05
Chemokines CXCR2	2	interleukin-8	0.04
Chemokines CCR1	−5	Macrophage inflammatory protein-1	0.08
TNFα	−3	TNFα	0.26
Histamine H_1	−7	pyrilamine	0.82
Histamine H_2	−12	cimetidine	270
Melanocortin MC_4	4	afamelanotide	0.2
Melatonin MT_1 (ML_{1A})	−6	melatonin	0.53
Muscarinic M_1	−4	pirenzepine	27
Muscarinic M_3	−7	4−DAMP	0.43
Muscarinic M_4	−3	4−DAMP	0.83
Muscarinic M_5	−12	4−DAMP	0.80
Neurokinin NK_1	−11	substance P	0.24
Neurokinin NK_2	−3	[Nleu10]-NKA (4−10)	2.5
Neurokinin NK_3	−7	SB−222200	8.8
NPY Y_1	−15	NPY	0.07
NPY Y_2	−18	NPY	0.09

Table 3.1. (continued)

Receptor/ Ion Channel/Enzyme	Inhibition by 10 μM E1R, %	Reference Compound	IC_{50}, nM
Neurotensin NTS_1	−3	neurotensin	0.24
Opioid and opioid-like δ	−4	enkephalin	1.9
Opioid and opioid-like κ	−5	U−50488	0.84
Opioid and opioid-like μ	−7	DAMGO	0.74
Opioid and opioid-like	4	nociceptin	0.29
Vasoactive intestinal peptide PAC_1	−2	pituitary adenylate cyclase-activating peptide (1−38)	0.05
Peroxisome proliferator-activated receptor gamma (PPARγ)	−9	rosiglitazone	12
Phencyclidine	6	MK−801	6.0
Prostanoid EP_2	−13	prostaglandin E2	2.2
Prostanoid IP (PGI_2)	−19	iloprost	13
Purinergic P2X	6	α,β-methyleneadenosine-5'-triphosphate	3.5
Purinergic P2Y	−4	2'-deoxyadenosine-5'-O-(1-thiotriphosphate)	25
Serotonin $5-HT_{1A}$	−4	8-hydroxy-2-(dipropylamino)tetralin	0.51
Serotonin $5-HT_{1B}$	0	serotonin	12
Serotonin $5-HT_{2A}$	5	ketanserin	0.77
Serotonin $5-HT_{2B}$	−11	2,5-dimethoxy-4-iodoamphetamine	3.2
Serotonin $5-HT_{2C}$	−2	RS−102221	5.5
Serotonin $5-HT_3$	−1	MDL−72222	4.9
Serotonin $5-HT_{5a}$	−11	serotonin	280
Serotonin $5-HT_6$	−10	serotonin	180
Serotonin $5-HT_7$	−4	serotonin	0.49
SigR (non-selective)	**−38**	**haloperidol**	**43**
Somatostatin sst	−5	somatostatin-14	0.14
Glucocorticoid	−11	dexamethasone	2.5
Vasoactive intestinal polypeptide receptor 1	−11	vasoactive intestinal polypeptide	0.12
$Vasopresin_{1a}$	2	vasopresin	1.9
Ca^{2+} channel (L, verapamil site) (phenylalkylamine)	−1	methoxyverapamil	20
K_V	−2	α-dendrotoxin	0.29
SK_{Ca} channel	−18	apamin	0.03
Na^+ channel (site2)	5	veratridine	7700
Cl^- channel (GABA-gated)	−3	picrotoxinin	250
Norepinephrine transporter	−17	protriptyline	2.5
Dopamine transporter	4	BTCP	2.6
Serotonin 5−HT transporter	5	imipramine	2.0

The results are expressed as percent inhibition of control specific binding (mean values; n = 2).

3.3. Activity of E1R on [^{3}H]-(+)-pentazocine binding (Publication II)

Based on screening results, the activity of E1R was evaluated in [^{3}H]-(+)-pentazocine binding assay. Unlike the selective Sig1R agonist PRE-084 (IC_{50} = 192 nM) or the non-selective SigR antagonist haloperidol (IC_{50} = 0.5 nM), E1R did not displace [^{3}H]-(+)-pentazocine from the Sig1R (Figure 3.2.). As seen in Figure 3.2. E1R did not modulate binding of [^{3}H]-(+)-pentazocine in this binding assay. It should be noted that we failed to demonstrate Sig1R modulatory effect also for phenytoin in this assay (Figure 3.2.).

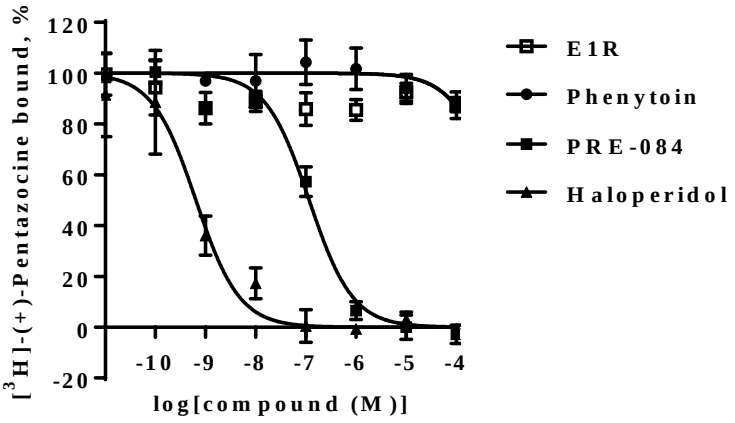


Figure 3.2. **The effects of E1R and sigma receptor ligands on the binding of [^{3}H]-(+)-pentazocine to a Sig1R**
Synaptosomes from rat brains were incubated with 1.5 nM [^{3}H]-(+)-pentazocine at 30 °C for 150 min. Haloperidol (10 µM) was used to define nonspecific binding. The data represent at least three experiments performed in duplicate.

3.4. Effects of E1R on the BDK-induced increase of $[Ca^{2+}]_i$ in NG108−15 cells (Publication II)

Selective Sig1R agonist PRE−084 at a dose of 2 µM significantly enhanced the BDK-induced $[Ca^{2+}]_i$ increase by 34 ± 4% in NG108−15 cells. Moreover, E1R at a dose of 10 µM significantly enhanced the $[Ca^{2+}]_i$ increase by 66 ± 3% (Figure 3.3.). In addition, PRE−084 effect on the $[Ca^{2+}]_i$ changes was potentiated 3 times after pre-incubation with E1R, reaching 212 ± 6% relative to the control. The effects of PRE−084, E1R and their combination were significantly antagonised by administering a selective Sig1R antagonist NE−100 at a dose of 40 µM (Figure 3.3.).

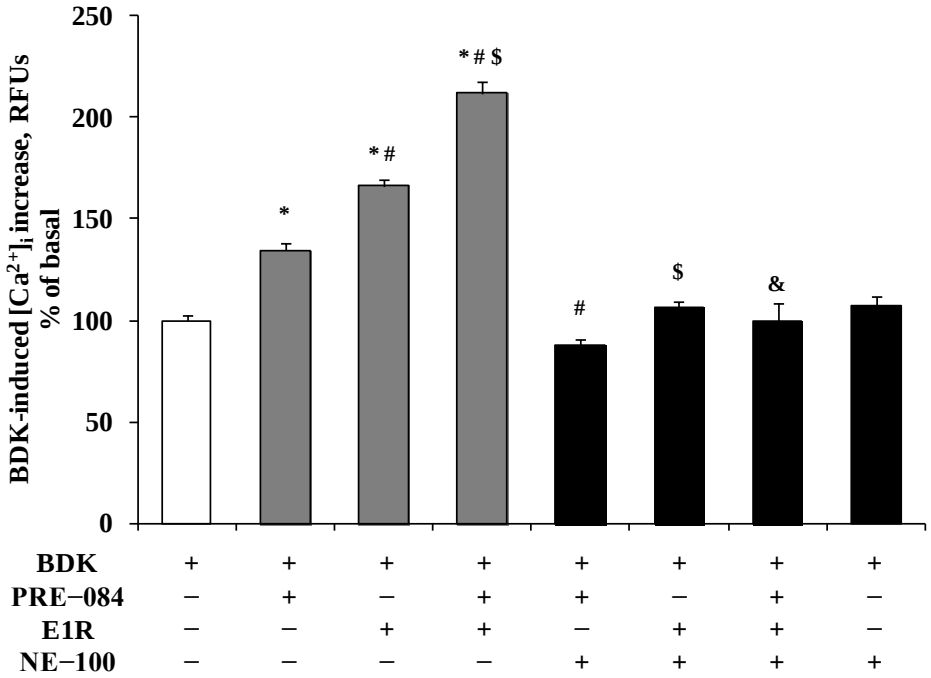


Figure 3.3. **The effects of Sig1R ligands and their combinations on 1 μM BDK-induced $[Ca^{2+}]_i$ increase in NG108−15 cells**

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 ± S.E.M. (n = 6−14). $^{*}P < 0.05$ vs. BDK, $^{\#}P < 0.05$ vs. PRE−084, $^{\$}P < 0.05$ vs. E1R, $^{\&}P < 0.05$ vs. E1R and PRE−084 combination (one-way ANOVA followed by Tukey's test).

3.5. Effects of E1R on Sig1R and Sig2R in an experimental model of isolated *vas deferens* (Publication I and Publication II)

The positive allosteric modulatory activities of E1R and other individual stereoisomers T1R, T1S and E1S were evaluated using the electrically stimulated rat *vas deferens* model. The addition of cumulative doses of E1R, T1R, T1S or E1S did not influence the contractions of electrically stimulated rat *vasa deferentia*. The intensity of electrically stimulated contractions of rat *vasa deferentia* in the presence of the Sig1R agonist PRE−084 (100 μM) was 122 ± 11% (Figure 3.4.A). Pre-incubation of *vasa deferentia* with a 10 μM solution of each tested stereoisomer 10 min before the addition of PRE−084 significantly increased the intensity of the contractions (Figures 3.4.A and B). Comparison between the activities of all stereoisimers of methylphenylpiracetam showed that E1R is the most effective positive allosteric Sig1R modulator (Figures 3.4.A and B).

The intensity of electrically stimulated contractions of rat *vasa deferentia* in the presence of the Sig2R agonist PB−28 were increased, but pre-treatment with E1R had no influence on the effects induced by PB−28 (Publication II).

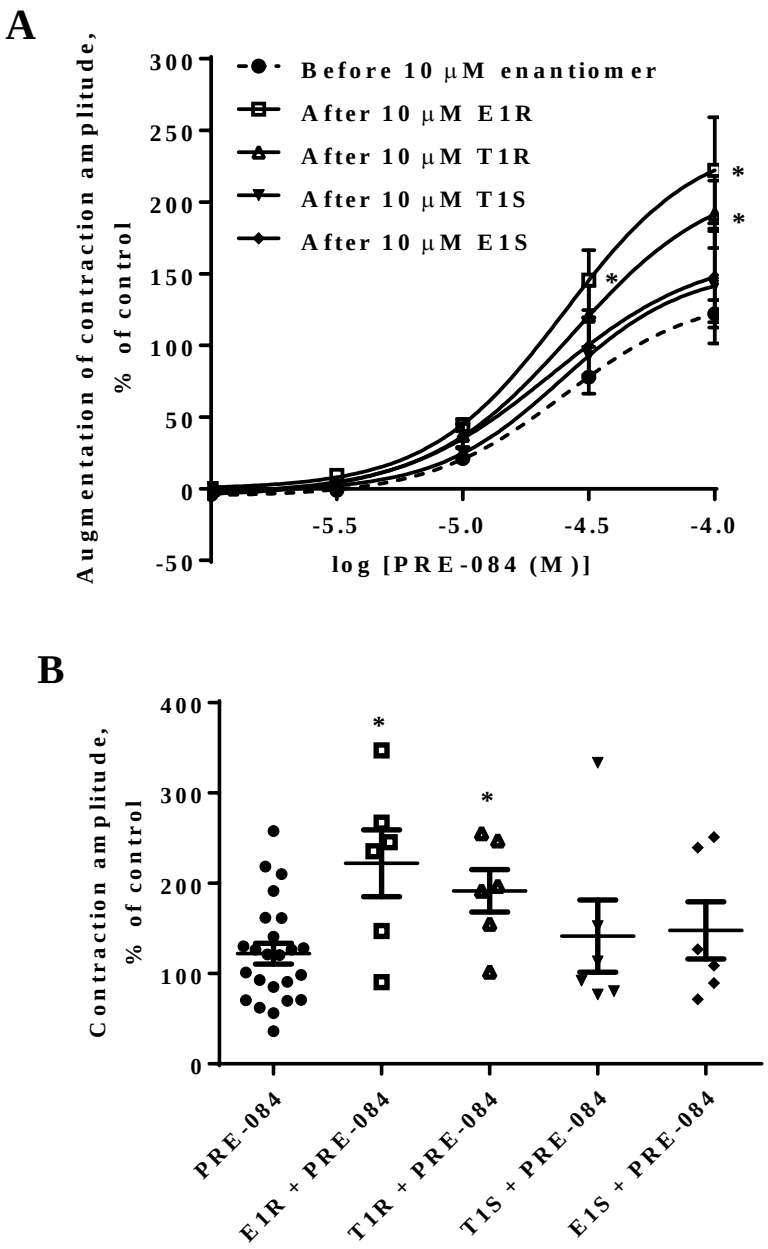


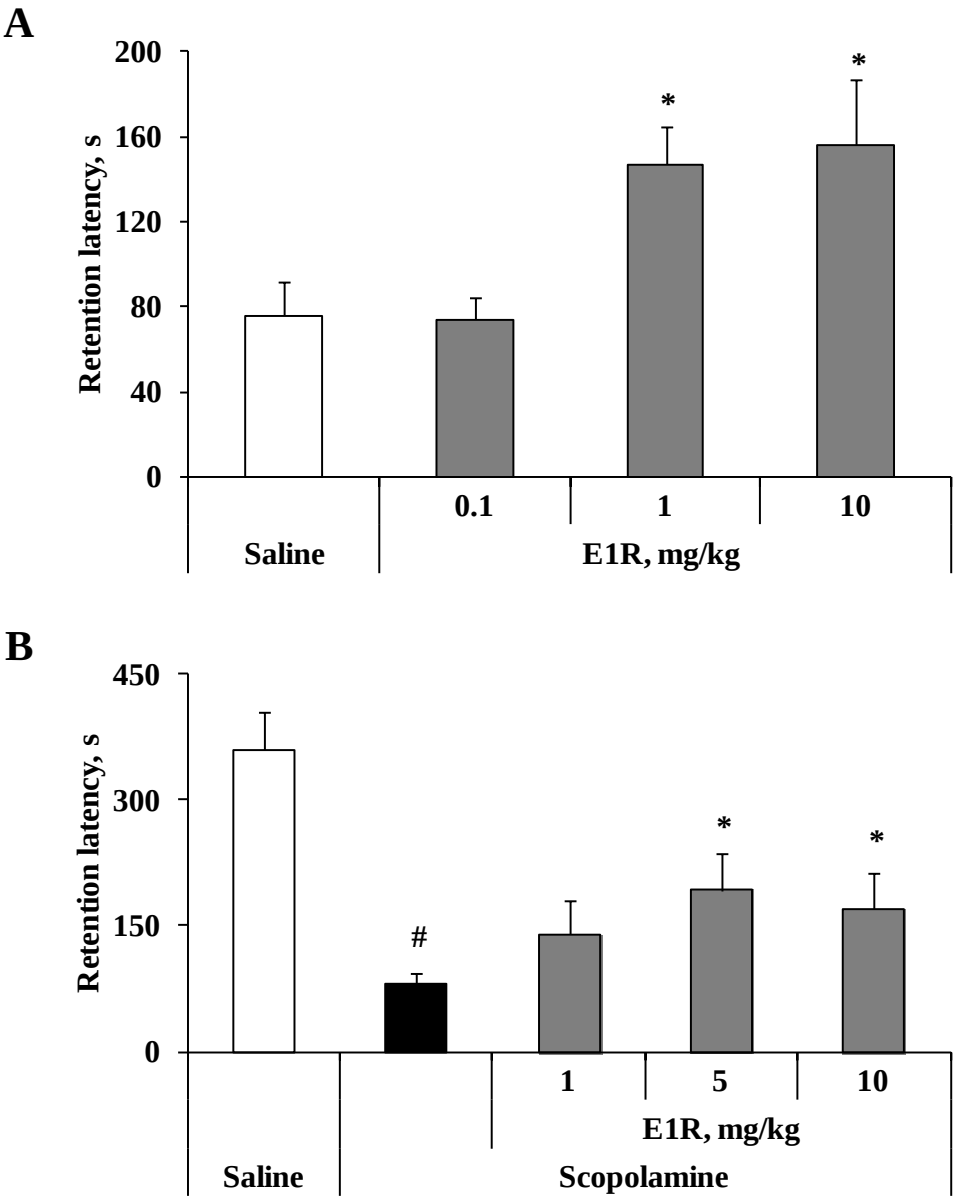
Figure 3.4. **The sigma receptor activity assay in the electrically stimulated rat *vas deferens* model** (**A**) Effects of E1R, T1R, T1S and E1S on contractions potentiated dose-dependently by the selective Sig1R agonist PRE−084 in the electrically stimulated rat *vas deferens*. (**B**) Comparison between the activities of stereoisomers at the highest tested PRE−084 concentration. The results are expressed as the percentage of control contraction height and represent the means ± S.E.M. (n = 6−24). **P* < 0.05 vs. PRE−084 treatment (two-way repeated ANOVA followed by the Bonferroni post-hoc test).

3.6. Effects of E1R on cognition in the PA test (Publication II)

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. As shown in Figure 3.5.A, treatment with E1R at doses of 1 and

10 mg/kg increased retention latency by 194 and 211%, respectively, as compared to the control group.

The PA test was also used to detect the effects of E1R on scopolamine-induced memory impairment. The retention latency in control animals was 360 ± 45 s, and pre-treatment with scopolamine significantly reduced the retention latency to 81 ± 13 s (Figure 3.5.B). Figure 3.5.B indicates that, E1R increased the retention latency at doses of 5 and 10 mg/kg by 237 and 209%, respectively, as compared to the scopolamine-treated group. Treatment with the selective Sig1R antagonist NE−100 significantly inhibited the cognition-enhancing activity of E1R at a dose of 5 mg/kg (Figure 3.5.C).



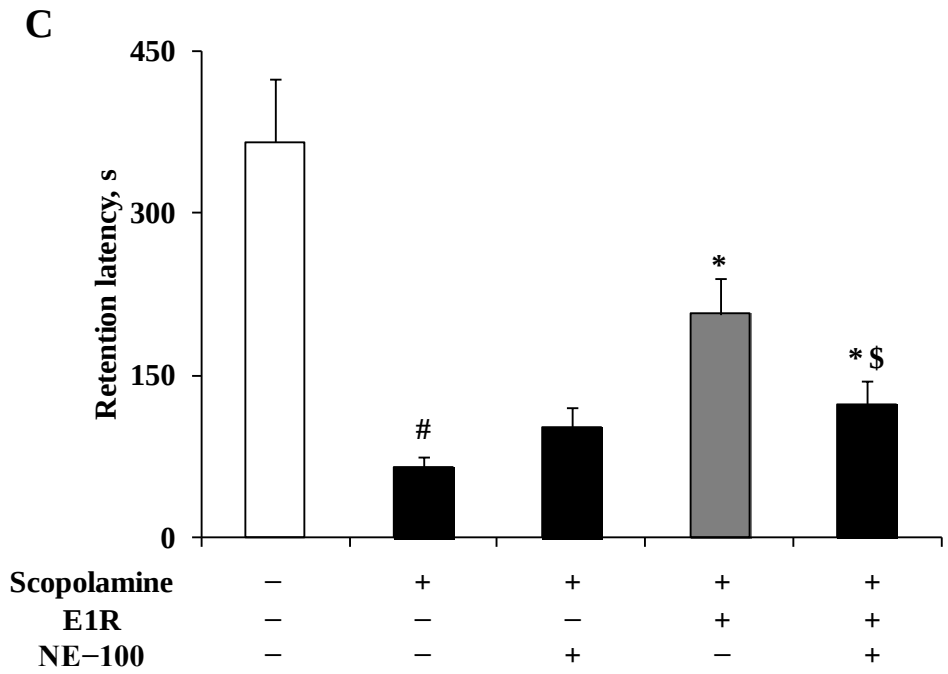


Figure 3.5. **Effects of E1R on memory and cognition in PA test**
(**A**) Dose-related effects of E1R on PA retention in mice. E1R was administered 60 min prior to the training session. The retention test was performed 24 h later. The vertical bars represent the means ± S.E.M. (n = 15−18). **P* < 0.05 vs. the saline group (one-way ANOVA followed by the Newman-Keuls multiple comparison test). (**B**) Dose-related effects of E1R on the scopolamine-induced impairment of PA retention in mice. The mice were injected with E1R 60 min prior to the training session. Scopolamine (0.3 mg/kg, s.c.) was administered 40 min prior to the training session. The vertical bars represent the means ± S.E.M. (n = 17−20). $^{\#}P$ < 0.05 of the scopolamine-treated group vs. the saline control group, **P* < 0.05 vs. the scopolamine-treated group (one-way ANOVA followed by the Newman-Keuls multiple comparison test). (**C**) The effect of E1R at a dose of 5 mg/kg was antagonised by the administration of the selective Sig1R antagonist NE−100 at a dose of 2 mg/kg. Each column represents the means ± S.E.M. (n = 20−25). $^{\#}P$ < 0.05 of the scopolamine-treated group vs. the saline control group, **P* < 0.05 vs. the scopolamine-treated group, and $^{\$}P$ < 0.05 vs. the E1R-treated group (one-way ANOVA followed by the Mann-Whitney U-test).

3.7. Effects of E1R on cognition in Y-maze test (Publication II)

The Y-maze test was used to detect the E1R's effect on scopolamine-induced working memory impairment (Figure 3.6.). The spontaneous alternation behaviour in the control animals was 59 ± 3%; pre-treatment with scopolamine significantly reduced the alternation behaviour to 42 ± 3%. As shown in Figure 3.6., treatment with E1R at dose of 10 mg/kg increased the spontaneous alternation behaviour by 31%, as compared to the scopolamine-treated group. Treatment with the selective Sig1R antagonist NE−100 (2 mg/kg) significantly inhibited the enhancement of working memory of E1R at a dose of 10 mg/kg.

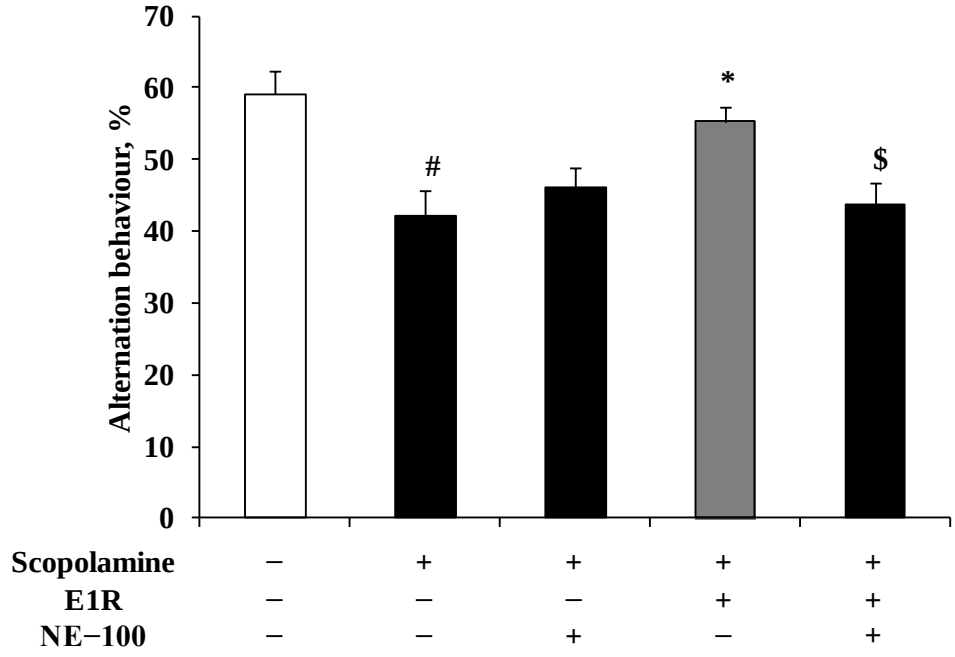


Figure 3.6. **The effect of E1R on scopolamine-induced impairment of spontaneous alternation behaviour in the Y-maze test in mice**

Mice were injected with E1R at a dose of 10 mg/kg i.p. 60 min prior to the training session. Scopolamine (0.5 mg/kg, s.c.) was administered 40 min prior to the training session. The effect of E1R was antagonised by the administration of the selective Sig1R antagonist NE−100 at a dose of 2 mg/kg 20 min prior to E1R. The data are presented as the means ± S.E.M. (n = 14−16). $^{\#}P < 0.05$ of the scopolamine-treated group vs. the saline group, $^{*}P < 0.05$ vs. the scopolamine-treated group, and $^{\$}P < 0.05$ vs. the E1R-treated group (one-way ANOVA followed by the Newman-Keuls multiple comparison test).

3.8. Effects of E1R on locomotion, muscle strength and coordination (Publication II)

The open-field test was used to determine the influence of the compound on locomotor activity. Doses of E1R up to 100 mg/kg did not affect the distance moved as compared to the control animals (Figure 3.7.). In the rota-rod, chimney and traction tests, the inhibitory activity of E1R on muscle function was observed at the following doses: ED_{50} (ED_{16}–ED_{84}) mg/kg = 453 (398−516), 349 (199−611) and 595 (409−866), respectively.

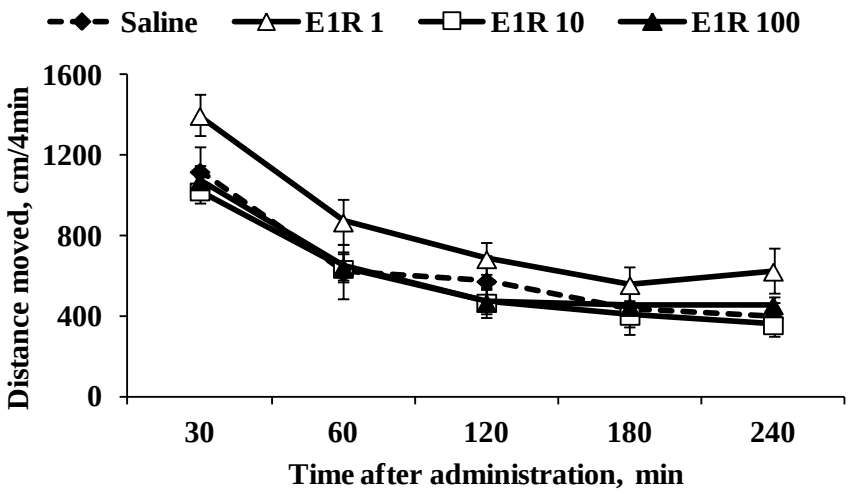


Figure 3.7. **The activity of E1R on moved distance in the open-field test**
E1R was administered at doses of 1, 10 and 100 mg⁄ kg (i.p.). The locomotor activities of mice were observed 30, 60, 120, 180 and 240 min after administration of E1R. The data are presented as the means ± S.E.M. (n = 10).

3.9. Activity of E1R in the PTZ- and BIC-induced seizure models (Publication IV)

The 1% PTZ i.v. infusion induced clonic and tonic seizures in control animals at a dose of 24 ± 1 mg/kg and 67 ± 8 mg/kg, respectively. To test activities of Sig1R ligands, compounds were administered 60 min before PTZ. E1R demonstrated dose-dependent anti-convulsive effects on PTZ-induced tonic seizures (Figure 3.8.B). E1R given i.p. at a dose of 10 mg/kg significantly increased the thresholds for clonic seizures by 20% and for tonic seizures by 47% (Figures 3.8.A and B). The thresholds on PTZ-induced clonic and tonic seizures increased by 23% and 75%, respectively, after the administration of E1R at a dose of 50 mg/kg (Figures 3.8.A and B). At a dose of 5 mg/kg NE−100 had no effect on seizure thresholds. The administration of NE−100 at a dose of 10 mg/kg showed a tendency for pro-convulsive activity on PTZ-induced clonic seizures. NE−100 at a dose of 25 mg/kg demonstrated significant pro-convulsive activity on PTZ-induced clonic seizures (Figure 3.8.A). NE−100 had no effect on tonic seizures (Figure 3.8.B). Selective Sig1R agonist PRE−084 did not change animal behaviour and the threshold for PTZ-induced seizures (Publication IV).

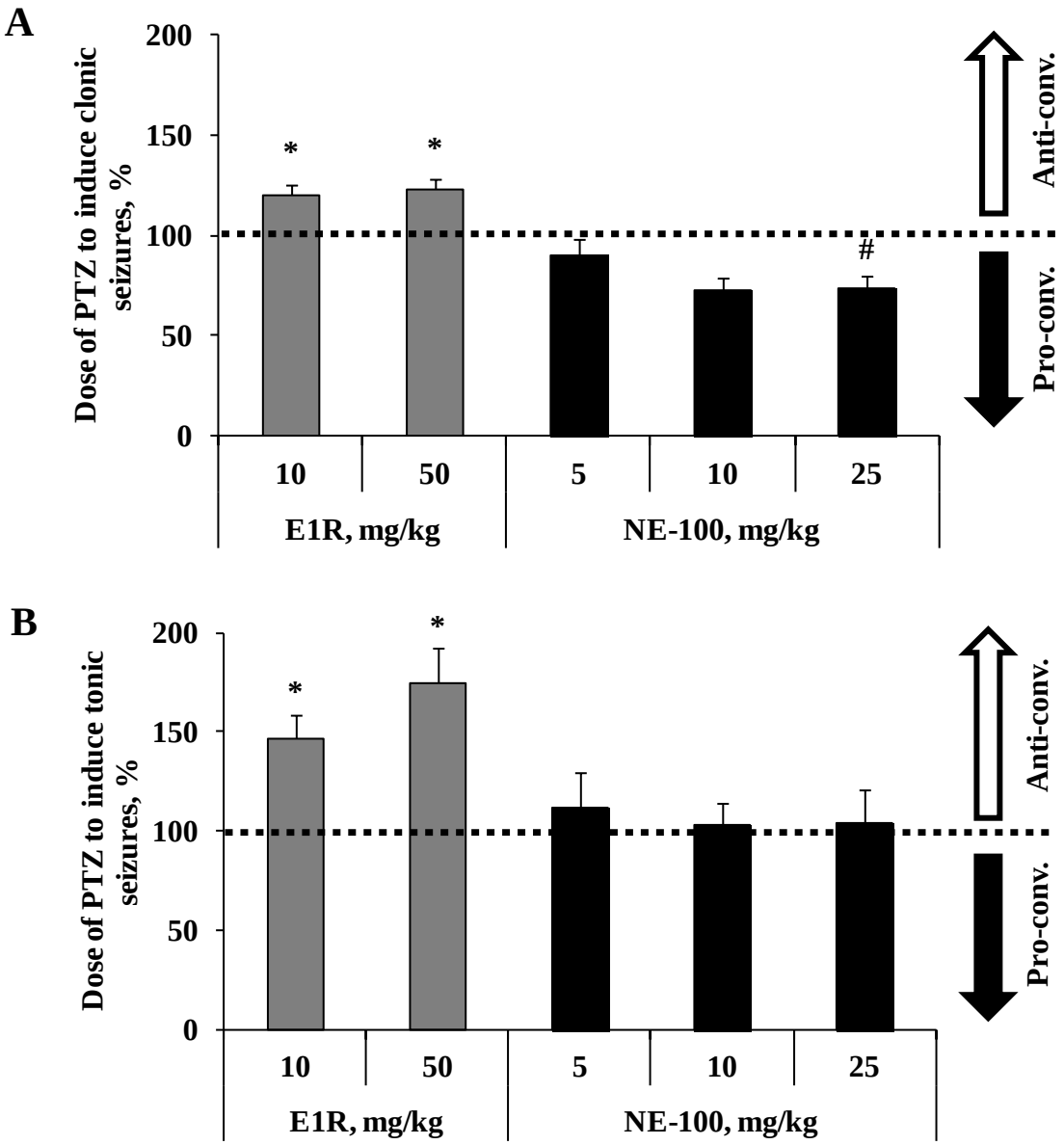


Figure 3.8. **Effects of E1R on PTZ-induced seizure thresholds**
Dotted line represents threshold for PTZ-induced seizures in control group (100%). (**A**) Activity of Sig1R ligands on PTZ-induced clonic seizures. (**B**) Activity of Sig1R ligands on PTZ-induced tonic seizures. Compounds were administered i.p. 60 min before PTZ injection. Data are expressed as the means ± S.E.M. (n = 8−10 in each group). **P* < 0.05 vs. control, #*P* = 0.05 vs. control (Student's t-test).
Anti-conv. – anti-convulsive effect; Pro-conv. – pro-convulsive effect.

The seizure-modulating activity of E1R and selective Sig1R ligands were also tested on BIC-induced seizures. Clonic seizures in control animals were induced at a dose of 0.49 ± 0.06 mg/kg of BIC. BIC-induced tonic seizures were induced at a dose of 0.96 ± 0.15 mg/kg. The administration of NE−100 at a dose of 10 mg/kg had no effect on the seizure thresholds. NE−100 at a dose of 25 mg/kg showed a slight tendency for pro-convulsive activity in the model of BIC-induced clonic seizures. E1R given at a dose of 50 mg/kg significantly elevated the thresholds on BIC-induced clonic and tonic seizures by 21 and 25%, respectively (Figures 3.9.A and B). Similarly as in PTZ-induced seizure model,

PRE−084 administered at a dose of 50 mg/kg demonstrated no differences compared with the control group on the BIC-induced seizure thresholds (Publication IV).

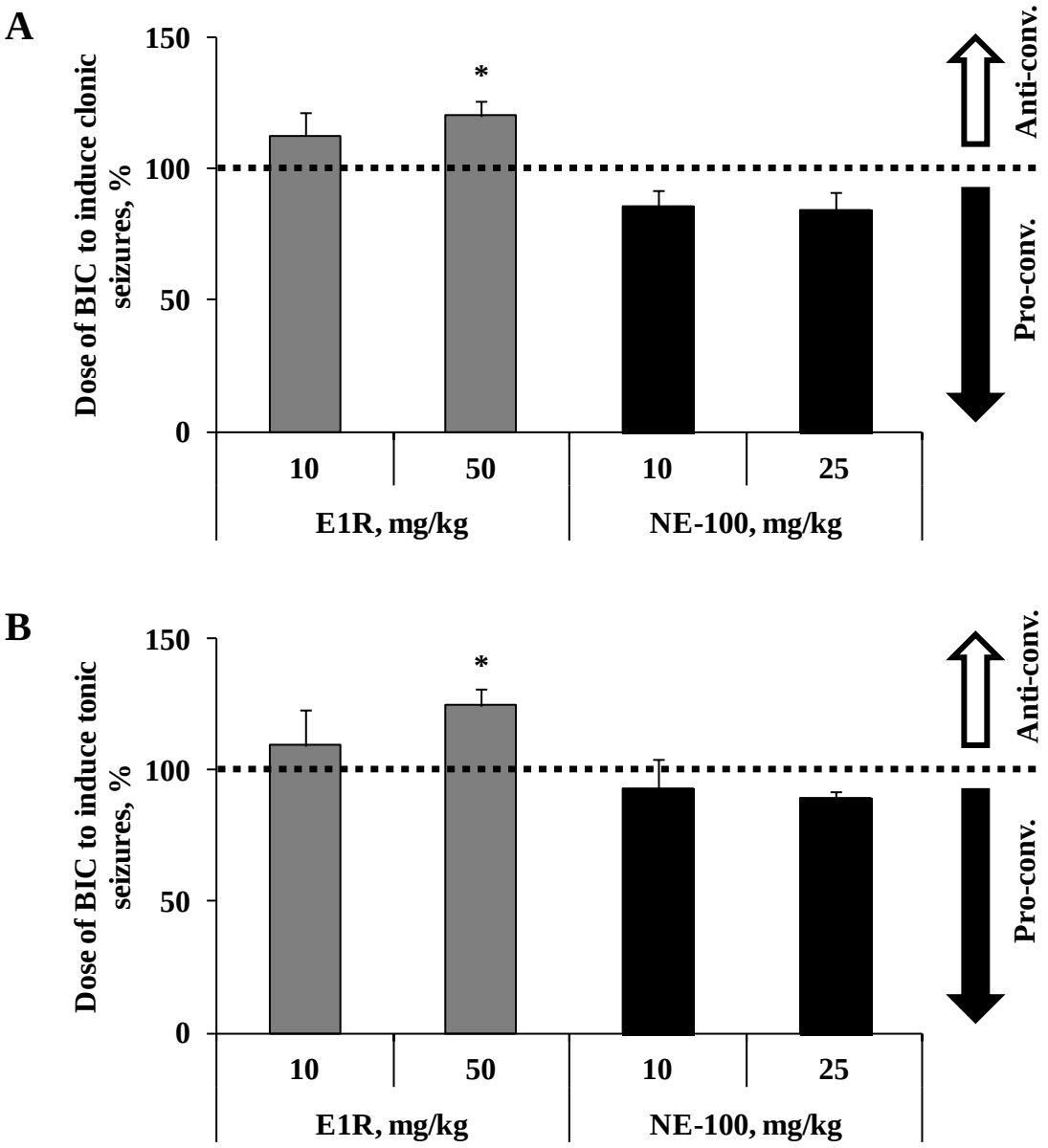


Figure 3.9. **Effects of E1R on BIC-induced seizure thresholds**
Dotted line represents threshold for PTZ-induced seizures in control group (100%). (**A**) Activity of Sig1R ligands on BIC-induced clonic seizures. (**B**) Activity of Sig1R ligands on BIC-induced tonic seizures. Compounds were administered i.p. 60 min before BIC injection. Data are expressed as the means ± S.E.M. (n = 7−10 in each group). **P* < 0.05 vs. control (Student's t-test). Anti-conv. – anti-convulsive effect; Pro-conv. – pro-convulsive effect.

3.10. Anti-seizure effects of E1R are Sig1R dependent (Publication IV)

To verify that Sig1R was involved in the anti-convulsive activity of E1R, selective Sig1R antagonist NE−100 was used. For PTZ-induced seizures, pre-treatment with NE−100 alone at a dose of 5 mg/kg had no significant effect on seizure thresholds (Figure 3.10.). E1R

given at a dose of 10 mg/kg significantly increased the threshold on PTZ-induced tonic seizures by 39% (Figure 3.10.). The administration of NE−100 (5 mg/kg) before E1R (10 mg/kg) significantly restored the tonic seizure threshold to the basal level (Figure 3.10.) and therefore, showed that the anti-seizure effect of E1R was mediated through Sig1R activity.

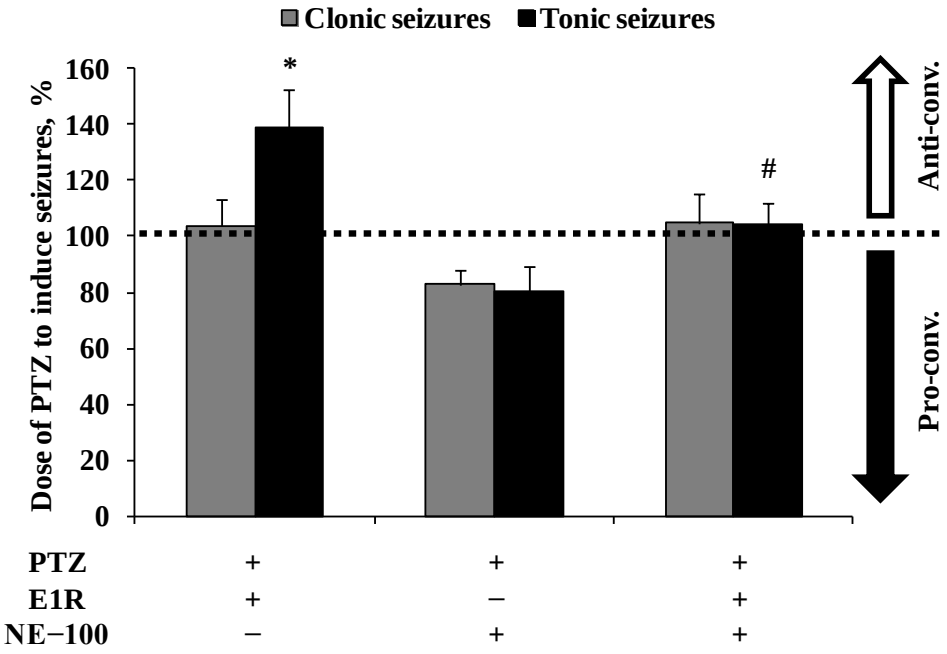


Figure 3.10. **Effect of selective Sig1R antagonist on the anti-convulsive activity of E1R on PTZ-induced seizures**
Dotted line represents threshold for PTZ-induced seizures in control group (100%). E1R was administered i.p. at a dose of 10 mg/kg 60 min before PTZ injection. NE−100 was given i.p. at a dose of 5 mg/kg 80 min before PTZ injection. Data are expressed as the means ± S.E.M. (n = 10 in each group). $^{*}P < 0.05$ vs. control, $^{\#}P < 0.05$ vs. E1R (one-way ANOVA followed by Newman-Keuls multiple comparison test). Anti-conv. – anti-convulsive effect; Pro-conv. – pro-convulsive effect.

3.11. The activity of E1R on NE-100-induced seizures (Publication IV)

Surprisingly, we discovered that the i.p. administration of selective Sig1R antagonist NE−100 at a dose of 50 mg/kg induced convulsions in mice before PTZ infusion. Convulsive activity after the administration of NE−100 at a dose of 50 mg/kg was observed for 5 from total of 7 animals, while NE−100 at a dose of 75 mg/kg induced generalised, tonic and clonic seizures for 11 from total of 11 animals. The convulsive behaviour of mice after administration of NE−100 is described in detail in Publication IV. E1R at a dose of 75 mg/kg partially prevented NE−100 induced seizures and showed lower average behavioural score (Figure 3.11.A and Table 3.2.). As demonstrated by the data expressed as areas under the curves (AUCs), E1R showed statistically significant effect (Figure 3.11.B).

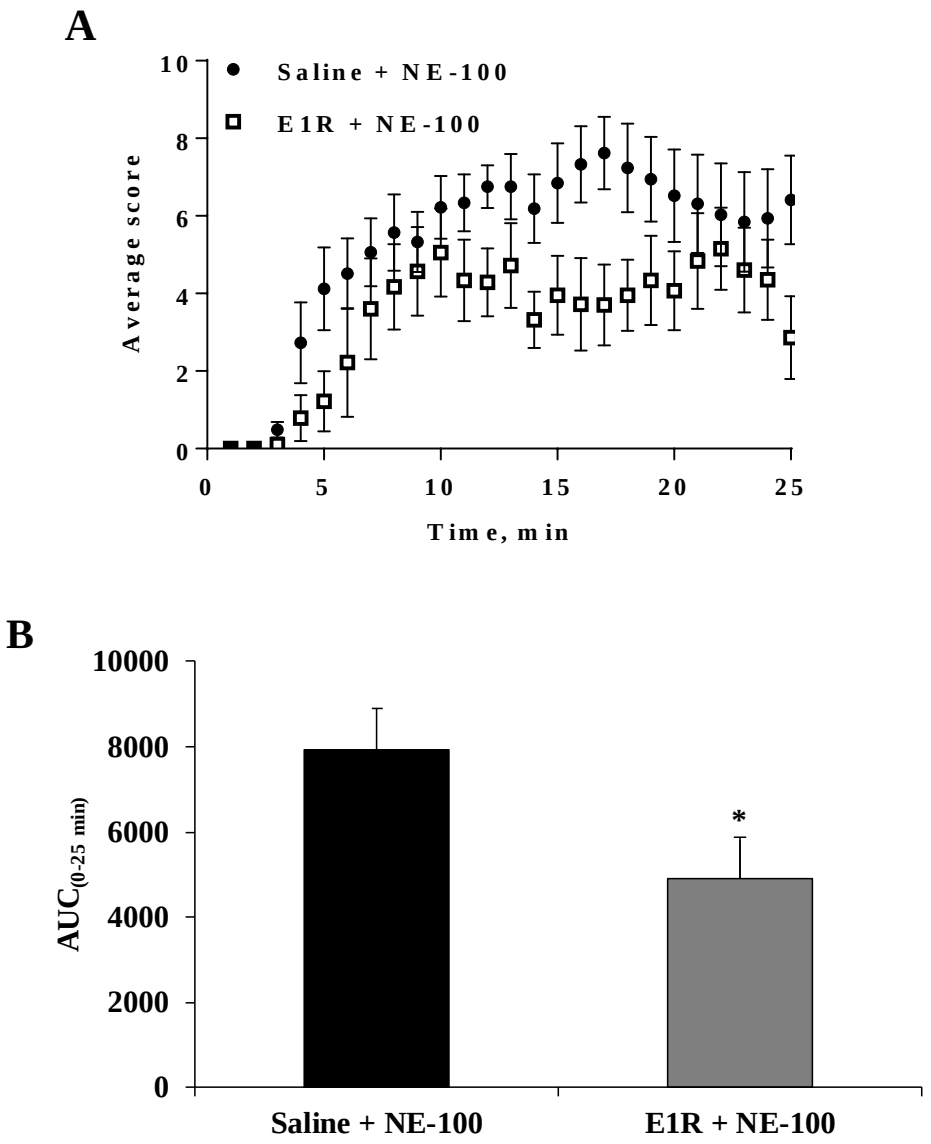


Figure 3.11. **The activity of E1R on NE−100-induced convulsive behaviour**
E1R (75 mg/kg, n = 6) or saline (n = 11) were administered i.p. 30 min prior to i.p. injection of NE−100 (75 mg/kg). (**A**) Average behavioural scores for each group during 25-min observation period. Data are expressed as the means for each 1 min period. (**B**) The area under curve ($AUC_{0–25}$ min) was calculated from behavioural scoring curve. Data are expressed as the means ± S.E.M. (n = 6−11). *P < 0.05 vs. the saline group (one-way ANOVA followed by Newman-Keuls multiple comparison test).

E1R significantly reduced also the number of animals with generalised seizures induced by NE−100 and reduced the generalised seizure count per animal (Figure 3.12.A). E1R also demonstrated higher latency times until the first occurrence of seizures induced by NE−100 (Table 3.2.) and increased the survival of animals (Figure 3.12.B). However, there was no significant difference when compared with the NE−100 treated animal group.

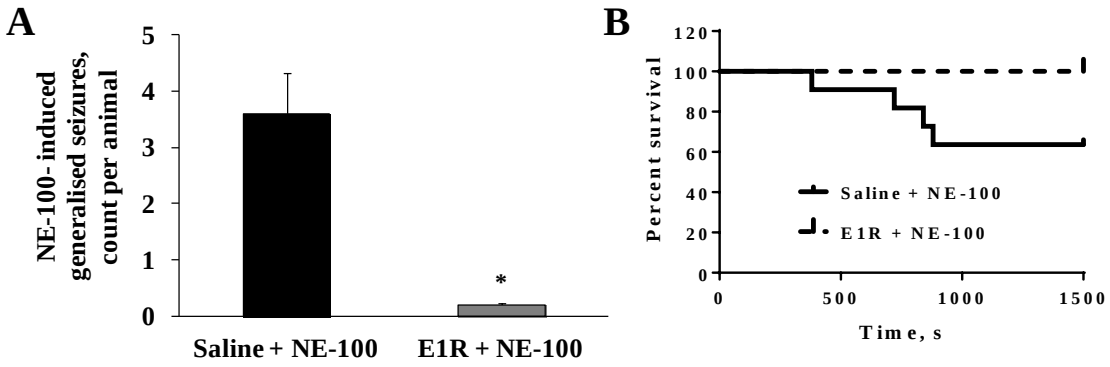


Figure 3.12. **The effects of E1R on NE−100-induced generalised seizures and survival**
E1R (75 mg/kg, n = 6) or saline (n = 11) were administered i.p. 30 min prior to i.p. injection of NE-100 (75 mg/kg). (**A**) Effects on NE−100-induced generalised seizures. Data are expressed as the means ± S.E.M. **P* < 0.05 vs. the saline group (one-way ANOVA followed by Newman-Keuls multiple comparison test). (**B**) Survival curve during 25-min observation period.

Table 3.2.

The activity of E1R on NE−100-induced convulsive behaviour

Observation	Saline + NE−100	E1R + NE−100
Animals with seizures, n/total n	11/11	5/6
Seizure onset time, s	238 ± 27	320 ± 34
Animals with generalised seizures, n/total n	11/11	1/5
Average maximal score (peak)	8.2 ± 0.8	5.8 ± 1.3
Average time to reach peak, s	569 ± 65	708 ± 166
Average behavioural score	5.3 ± 0.3	3.3 ± 0.2

E1R (75 mg/kg; n = 6) or saline (n = 11) were administered i.p. 30 min prior to i.p. injection of NE−100 (75 mg/kg).

4. DISCUSSION

Sig1R is an exciting novel drug target for neurological applications. We have discovered the novel compound E1R, which has cognition-enhancing and anti-seizure properties that are related to its positive allosteric modulatory activity at Sig1R. E1R is the first described selective PAM of Sig1R that demonstrates a novel mechanism for the improvement of memory and cognition.

4.1. Allosteric modulators of Sig1R

The Sig1R site was the only site that E1R was discovered to target in *in vitro* pharmacological profiling assays, which included a number of ion channels, G protein-coupled receptors and CNS transporters (Publication II). The selected *in vitro* assays revealed that E1R did not bind directly to Sig1R but rather acted as a PAM of the receptor. It should be noted that E1R enhanced the binding of an unselective sigma receptor radioligand, [^{3}H]-DTG; however, we failed to demonstrate a Sig1R modulatory effect of E1R in a selective Sig1R radioligand [^{3}H]-(+)-pentazocine binding assay using rat brain tissues (Publication II). At the same time, phenytoin also did not change the binding of [^{3}H]-(+)-pentazocine, whereas PRE−084 and haloperidol dose-dependently competed with [^{3}H]-(+)-pentazocine to bind to Sig1R (Publication II). The allosteric modulatory activity of phenytoin has previously been described in rat and guinea pig brains (Cobos *et al*., 2006; DeHaven-Hudkins *et al*., 1993; Guo *et al*., 2013), rat livers (McCann and Su, 1991), and mice lung tissues (Lever *et al*., 2015). In turn, it has been shown that phenytoin could modulate Sig1R ligand binding in rat brain tissue but not in rat liver tissue (Guo *et al*., 2013). For example, SCH−23390 and SKF−38393 modulated the binding of [^{3}H]-(+)-pentazocine only in liver tissues, while no detectable effects were observed in brain tissues (Guo *et al*., 2013). In the same study, SKF−83959 was shown to allosterically modulate the binding of [^{3}H]-(+)-pentazocine in both rat brain and liver tissues (Guo *et al*., 2013). The different effects of these Sig1R allosteric modulators in rat and guinea pig brain tissues have been described previously and could be explained by the variation in the size of the binding site between the two species (Klein and Musacchio, 1992). It seems that not only different species but also different tissues from the same animal species can respond differently to allosteric Sig1R modulators. SKF−83959 and its analogues failed to change the binding activity of [^{3}H]-(+)-pentazocine at Sig1R in human embryonic kidney (HEK)293 cells that stably expressed the receptor (Guo *et al*., 2013). The lack of binding modulatory activity of

[^{3}H]-(+)-pentazocine in HEK293 cells was also observed for phenytoin (Guo *et al*., 2013). Although [^{3}H]-(+)-pentazocine displayed similar affinity for Sig1R in transfected HEK293 cells and rat brain tissues, the absence of allosteric modulation of Sig1R in the constructed system *in vitro* could be attributed to differences in Sig1R structure, cellular contents, or auxiliary proteins (Guo *et al*., 2013), which should be taken into account when studying mechanisms of Sig1R *in vitro*.

Despite the lack of [^{3}H]-(+)-pentazocine binding modulatory activity in rat brain tissue, E1R potentiated the contractions of rat *vasa deferentia* in the presence of the Sig1R agonist PRE−084 but not in the presence of the Sig2R agonist PB−28 (Publication II). In addition, E1R enhanced the effect of PRE−084 on the BDK-induced $[Ca^{2+}]_i$ increase, thus confirming its Sig1R positive allosteric modulatory effect *in vitro*. For comparison, the *in vitro* activities of allosteric Sig1R modulators are summarized in the Table 4.1.

Table 4.1.

The comparison of *in vitro* allosteric effects of positive allosteric Sig1R modulators

Compound	[μM]	Activity	SigR ligand	Tissues/cells (species)	References
Phenytoin	10−100	increases the binding	[^{3}H]-DM	brain tissues (guinea pig)	(Craviso *et al*., 1983; Musacchio *et al*., 1988 and 1989)
	10−100	increases the binding	[^{3}H]-(+)-3-PPP	brain tissues (guinea pig)	(Musacchio *et al*., 1989)
	300	increases the binding	[^{3}H]-(+)-SKF−10,047	brain tissues (guinea pig)	(Karbon *et al*., 1991)
	300	increases the binding	[^{3}H]-(+)-SKF−10,047	liver tissues (rat)	(McCann and Su, 1991)
	0.1−250	increases the binding	[^{3}H]-(+)-Pentazocine	brain tissues (guinea pig)	(DeHaven-Hudkins *et al*., 1993; Cobos *et al*., 2005 and 2006)
	100−10000	increases the binding	[^{3}H]-(+)-Pentazocine	brain tissues (rat)	(Guo *et al*., 2013)
	1000	increases the binding affinity of DM	[^{3}H]-(+)-Pentazocine	lung tissues (mice)	(Lever *et al*., 2015)
	0.0001−100	no effect	[^{3}H]-(+)-Pentazocine	brain tissues (rat)	Publication II
	1−10000	no effect	[^{3}H]-(+)-Pentazocine	liver tissues (rat)	(Guo *et al*., 2013)
	10, 100	no effect	[^{3}H]-(+)-Pentazocine	constructed HEK293 cells	(Guo *et al*., 2013)

Table 4.1. (continued)

Compound	[μM]	Activity	SigR ligand	Tissues/cells (species)	References
Phenytoin	10, 100/ 300	no effect	[^{3}H]-DTG	brain tissues (guinea pig/ rat)	(Karbon *et al*., 1991/ Guo *et al*., 2013)
	0.1−250	decreases the binding	[^{3}H]-NE−100	brain tissues (guinea pig)	(Cobos *et al*., 2006)
	10, 100	no effect	[^{3}H]-Progesterone	brain and liver tissues (rat)	(Guo *et al*., 2013)
Ropizine (SC−13504)	0.1−10	increases the binding	[^{3}H]-DM	brain tissues (guinea pig)	(Musacchio *et al*., 1988 and 1989; Klein and Musacchio, 1992)
	0.1−10	increases the binding	[^{3}H]-(+)-3-PPP	brain tissues (guinea pig)	(Musacchio *et al*., 1989; Klein and Musacchio, 1990 and 1992)
SKF−83959	0.1−100	increases the binding	[^{3}H]-(+)-Pentazocine	brain and liver tissues (rat)	(Guo *et al*., 2013)
	0.1−10	increases the binding affinity of DHEA	[^{3}H]-(+)-Pentazocine	brain tissues (rat)	(Wu *et al*., 2015)
	0.1	enhances the anti-inflammatory effect on LPS induced inflammation	DHEA	microglial BV−2 cells (mice)	(Wu *et al*., 2015)
	1	enhances the anti-inflammatory effect on LPS induced inflammation	PRE−084	microglial BV−2 cells (mice)	(Wu *et al*., 2015)
	10, 100	no effect	[^{3}H]-(+)-Pentazocine	constructed HEK293 cells	(Guo *et al*., 2013)
	10, 100	no effect	[^{3}H]-Progesterone	brain and liver tissues (rat)	(Guo *et al*., 2013)
	10, 100	no effect	[^{3}H]-DTG	brain and liver tissues (rat)	(Guo *et al*., 2013)
SCH−23390	0.1−100	increases the binding	[^{3}H]-(+)-Pentazocine	liver tissues (rat)	(Guo *et al*., 2013)
	0.001−100	no effect	[^{3}H]-(+)-Pentazocine	brain tissues (rat)	

Table 4.1. (continued)

Compound	[μM]	Activity	SigR ligand	Tissues/cells (species)	References
SCH−23390	10, 100	no effect	[^{3}H]-(+)-Pentazocine	constructed HEK293 cells	(Guo *et al*., 2013)
	10, 100	no effect	[^{3}H]-Progesterone	brain and liver tissues (rat)	
	10, 100	no effect	[^{3}H]-DTG	brain and liver tissues (rat)	
SKF−38393	0.1−100	increases the binding	[^{3}H]-(+)-Pentazocine	liver tissues (rat)	(Guo *et al*., 2013)
	0.001−100	no effect	[^{3}H]-(+)-Pentazocine	brain tissues (rat)	
	10, 100	no effect	[^{3}H]-(+)-Pentazocine	constructed HEK293 cells	
	10, 100	no effect	[^{3}H]-Progesterone	brain and liver tissues (rat)	
	10, 100	no effect	[^{3}H]-DTG	brain and liver tissues (rat)	
SOMCL−668	100	increases the binding	[^{3}H]-(+)-Pentazocine	brain tissues (rat)	(Guo *et al*., 2015)
	10	enhances the translocation of Sig1R from Bip	(+)-SKF−10,047	hippocampal neuronal HT−22 cells (mice)	(Wang *et al*., 2016)
	10	enhances stimulated neurite growth and BDNF secretion	(+)-SKF−10,047	primary cortical/ hippocampal neurons (mice)	(Wang *et al*., 2016)
E1R	10	increases the binding	[^{3}H]-DTG	Jurkat cells (human)	Publication II
	0.0001−100	no effect	[^{3}H]-(+)-Pentazocine	brain tissues (rat)	Publication II
	10	enhances the activity on electrically stimulated contractions	PRE−084	*vasa deferentia* (rat)	Publication I
	10	no effect	PB−28	*vasa deferentia* (rat)	Publication I
	10	enhances the activity on the BDK-induced $[Ca^{2+}]_i$ increase	PRE−084	NG108-15 cells (rat and mice)	Publication II

PAMs are compounds that differ from orthosteric Sig1R ligands in molecular structure but share some similarities with each other. Allosteric modulators are small heterocyclic compounds with two hydrophobic substituents (phenyl and/or methyl groups) on the heterocyclic ring. The difference in molecular structure from prototypical Sig1R ligands explains why allosteric modulators do not compete for binding in the active site. Sig1R is spread throughout the cells in the body; therefore, allosteric modulatory activity has been observed in different cells and tissues, which suggests these compounds as possible drugs for multiple pharmacological applications.

4.2. Modulation of Sig1R and regulation of memory and cognition

Several lines of evidence have suggested that the activation of Sig1R ameliorates cognitive deficits in animal models of cholinergic dysfunction that mimic the cognitive symptoms of Alzheimer's disease (Antonini *et al*., 2009; Earley *et al*., 1991; Matsuno *et al*., 1994; Maurice *et al*., 1998; Maurice and Su, 2009). In addition, some Sig1R agonists have been shown to increase acetylcholine release (Matsuno *et al*., 1993; van Waarde *et al*., 2011). Because our *in vitro* studies identified E1R as a PAM of the Sig1R, we hypothesized that E1R might protect against scopolamine-induced cognitive deficits. E1R successfully alleviated scopolamine-induced cognitive impairment in mice, as assessed using the PA and Y-maze tests. The effects of E1R were antagonized by the selective Sig1R antagonist NE−100, thus confirming the Sig1R modulatory activity of E1R *in vivo*.

Neurosteroids are considered the most likely endogenous Sig1R ligands (Cobos *et al*., 2008; Niitsu *et al*., 2012). Neurosteroids such as pregnenolone and DHEA are known to bind to Sig1R under physiological conditions, and Sig1R constitutes one of the key targets in their trophic, neuromodulatory and behavioural effects (Monnet and Maurice, 2006; Su *et al*., 1988a). Pregnenolone, DHEA and other nonsteroidal Sig1R agonists, influence the learning and memory processes in cholinergic and NMDA receptor-dependent models of amnesia and ageing (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). Therefore, we propose that the cognition-enhancing effects of E1R may involve the modulation of the activity of some endogenous agonists of Sig1R.

The memory improving effects of E1R on both drug-naive and scopolamine-treated mice in the PA test are of particular interest due to the piracetam-like structure of E1R. Many racetams share piracetam's nootropic properties in several mammalian species, ranging from

rodents to humans (Frostl and Maitre, 1989; Gouliaev *et al.*, 1995; Malykh and Sadaie, 2010). Racetams enhance performance in various learning and memory tasks, particularly in the PA test in mice and rats (Krylova *et al.*, 1991; Zvejniece *et al.*, 2011). Piracetam and its derivatives are known to alleviate the memory deficits caused by scopolamine and other amnesic drugs (Malykh and Sadaie, 2010; Zvejniece *et al.*, 2011). Phenylpiracetam and its most active (R)-enantiomer (R)-phenylpiracetam possess 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; Publication I), suggesting that E1R may exhibit similar behavioural effects. However, our present data show that although E1R possesses cognition-enhancing activity similar to (R)-phenylpiracetam, E1R does not affect performance in the open-field test at doses up to 100 mg/kg (Publication II). Therefore, even minor structural alterations may contribute to rather significant differences in the biological (pharmacological) activity of piracetam-like compounds. While different molecular targets have been suggested for racetams, Sig1R is not mentioned among them, even though they share common activities with Sig1R (Figure 4.1.).

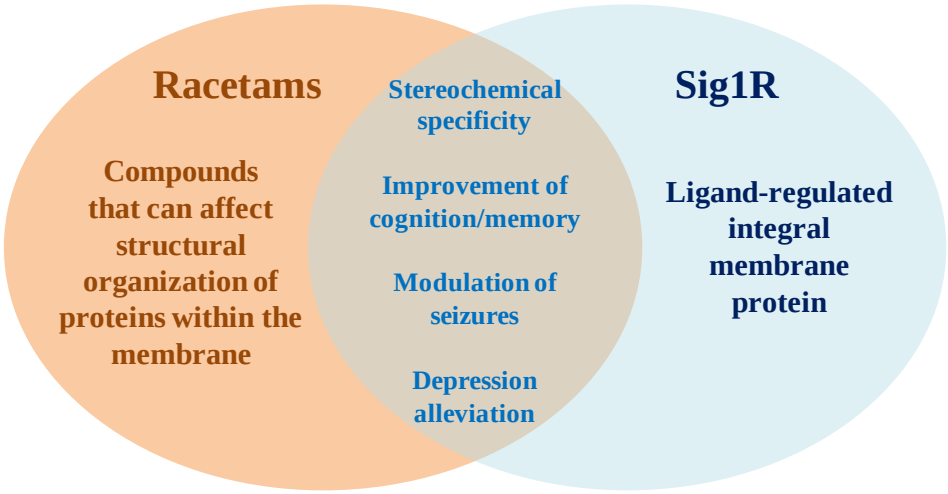


Figure 4.1. **Links between the pharmacological activities of racetams and roles of Sig1R.**

Among all the positive allosteric Sig1R modulators, E1R is the only modulator known to exert memory improving effects. Phenytoin has been reported to decrease motor activity in mice (Poncelet *et al.*, 1984), reduce increases in extracellular K^+ concentration (Nobile and Lagostena, 1998) and inhibit both Na^+ (Rush and Elliott, 1997) and T-type Ca^{2+} currents (Todorovic and Lingle, 1998). In contrast to E1R, treatment with phenytoin triggered memory impairment during the PA task (Reeta *et al.*, 2009). Treatment of epilepsy with phenytoin has been shown to induce learning and memory deficits in patients as well (Mishra and Goel,

2015). Supplementary approaches for the management of memory deficits associated with conventional anti-epileptic drugs are needed (Mishra and Goel, 2015). The effects of E1R indicate that it might be a promising novel anti-seizure drug with none of the negative influences on memory typically encountered with many anti-epileptic drugs. Unlike phenytoin, E1R does not affect locomotion at doses up to 100 mg/kg and does not influence Na^+ and K^+ channels, as shown in pharmacological profiling assays. In addition, E1R was found to be free of potential motor side effects due to the absence of effects of the compound at doses up to 200 mg/kg on locomotor activity, muscle tone or coordination. Therefore, E1R is the first reported PAM of Sig1R that enhances cognition without affecting locomotor activity, and similar to other piracetam-like compounds, E1R demonstrates no serious side effects.

4.3. Sig1R ligands and seizures

All positive allosteric Sig1R modulators demonstrate anti-seizure activity. However, the anti-seizure activity is not associated directly with Sig1R for all allosteric Sig1R modulators (Table 4.2.). It was shown that combined treatment with phenytoin and the selective Sig1R antagonist BD−1047 did not alter the seizure threshold in the maximal electroshock-induced seizure model compared with treatment with phenytoin alone (Guo *et al*., 2015). The primary mechanism of the anti-seizure activity of phenytoin involves the inhibition of voltage-gated sodium channels (Tunnicliff, 1996). SCH−23390 has been shown to modulate seizures evoked by chemoconvulsants (Bourne *et al*., 2001), but the anti-seizure activity was demonstrated to be D_1 receptor dependent (Bourne *et al*., 2001). This suggests that the pharmacological activity of PAMs of Sig1R may depend on the cumulative effect of several molecular mechanisms.

The seizure modulating activity is not unique to positive allosteric Sig1R modulators. For example, the high affinity Sig1R agonists DM (24 mg/kg, s.c.) and dimemorfan (24 mg/kg, s.c.) have prevented kainic acid-induced seizures in rats (Shin *et al*., 2005). A similar effect has been shown for another Sig1R agonist, pentoxyverine, on kainic acid-induced neurotoxicity in rats (Kim *et al*., 2001). Racemic (±)-pentazocine co-administered with naloxone dose-dependently (20−100 mg/kg, s.c.) reduced tonic seizures induced by N-methyl-DL-aspartic acid in mice (Singh *et al*., 1990). In turn, it has been shown that the Sig1R agonists SA−4503 and DTG cannot protect against cocaine-induced seizures, while in the same study, the Sig1R antagonist panamesine demonstrated anti-convulsive activity (Skuza, 1990). Similar activity has been shown for the Sig1R antagonists

AC−927 (1−10 mg/kg, i.p.), LR−172 (1−30 mg/kg, i.p.) and BD−1047 (1−40 mg/kg, i.p.) against cocaine-induced seizures (Matsumoto *et al*., 2011; McCracken *et al*., 1999). However, there is limited information available concerning the activity of Sig1R antagonists in other seizure models. For example, in kainic acid-, maximal electroshock- and PTZ-induced seizure models, the Sig1R antagonist BD−1047 possess seizure modifying activity, but the compound was used only at low doses (1 and 2 mg/kg, i.p.), while Sig1R agonists in the same seizure models have been studied in wider concentration ranges. NE−100 is a selective Sig1R antagonist (K_i = 0.86 nM) displaying more than 55-fold selectivity for Sig1R over Sig2R and more than 6000-fold selectivity for Sig1R over dopamine, serotonin and phencyclidine receptors (Okuyama *et al*., 1993). We found that NE−100 presents a dose-dependent pro-convulsive activity in PTZ- and BIC-induced seizure models and induces convulsions in mice after only a single injection (Publication IV). NE−100-induced convulsive behaviour was partially attenuated by E1R. The interaction between E1R and NE−100 allows us to confirm the role of Sig1R in the seizure modulating activity of these compounds.

Table 4.2.

Sig1R-dependent anti-seizure activity of positive allosteric Sig1R modulators

Compound	Dose, mg/kg	Seizure model	Effects	References
SKF−83959	2	maximal electroshock seizure threshold test	no significant effect	(Guo *et al*., 2015)
	10		increased the seizure threshold	
	20			
	40			
	2	PTZ (80 mg/kg, s.c.)	no significant effect	
	10		no significant effect	
	20		prolonged the latencies of clonic and generalized clonic-tonic seizures, survival time, and significantly lowered seizure scores	
	40			
	2	kainic acid (30 mg/kg, i.p.)	no significant effect	
	10		no significant effect	
	20		no significant effect	
	40		significantly reduced seizure incidence, prolonged the latency to seizures, and shortened the duration of seizures	
SOMCL−668	**40**	maximal electroshock seizure threshold test	increased the seizure threshold	(Guo *et al*., 2015)
	40	PTZ (80 mg/kg, s.c.)	prolonged the latency time to the generalized clonic-tonic seizures and survival time	

Table 4.2. (continued)

Compound	Dose, mg/kg	Seizure model	Effects	References
SOMCL−668	**40**	kainic acid (30 mg/kg, i.p.)	prolonged the latency time and shortened the duration of seizures	(Guo *et al*., 2015)
E1R	**10**	PTZ (i.v. infusion)	increased the threshold for clonic and tonic seizures	Publication IV
	50			
	10	BIC (i.v. infusion)	no significant effect	
	50		increased the threshold for clonic and tonic seizures	
	75	NE−100 (75 mg/kg, i.p.)*	significantly reduced generalised seizure count and average behavioural score	

*NE−100 induced seizures in 100% of animals at a dose of 75 mg/kg. Phenytoin, ropizine, SCH−23390 and SKF−38393 are not included in the table because the seizure modulating activities of these compounds are not shown to be significantly blocked by selective Sig1R antagonists.

Some derivatives of piracetam demonstrate anti-seizure activity and are used in clinical practice to treat epilepsy. However, the anti-seizure activity of these compounds is not related to the modulation of Sig1R. Brivaracetam, levetiracetam and seletracetam are anticonvulsants that bind to SV2A with high affinity (Malykh and Sadaie, 2010). Piracetam itself demonstrates poor anti-convulsive activity and has lower affinity for SV2A than levetiracetam (Noyer *et al*., 1995). To date, Sig1R has been the only established target involved in the pharmacological activity of E1R (Publication II), and E1R is the first known derivative of piracetam demonstrating anti-seizure activity, which is due to its selective positive allosteric modulatory activity at Sig1R.

4.4. Sig1R allosteric modulators: possible molecular mechanisms

There are no clearly defined molecular mechanisms that could fully describe the function of Sig1R and the activity of Sig1R ligands. The crystal structure of Sig1R shows that the ligand-binding domain in the protein is highly conserved, and how ligands enter and exit this site remains unclear (Schmidt *et al*., 2016). The binding site for allosteric Sig1R modulators probably is located outside the orthosteric ligand-binding domain. Since allosteric modulators are compounds that induce a conformational change within the protein structure, they should reorganize the Sig1R protein in a way that would allow agonists to freely enter the ligand-binding site. It has been discussed previously that phenytoin might induce a conformational change in the receptor and thus enhance the affinity of the orthosteric ligand [^{3}H]-(+)-pentazocine for its binding site on Sig1R (Cobos *et al*., 2006). However, it is not

clear how positive allosteric Sig1R modulators can distinguish between agonists and antagonists and then selectively enhance the activity of agonists, even though the agonists and antagonists sometimes contain the same structural moieties.

The activity of Sig1R might depend on the receptor's oligomerization states (Chu and Ruoho, 2016). It has been shown that the Sig1R agonist (+)-pentazocine increased the relative ratio of dimers and monomers, while the inhibitor haloperidol increased the incidence of higher oligomeric forms (Chu and Ruoho, 2016). This indicates that higher oligomeric forms of S1R might be functionally inactive (Figure 4.2.A). Since all Sig1R allosteric modulators known thus far are PAMs and enhance the activity of Sig1R agonists, they might modulate Sig1R by stabilizing the agonist state of the receptor (Figure 4.2.) providing an increase in the dimeric (Figure 4.2.B) and/or monomeric (Figure 4.2.C) protein forms.

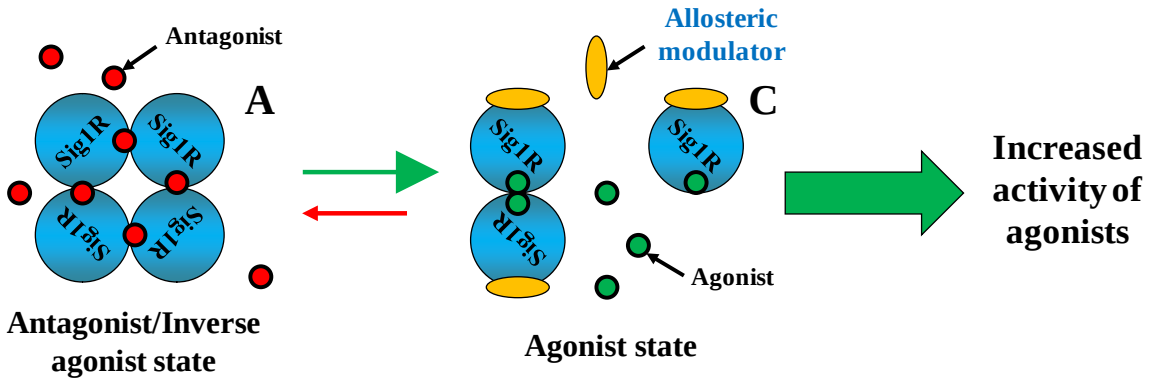


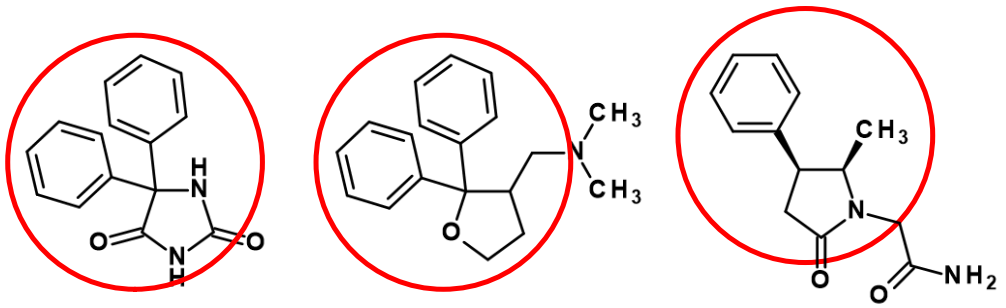
Figure 4.2. **Stabilization of the agonist state of Sig1R by positive allosteric modulators**
A model showing possible mechanisms of Sig1R ligand activity. **A** – oligomeric form. For the agonist state, **B** and **C** represent the dimeric and monomeric forms of Sig1R, respectively.

The hypothetical model of the activity of positive allosteric Sig1R modulators presented in Figure 4.2. explains the dual interaction between E1R and NE−100 in the seizure modulation experiments. When injected before E1R, NE−100 at low doses blocks the activity of E1R in a chemoconvulsant-induced seizure model because it can promote the oligomerization of Sig1R. This provides evidence that the effects of E1R are Sig1R related (Publication IV). On the other hand, seizures induced by NE−100 at high doses could be attenuated by E1R when it is injected before NE−100, possibly by promoting agonist state formation. In this case, the distribution between the agonist and the antagonist state of Sig1R might depend on the time and order of administration and binding affinity (on-off rate) of each Sig1R ligand, which probably could shift the equilibrium between different states of Sig1R oligomerization towards the preferred state of the first administered compound.

The enhancing effect of E1R on the BDK-stimulated $[Ca^{2+}]_i$ increase could be explained either by the presence of a possible Sig1R endogenous agonist in NG108−15 cells

or ago-allosteric modulatory activity of E1R in the respective test system. Ago-allosteric modulators are both allosteric agonists and allosteric modulators. An ago-allosteric modulator acts as both an agonist and an enhancer of agonist potency and provides “superagonism”, which would result in an efficacy greater than 100% (Schwartz and Holst, 2007). For example, in our studies, both PRE−084 and E1R increased the BDK-induced $[Ca^{2+}]_i$ increase, while the combination of both compounds resulted in an even more pronounced cellular response.

Interestingly, the chemical structure of ANAVEX™ 2−73 (tetrahydro-N,N-dimethyl-2,2-diphenyl-3-furanmethanamine) is somewhat similar to that of positive allosteric Sig1R modulators (Figure 4.3.). However, ANAVEX™ 2−73 is not only a Sig1R agonist but also M_1 muscarinic acetylcholine receptor agonist and an M_2/M_3 receptor antagonist. ANAVEX™ 2−73 administered i.p. at doses of 0.3−1 mg/kg reversed alternation deficits and passive avoidance deficits in the scopolamine model in mice (Villard *et al.*, 2011). ANAVEX™ 2−73 also showed dose-dependent anti-convulsive activity against maximal electroshock- and PTZ-induced seizures (Vamvakidès, 2002). In addition, ANAVEX™ 2−73 has been studied in clinical trials for the treatment of Alzheimer’s disease and epilepsy. It seems that the similarities in molecular structure between the ANAVEX™ 2−73 and PAMs of Sig1R most likely accounts for the anti-seizure activity of the compound because not all PAMs have been shown to improve memory function, while all PAMs of Sig1R have been shown to act as anti-epileptics.



Phenytoin **ANAVEX™2−73** **E1R**

Figure 4.3. **Similarities between the molecular structures of allosteric Sig1R modulators and the Sig1R agonist ANAVEX™ 2−73**

The pharmacological profile of ANAVEX™ 2−73 demonstrates the synergy between muscarinic and Sig1R sites (Villard *et al.*, 2011). The mechanism for the memory improving activity of ANAVEX™ 2−73 could be the supersensitization of M_1 muscarinic receptor through heterodimerization with Sig1R (Fisher *et al.*, 2013). Sig1R has already been described as a chaperone that modulates other receptor systems through protein-protein

interactions (Pabba, 2013). Therefore, it is possible that heteromeric complexes formed by Sig1R and its target proteins could be regulated by PAMs of Sig1R. Compared to E1R, ANAVEX™ 2−73 demonstrates a similar pharmacological profile, including both memory improvement and anti-seizure activity; thus, E1R might also act on different complexes of Sig1R and its target proteins by activating Sig1R to initiate the formation of complexes and possibly by stabilizing Sig1R-protein heteromers after they are formed.

Overall, it is clear that the effects of allosteric Sig1R modulators cannot be explained by simple rules or models and that a more complex system is involved in the modulation of Sig1R activity by Sig1R ligands. E1R has unique pharmacological and behavioural profiles as well as low toxicity, thus indicating its potential use as a tool compound for detailed studies of CNS targeted Sig1R molecular mechanisms and pharmacology.

5. CONCLUSIONS

1. Enantiomers E1R and T1R of the novel 4,5-disubstituted piracetam derivative methylphenylpiracetam, which have an R-configuration at the C-4 chiral centre in the 2-pyrrolidone ring, are more effective PAMs of Sig1R than their optical antipodes.
2. E1R does not compete with selective Sig1R ligands to bind in the Sig1R orthosteric binding site. The *in vitro* and *ex vivo* activity of E1R confirms that it acts as positive allosteric Sig1R modulator.
3. E1R enhances cognition and alleviates scopolamine-induced cholinergic dysfunction without affecting locomotor activity in mice and possesses significant anti-seizure activity in chemoconvulsant-induced seizure models.
4. The pharmacological activity of E1R is of particular relevance as a new approach for the treatment of cognitive disorders, including those that are associated with neurodegenerative diseases, and epilepsy.

6. APPROBATION OF THE STUDY – PUBLICATIONS AND THESIS

Doctoral thesis is based on following SCI publications:

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SUPPLEMENTS

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Synthesis and biological evaluation of 2-(5-methyl-4-phenyl-2-oxopyrrolidin-1-yl)-acetamide stereoisomers as novel positive allosteric modulators of sigma-1 receptor

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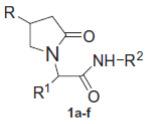
ABSTRACT

Novel positive allosteric modulators of sigma-1 receptor represented by 2-(5-methyl-4-phenyl-2-oxopyrrolidin-1-yl)-acetamide enantiomers were synthesised using an asymmetric Michael addition of 2-nitroprop-1-enylbenzene to diethyl malonate. Following the chromatographic separation of the methyl *erythro*- and *threo*-4-nitro-3*R*- and 3*S*-phenylpentanoate diastereoisomers, target compounds were obtained by their reductive cyclisation into 5-methyl-4-phenylpyrrolidin-2-one enantiomers and the attachment of the acetamide group to the heterocyclic nitrogen. Experiments with electrically stimulated rat vas deference contractions induced by the PRE-084, an agonist of sigma-1 receptor, showed that (4*R*,5*S*)- and (4*R*,5*R*)-2-(5-methyl-4-phenyl-2-oxopyrrolidin-1-yl)-acetamides with an *R*-configuration at the C-4 chiral centre in the 2-pyrrolidone ring were more effective positive allosteric modulators of sigma-1 receptor than were their optical antipodes.

1. Introduction

During the past decade, there has been significant interest in the investigation of Structure–Activity Relationship (SAR) aimed at searching for new nootropic pharmaceuticals for the treatment of cognition/memory disorders. Currently, drugs **1a–f** containing the pyrrolidin-2-one pharmacophore and representing the so-called racetam family play a key role in the treatment of these disorders. For example, piracetam (**1a**), pramiracetam (**1b**), etiracetam (**1c**), nefiracetam (**1d**), oxiracetam (**1e**) and phenylpiracetam (**1f**) are drugs of choice for the specific therapy of cognition/memory disorders.[1] However, the discovery of new effective pharmaceuticals that improve neurotransmission in the human brain is critical because it affects mental and cognitive abilities.



1a-f

a) piracetam (R=R^1=R^2=H);
b) pramiracetam (R=R^1=H, R^2=(CH$_2$)$_2$N(Pr-*i*)$_2$);
c) etiracetam (R=R^2=H, R^1=Et);
d) nefiracetam (R=R^2=H, R^1=2,6-Me$_2$C$_6$H$_3$);
e) oxiracetam (R=OH, R^1=R^2=H);
f) phenylpiracetam (R=Ph, R^1=R^2=H)

Our previous investigations in this field of medicinal chemistry were aimed at the resolution of racemic phenylpiracetam **1f** into individual stereoisomers and their subsequent pharmaceutical analysis. The *R*-phenylpiracetam 4*R*-**1f** was found to be a more effective antidepressant, analgesic, muscle relaxant and psycho-stimulating compound than was the *S*-antipode 4*S*-**1f**.[2] These results were in good agreement with pharmacological data demonstrating the effectiveness of employing the conformational variation of chiral centre(s) of racemic molecules, wherein the enantiomeric resolution can be used to afford the optimal biological effect.

The objective of this investigation is the resolution of a known racemic 4,5-disubstituted piracetam analogue, 2-(5-methyl-4-phenyl-2-oxopyrrolidin-1-yl)-acetamide (**2**),[3] whose nootropic properties could be improved by regulating the stereochemistry of the C4 and C5 positions of the pyrrolidin-2-one ring.

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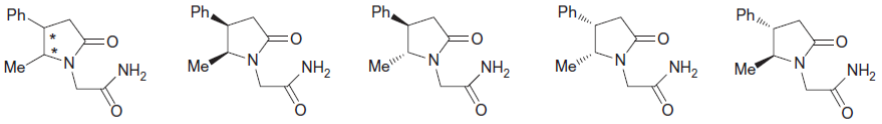


2 (4*R*,5*S*)-**2a** (4*R*,5*R*)-**2b** (4*S*,5*R*)-**2c** (4*S*,5*S*)-**2d**

They attracted our attention due to the unexpected activity discovered for compound (4*R*,5*S*)-**2a** during in vitro CNS pharmacological target profiling, which was performed in a commercially available panel of 77 radioligand binding assays (CEREP, France).[4] The only target site for its activity (inhibition or enhancement of radioligand binding for more than 20%) was sigma receptor, where this compound increased the specific binding of radioligand, thus suggesting a positive modulatory activity of (4*R*,5*S*)-**2a** on sigma receptors.

Recent studies consider sigma-1 receptor (sig-1R) as an emerging new CNS drug target. This receptor plays an important role in neuronal plasticity, a process implicated in the pathophysiology of neuropsychiatric diseases, such as Alzheimer's disease, major depressive disorders, and schizophrenia.[5,6] Sig-1R has been thoroughly studied to elucidate possible neuropharmacological applications, mainly in learning and memory processes, including depression, anxiety, schizophrenia, analgesia and some effects of drug abuse.[7] As a chaperone protein, sig-1R modulates the intracellular calcium signalling of the endoplasmatic reticulum. Several studies suggest that sig-1R receptor is involved in memory processes and their agonists have demonstrated effectiveness in the treatment of cognitive impairments in experimental animal models.[8]

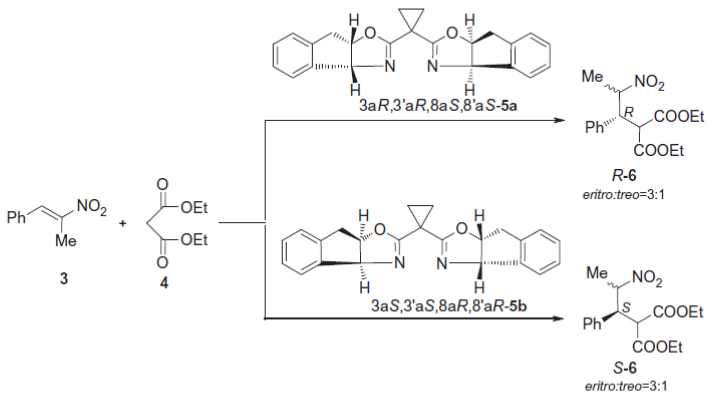
To date, we have not found any data about the effect of piracetam and its structural analogues on sig-1R. Therefore, our research is aimed at the synthesis of 2-(5-methyl-4-phenyl-2-oxopyrrolidin-1-yl)-acetamide (**2**) four individual enantiomers 4*R*,5*S*-**2a**, 4*R*,5*R*-**2b**, 4*S*,5*R*-**2c**, and 4*S*,5*S*-**2d** for the purpose to compare the modulatory activity of these compounds with respect to sig-1R using the isolated vas deferens experimental model.

2. Chemistry

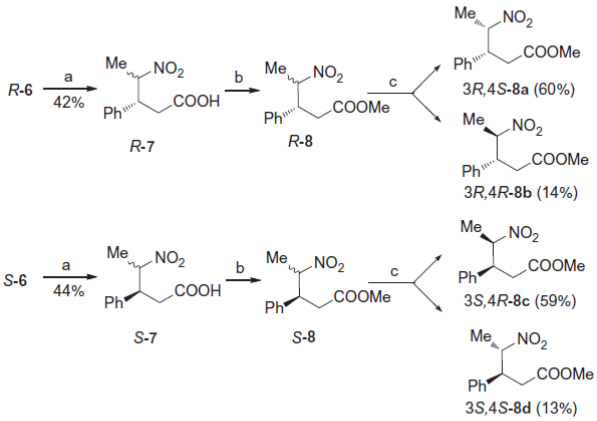
No evidence was found in the literature related to the chiral resolution of racemic 2-(5-methyl-4-phenyl-2-oxopyrrolidin-1-yl)-acetamide (**2**) or the asymmetric synthesis of its separate enantiomers from chiral or nonchiral reagents. Accordingly, we describe the special methodology for the preparation of these compounds. The crucial part of the synthesis consists of the asymmetric Michael addition of 2-nitroprop-1-enylbenzene (**3**) to diethyl malonate (**4**) catalysed by the chiral 2,2′-cyclopropylidene-bis-oxazoline compound **5**, magnesium triflate and an organic base according to the methodology developed by Barnes et al.[9] As a result, diethyl (1*R*)-2-(2-nitro-1-phenylpropyl)-malonate (*R*-**6**) was obtained as a mixture of *erythro*- and *threo*-diastereoisomers in 87% yield and with optical purity 94% in the case of (3a*R*,3′a*R*,8a*S*,8′a*S*)-2,2′-cyclopropylidenebis-[3a,8a]-dihydro-8*H*-indeno-[1,2-*d*]-oxazole ((3a*R*,3′a*R*,8a*S*,8′a*S*)-**5a**). The substitution of the catalyst by its optical antipode (3a*S*,3′a*S*,8a*R*,8′a*R*)-**5b** resulted in the preparation of a second pair of diastereoisomers *S*-**6** in 83% yield and with optical purity 95%. The introduction of the methyl group in nitrostyrene at least 10 times hindered the rate of the condensation compared with that of 2-nitrovinylbenzene, as discussed in the study.[9] The usage of twofold amount of the chiral catalyst **5** allowed to reduce the reaction time up to 72 h (Scheme 1).

Subsequent chemical manipulations with diastereoisomeric mixtures *R*-**6** and *S*-**6** were aimed at obtaining suitable derivatives for resolution by column chromatography. Methyl (3*R*)- and (3*S*)-4-nitro-3-phenylpentanoates *R*-**8** and *S*-**8** were found to be the most suitable candidates for this purpose. According to this precondition, compounds *R*-**6** and *S*-**6** were subjected to acidic hydrolysis and decarboxylation. Intermediate (3*R*)- and (3*S*)-4-nitro-3-phenylpentanoic acids *R*-**7** and *S*-**7** were esterified by methanol, and the obtained diastereoisomeric mixtures of esters *R*-**8** and *S*-**8** were separated by column chromatography on silica gel, affording the *erythro*- and *threo*-isomers of 4-nitro-3-phenylpentanoate: 3*R*,4*S*-**8a**, 3*R*,4*R*-**8b**, 3*S*,4*R*-**8c** and 3*S*,4*S*-**8d** (Scheme 2).

The resulting methyl 4-nitro-3-phenylpentanoates **8a–d** were converted into target compounds 4*R*,5*S*-**2a**, 4*R*,5*R*-**2b**, 4*S*,5*R*-**2c**, and 4*S*,5*S*-**2d** using typical reactions for the preparation of 2-(2-oxopyrrolidin-1-yl)-acetamide analogues:

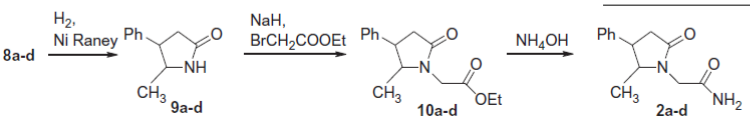


Scheme 1. The preparation of diethyl (1*R*)- and (1*S*)-2-(2-nitro-1-phenylpropyl)-malonates *R*-**6** and *S*-**6**.



Scheme 2. The preparation of methyl (3*R*)- and (3*S*)-4-nitro-3-phenylpentanoates **8a–d**. Reagents and conditions: (a) 36% HCl and CH_3COOH mixture (1:3), reflux, 18 h; (b) MeOH, $SOCl_2$ (cat) 20 h, reflux; (c) chromatographic separation on silica gel.

(a) Hydrogenation of each stereoisomer **8a–d** in the presence of Ni Raney catalyst accompanied by the cyclisation of intermediate ethyl 4-amino-3-phenylpentanoates into the appropriate 5-methyl-4-phenylpyrrolidin-2-ones: 4*R*,5*S*-**9a**, 4*R*,5*R*-**9b**, 4*S*,5*R*-**9c**, and 4*S*,5*S*-**9d**.
(b) The treatment of the individual 5-methyl-4-phenylpyrrolidin-2-ones **9a–d** with sodium hydride and ethyl bromoacetate.
(c) Carbamoylation of the ethyl 2-(5-methyl-2-oxo-4-phenylpyrrolidin-1-yl)-acetates 4*R*,5*S*-**10a**, 4*R*,5*R*-**10b**, 4*S*,5*R*-**10c**, and 4*S*,5*S*-**10d** with ammonium hydroxide.[3,10]



2, **8**, **9**, **10** a = 4*R*,5*S*; b = 4*R*,5*R*; c = 4*S*,5*R*; d = 4*S*,5*S*

The target enantiomers 4*R*,5*S*-**2a**, 4*R*,5*R*-**2b**, 4*S*,5*R*-**2c**, and 4*S*,5*S*-**2d** were recrystallised from water and according to chiral chromatography data their optical purity was in the range 90–99%. The angles of optical rotation for these compounds were in agreement with the stereochemistry of phenyl and methyl groups (Table 1) and consistent with the X-ray analysis of the two enantiomers 4*R*,5*S*-**2a** and 4*R*,5*R*-**2b** (Figs. 1 and 2). The ORTEP diagram of these compounds demonstrates that the crystal structure of 4*R*,5*R*-**2b** features two independent molecules in the asymmetric unit. Both molecules are connected by a centre of pseudoinversion. The main bond lengths and angles of 4*R*,5*S*-**2a** and 4*R*,5*R*-**2b** are provided in Table 2.

Table 1
Optical rotation angles for 2-(5-methyl-2-oxo-4-phenyl-pyrrolidin-1-yl)-acetamide (**2**) stereoisomers

Compound	$[\alpha]_D^{20}$	Solvent, concentration
4*R*,5*S*-**2a**	−96.7°	*c* 0.05, MeOH
4*R*,5*R*-**2b**	+22.9°	*c* 0.05, MeOH
4*S*,5*R*-**2c**	+94.1°	*c* 0.05, MeOH
4*S*,5*S*-**2d**	−26.0°	*c* 0.05, MeOH

3. Results and discussion

The positive allosteric modulatory activity of the 2-(5-methyl-4-phenyl-2-oxopyrrolidin-1-yl)-acetamide enantiomers **2a–d** with respect to sig-1R was evaluated using the electrically stimulated rat vas deferens model.[11] The action was determined by comparing the vas deferens contraction heights induced by selective sig-1R agonist PRE-084 (2-morpholin-4-ylethyl-1-phenylcyclohexane-1-carboxylate) in the absence of the tested compound (control) and after preincubation with a 10 μM solution of each enantiomer.

The intensity of the electrically stimulated contractions of rat vas deferens in the presence of 100 μM of PRE-084 was 122 ± 11%. The tested compounds alone did not alter the height of contractions of electrically stimulated vas deferens. In contrast, the preincubation of vas deferens in 10 μM solution of tested enantiomers 10 min before the addition of the PRE-084 increased the

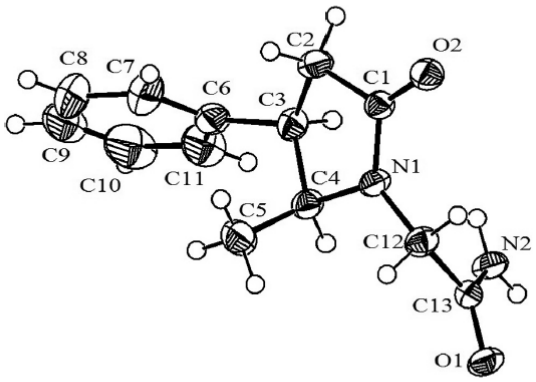


Figure 1. ORTEP diagram of the crystal structure of 4*R*,5*S*-**2a**.

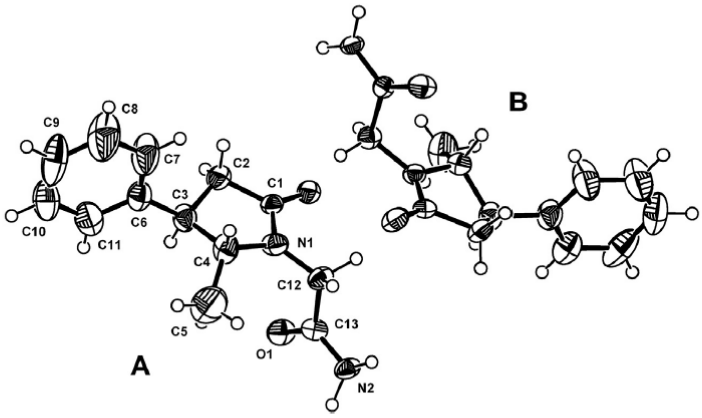


Figure 2. ORTEP diagram of the crystal structure of 4*R*,5*R*-**2b**.

Table 2
Principal bond lengths (l) and valence angles (ω) for 4*R*,5*S*-**2a** and 4*R*,5*R*-**2b** compounds

Bond	l (Å)		
	4*R*,5*S*-**2a**	4*R*,5*R*-**2b**	
		Molecule A	Molecule B
N1–C1	1.345 (2)	1.354 (6)	1.325 (6)
N1–C12	1.449 (2)	1.437 (6)	1.457 (6)
C1–C2	1.512 (2)	1.499 (7)	1.521 (7)
C2–C3	1.524 (2)	1.523 (7)	1.531 (7)
C3–C4	1.551 (2)	1.528 (7)	1.536 (7)
C3–C6	1.513 (2)	1.508 (7)	1.525 (7)
C4–N1	1.476 (2)	1.480 (6)	1.472 (6)
C4–C5	1.518 (2)	1.502 (8)	1.490 (7)
C12–C13	1.520 (2)	1.530 (7)	1.514 (7)
C13–O1	1.235 (2)	1.231 (5)	1.244 (5)
C13–N2	1.319 (2)	1.318 (7)	1.332 (7)
Angle		ω (°)	
	4*R*,5*S*-**2a**	4*R*,5*R*-**2b**	
		Molecule A	Molecule B
C1–N1–C4	113.9 (1)	112.2 (4)	115.0 (4)
C1–N1–C12	123.4 (1)	122.8 (4)	121.3 (4)
C4–N1–C12	122.1 (1)	125.0 (5)	121.1 (4)
N1–C1–C2	107.7 (1)	108.4 (5)	107.1 (5)
C1–C2–C3	103.6 (1)	104.5 (5)	102.9 (5)
C2–C3–C4	103.0 (1)	102.9 (5)	103.0 (5)
C3–C4–N1	100.7 (1)	103.0 (5)	100.0 (5)
N1–C12–C13	113.9 (1)	111.2 (5)	112.0 (5)
C12–C13–O1	119.0 (1)	120.8 (5)	120.5 (5)
C12–C13–N2	118.0 (1)	115.9 (5)	116.3 (5)

Table 3
Positive allosteric modulating effect of enantiomers **2a–d** in rat vas deferens contraction experiments

Enantiomers	% Of increase*
4*R*,5*S*-**2a**	222 ± 37**
4*R*,5*R*-**2b**	191 ± 23**
4*S*,5*R*-**2c**	141 ± 40
4*S*,5*S*-**2d**	147 ± 31

* The response of isolated vas deferens to selective sig-1R agonist PRE-084 was compared without (control) or in the presence of enantiomers **2a–d** at the concentration of 10 μM. All results are expressed as a mean ± SEM of six vas deferens.
** P <0.01 paired student t-test.

intensity of observed contractions. As shown in Table 3, this increase in activity was inherent to all enantiomers. However, the contractions in the case of 4*R*,5*S*-**2a** and 4*R*,5*R*-**2b** were observed to be at least two times higher than those of the control (Table 3).

4. Conclusions

In summary, four individual enantiomers of 2-(5-methyl-4-phenyl-2-oxopyrrolidin-1-yl)-acetamide 4*R*,5*S*-**2a**, 4*R*,5*R*-**2b**, 4*S*,5*R*-**2c**, and 4*S*,5*S*-**2d** were obtained using an asymmetric Michael addition of 2-nitroprop-1-enylbenzene to diethyl malonate catalysed by the chiral 2,2′-cyclopropylidene-bis-oxazoline. This reaction was followed by the chromatographic separation of the diastereoisomeric mixtures of methyl *erythro*- and *threo*-4-nitro-3*R*-phenylpentanoate (*R*-**8**) and methyl *erythro*- and *threo*-4-nitro-3*S*-phenylpentanoate (*S*-**8**), the reductive cyclisation of each enantiomer into the appropriate pyrrolidin-2-one and the final conversion of these compounds into target N-acetamide derivatives. The evaluation of the in vitro biological effect of these compounds using electrically stimulated rat vas deferens contractions induced by selective sigma-1 receptor agonist PRE-084 demonstrated that enantiomers 4*R*,5*S*-**2a** and 4*R*,5*R*-**2b** with the *R*-configuration at the C-4 chiral centre in the 2-pyrrolidone ring are more effective positive allosteric modulators of sigma-1 receptor than are their optical antipodes.

5. Experimental

5.1. Chemistry

All chemicals were supplied by Acros and Aldrich. The melting points were determined using a Boetius PHMK melting point apparatus and are reported uncorrected. ^{1}H and ^{13}C NMR spectra were obtained using a Varian 400 MHz Mercury-400 spectrometer and $CDCl_3$ as the solvent. The chemical shifts are reported in δ values (ppm) relative to an internal TMS standard. The abbreviations are s = singlet, d = doublet, t = triplet, m = multiplet, and br = broad. High-resolution mass spectra were obtained using a Micromass Quatro Micro™ API with MeCN as the solvent. Optical rotation values at 405 nm were measured on a Perkin–Elmer 141 Polarimeter. Elemental analyses (C, H, N) were performed on a Carlo Erba 1108 analyser and were found to be within ±0.4% of the theoretical values. The purity of the compounds was determined by TLC using Merck 60F254 silica gel, whereas Merck Kieselgel (silica gel 0.063–0.230 mm) was used for column chromatography.

5.1.1. Diethyl (1*R*)-2-(2-nitro-1-phenylpropyl)-malonate (*R*-6)

To a solution of (3a*R*,3′a*R*,8a*S*,8′a*S*)-2,2′-cyclopropylidenebis-[3a,8a]-dihydro-8*H*-indeno-[1,2-*d*]-oxazole (**5a**) (420 mg, 1.18 mM) in chloroform (amylene stabilised) (5 ml) in a 250 ml reaction flask, magnesium triflate (378 mg, 1.18 mM) and water (25 μl) were added at room temperature, and the mixture was allowed to stir under argon for 1 h. Molecular sieves (1.0 g) were added to the mixture, and the mixture was stirred for an additional 30 min. The obtained suspension was diluted with 45 ml of a chloroform solution containing diethylmalonate (1.67 g, 10.2 mM), 2-nitroprop-1-enylbenzene (1.63 g, 10.0 mM) and a mixture of morpholine (46 μl) and tetra-methylguanidine (46 μl). The reaction mixture was stirred at 20–25 °C for 72 h. The degree of conversion and the selectivity were determined by a chiral HPLC analysis [Chiralpak IC, 4.6 × 250 mm, 1.0 ml/min, eluent *i*-PrOH–hexane (1:9)] every 12 h. After the completion of the reaction, the reaction mixture was diluted with hexane (50 ml) and stirred for 20 min. Subsequently, the solid was filtered off and the filtrate was washed with 5% aqueous HCl (2 × 50 ml), brine (2 × 50 ml), dried over anhydrous Na_2SO_4. The drying reagent was removed by filtration and the solution was concentrated under reduced pressure. The residue was purified by silica chromatography using ethylacetate/hexane (1:10) and fractions with R_f 0.28 were collected. Yield: 87% (2.8 g). According to the chiral HPLC, the obtained low-melting yellow solid was a mixture of *erythro*- and *threo*-isomers of diethyl (1*R*)-2-(2-nitro-1-phenylpropyl)-malonate in a ratio 3:1. Optical purity: 94%. *erythro*-Isomer ^{1}H NMR ($CDCl_3$); δ = 0.85 (t, 3H, J = 7.0 Hz, CH_2CH_3), 1.15–1.27 (m, 3H, CH_2CH_3), 1.37 (d, 3H, J = 6.8 Hz, CH_3CHNO_2), 3.63–3.93 (m, 3H, CH_2CH_3, COC*H*CO), 4.07–4.29 (m, 3H, CH_2CH_3), 5.07–5.16 (m, 1H, C*H*NO_2), 6.99–7.28 (m, 5H, C_6H_5); *threo*-isomer ^{1}H NMR ($CDCl_3$); δ = 0.93 (t, 3H, J = 7.0 Hz, 3CH_2CH_3), 1.15–1.27 (m, 3H, CH_2CH_3), 1.29 (d, 1H, J = 6.8 Hz, CH_3CHNO_2), 3.63–3.93 (m, 3H, CH_2CH_3, COC*H*CO), 4.07–4.29 (m, 3H, CH_2CH_3, PhC*H*), 4.29–5.06 (m, 1H, C*H*NO_2), 6.99–7.28 (m, 5H, C_6H_5); HRMS: calculated for $[C_{16}H_{21}NO_6+H]^+$: 324.1444; found: 324.1442. Anal. Calcd for $C_{16}H_{21}NO_6$ (323.35): C, 59.43; H, 6.55; N, 4.33. Found: C, 59.51; H, 6.46; N, 4.27.

5.1.2. Diethyl (1*S*)-2-(2-nitro-1-phenylpropyl)-malonate (*S*-6)

The substitution of chiral catalyst **5a** with its optical antipode **5b** in Section 5.1.1 afforded the diastereoisomeric mixture ***S*-6**. Yield: 83% (2.67 g). According to the chiral HPLC, the obtained low-melting yellow solid was a mixture of *erythro*- and *threo*-isomers of diethyl 2-(2-nitro-1(*S*)-phenylpropyl)-malonate in a ratio of 3:1. Optical purity: 95%. *erythro*-Isomer ^{1}H NMR ($CDCl_3$); δ = 0.85 (t, 3H, J = 7.0 Hz, CH_2CH_3), 1.15–1.27 (m, 3H, CH_2CH_3), 1.37 (d, 3H, J = 6.8 Hz, CH_3CHNO_2), 3.63–3.93 (m, 3H, CH_2CH_3, COC*H*CO), 4.07–4.29 (m, 3H, CH_2CH_3, PhC*H*), 5.07–5.16 (m, 1H, C*H*NO_2), 6.99–7.28 (m, 5H, C_6H_5); *threo*-Isomer ^{1}H NMR ($CDCl_3$); δ = 0.93 (t, 1H, J = 7.0 Hz, CH_2CH_3), 1.15–1.27 (m, 3H, CH_2CH_3), 1.29 (d, 1H, J = 6.8 Hz, CH_3CHNO_2), 3.63–3.93 (m, 3H, CH_2CH_3, COC*H*CO), 4.07–4.29 (m, 3H, CH_2CH_3, PhC*H*), 4.29–5.06 (m, 1H, C*H*NO_2), 6.99–7.28 (m, 5H, C_6H_5); HRMS: calculated for $[C_{16}H_{21}NO_6+H]^+$: 324.1444; found: 324.1443. Anal. Calcd for $C_{16}H_{21}NO_6$ (323.35): C, 59.43; H, 6.55; N, 4.33. Found: C, 59.49; H, 6.54; N, 4.27.

5.1.3. (3*R*)-4-Nitro-3-phenylpentanoic acid (*R*-7)

Diethyl (1*R*)-2-(2-nitro-1-phenylpropyl)-malonate (2.00 g, 6.18 mM) was refluxed in a mixture of acetic and 36% hydrochloric acids at a ratio of 1:3 (30 ml) for 18 h. After the completion of the reaction, the reaction mixture was cooled and concentrated under reduced pressure. The residue was purified by silica column chromatography using ethylacetate/hexane (1:5), and fractions with R_f 0.22 were collected. The obtained yellow solid was a mixture of *erythro*- and *threo*-isomers of (3*R*)-4-nitro-3-phenylpentanoic acid in a ratio of 4:1. Yield: 42% (579 mg). *erythro*-Isomer ^{1}H NMR ($CDCl_3$); δ = 1.48 (d, 3H, J = 6.6 Hz, 5-CH_3), 2.61–2.91 (m, 2H, 2-CH_2), 3.54–3.70 (m, 1H, 3-H), 4.77–4.87 (m, 1H, 4-H), 7.05–7.48 (m, 5H, C_6H_5); *threo*-isomer ^{1}H NMR ($CDCl_3$); δ = 1.27 (d, 3H, J = 6.6 Hz, 5-CH_3), 2.61–2.91 (m, 2H, 2-CH_2), 3.54–3.70 (m, 1H, 3-H), 4.66–4.70 (m, 1H, 4-H), 7.05–7.48 (m, 5H, C_6H_5); HRMS: calculated for $[C_{11}H_{13}NO_4+H]^+$: 224.0921; found: 224.0917.

5.1.4. (3*S*)-4-Nitro-3-phenylpentanoic acid (*S*-7)

The substitution of ***R*-6** with its optical antipode ***S*-6** in Section 5.1.3 afforded a diastereoisomeric mixture of *erythro*- and *threo*-isomers of (3*S*)-4-nitro-3-phenylpentanoic acid in a ratio of 4:1. Yield: 44% (606 mg). *erythro*-Isomer ^{1}H NMR ($CDCl_3$); δ = 1.48 (d, 3H, J = 6.6 Hz, 5-CH_3), 2.61–2.91 (m, 2H, 2-CH_2), 3.54–3.70 (m, 1H, 3-H), 4.77–4.87 (m, 1H, 4-H), 7.05–7.48 (m, 5H, C_6H_5); *threo*-isomer ^{1}H NMR ($CDCl_3$); δ = 1.27 (d, 3H, J = 6.6 Hz, 5-CH_3), 2.61–2.91 (m, 2H, 2-CH_2), 3.54–3.70 (m, 1H, 3-H), 4.66–4.70 (m, 1H, 4-H), 7.05–7.48 (m, 5H, C_6H_5); HRMS: calculated for $[C_{11}H_{13}NO_4+H]^+$: 224.0921; found: 224.0919.

5.1.5. Methyl *erythro*-(3*R*,4*S*)-4-nitro-3-phenylpentanoate (3*R*,4*S*-8a)

A mixture of *erythro*- and *threo*-isomers of (3*R*)-4-nitro-3-phenylpentanoic acid (*R*-7) (500 mg, 2.42 mM) and thionyl chloride (61 μl, 1.0 mM) in methanol (20 ml) was refluxed for 6 h. The reaction mixture was cooled and concentrated under reduced pressure. The residue was purified by silica column chromatography using ethylacetate/hexane (1:15). The fractions with R_f 0.18 containing methyl *erythro*-(3*R*,4*S*)-4-nitro-3-phenylpentanoate were collected and evaporated under reduced pressure. Yield: 345 mg (60%) of low melting yellow solid. Optical purity 93% according to chiral HPLC. ^{1}H NMR ($CDCl_3$); δ = 1.48 (d, 3H, J = 6.6 Hz, 5-CH_3), 2.58–2.85 (m, 2H, CH_2), 3.53 (s, 3H, OCH_3), 3.56–3.71 (m, 1H, 3-H), 4.79–4.88 (m, 1H, 4-H), 7.07–7.31 (m, 5H, C_6H_5); HRMS: calculated for $[C_{12}H_{15}NO_4+H]^+$: 238.1077; found: 238.1074. Anal. Calcd for $C_{12}H_{15}NO_4$ (237.26): C, 60.75; H, 6.37; N, 5.90. Found: C, 60.53; H, 6.24; N, 5.82.

5.1.6. Methyl *threo*-(3*R*,4*R*)-4-nitro-3-phenylpentanoate (3*R*,4*R*-8b)

The fractions with R_f 0.26 containing methyl *threo*-(3*R*,4*R*)-4-nitro-3-phenylpentanoate obtained during the chromatographic separation in Section 5.1.5 were collected and evaporated under reduced pressure. Yield: 80 mg (14%) of low melting yellow solid *threo*-(3*R*,4*R*)-4-nitro-3-phenylpentanoate. Optical purity 82% according to chiral HPLC. ^{1}H NMR ($CDCl_3$); δ = 1.27 (d, 3H, J = 6.6 Hz, 5-CH_3), 2.58–2.85 (m, 2H, 2-CH_2), 3.46 (s, 3H, OCH_3), 3.56–3.71 (m, 1H, 3-H), 4.68–4.77 (m, 1H, 4-H), 7.07–7.31 (m, 5H, C_6H_5); HRMS: calculated for $[C_{12}H_{15}NO_4+H]^+$: 238.1077; found: 238.1075. Anal. Calcd for $C_{12}H_{15}NO_4$ (237.26): C, 60.75; H, 6.37; N, 5.90. Found: C, 60.39; H, 6.28; N, 5.75.

5.1.7. Methyl *erythro*-(3*S*,4*R*)-4-nitro-3-phenylpentanoate (3*S*,4*R*-8c)

A mixture of *erythro*- and *threo*-isomers of (3*S*)-4-nitro-3-phenylpentanoic acid (*S*-7) (500 mg, 2.42 mM) and thionyl chloride (61 μl, 1.0 mM) in methanol (20 ml) was refluxed for 6 h. The reaction mixture was cooled and concentrated under reduced pressure. The residue was purified by silica column chromatography using ethylacetate/hexane (1:15). The fractions with R_f 0.18 containing methyl *erythro*-(3*S*,4*R*)-4-nitro-3-phenylpentanoate were collected and evaporated under reduced pressure. Yield: 340 mg (59%) of low melting yellow solid. Optical purity 93% according to chiral HPLC. ^{1}H NMR ($CDCl_3$); δ = 1.48 (d, 3H, J = 6.6 Hz, 5-CH_3), 2.58–2.85 (m, 2H, CH_2), 3.53 (s, 3H, OCH_3), 3.56–3.71 (m, 1H, 3-H), 4.79–4.88 (m, 1H, 4-H), 7.07–7.31 (m, 5H, C_6H_5); HRMS: calculated

for $[C_{12}H_{15}NO_4+H]^+$: 238.1077; found: 238.1074. Anal. Calcd for $C_{12}H_{15}NO_4$ (237.26): C, 60.75; H, 6.37; N, 5.90. Found: C, 60.63; H, 6.29; N 5.78.

5.1.8. Methyl *threo*-(3*S*,4*S*)-4-nitro-3-phenylpentanoate (3*S*,4*S*-8d)

The factions with R_f 0.26 containing methyl *threo*-(3*S*,4*S*)-4-nitro-3-phenylpentanoate obtained during the chromatographic separation in Section 5.1.7 were collected and evaporated under reduced pressure. Yield: 78 mg (13%) of low melting yellow solid. Optical purity 94% according to chiral HPLC. ^{1}H NMR ($CDCl_3$); δ = 1.27 (d, 3H, J = 6.6 Hz, 5-CH_3), 2.58–2.85 (m, 2H, 2-CH_2), 3.46 (s, 3 H, OCH_3), 3.56–3.71 (m, 1H, 3-H), 4.68–4.77 (m, 1H, 4-H); 7.07–7.31 (m, 5H, C_6H_5); HRMS: calculated for $[C_{12}H_{15}NO_4+H]^+$: 238.1077; found: 238.1073. Anal. Calcd for $C_{12}H_{15}NO_4$ (237.26): C, 60.75; H, 6.37; N, 5.90. Found: C, 60.60; H, 6.31; N, 5.77.

5.1.9. *erythro*-(4*R*,5*S*)-5-Methyl-4-phenylpyrrolidin-2-one (4*R*,5*S*-9a)

The hydrogenation was performed using a stirring suspension of methyl *erythro*-(3*R*,4*S*)-4-nitro-3-phenylpentanoate (3*R*,4*S*-**8a**) (600 mg, 2.52 mM) in ethanol (40 ml) and 1 ml of 50% Ni Raney slurry in water at 50 °C and 50 atm for 18 h. After the completion of the reaction, the reaction mixture was cooled, and the catalyst was filtered off and washed with 30 ml of ethanol. The filtrate was concentrated under reduced pressure. The purification of the residue by column chromatography on silica gel using CH_2Cl_2/EtOH (20:1) and collecting fractions with R_f 0.40 afforded *erythro*-(4*R*,5*S*)-5-methyl-4-phenylpyrrolidin-2-one as a white solid. Yield: 80% (353 mg). ^{1}H NMR ($CDCl_3$); δ = 0.75 (d, 3H, J = 6.5 Hz, 5-CH_3), 2.55–2.69 (m, 2H, 3-CH_2), 3.64–3.72 (m, 1H, 4-H), 3.96–4.04 (m, 1H, 5-H), 6.78 (br s, 1H, NH), 7.07–7.33 (m, 5H, C_6H_5); ^{13}C NMR ($CDCl_3$); δ = 17.50, 35.22, 44.08, 53.72, 126.51, 127.12, 127.92, 128.50, 128.80, 138.81, 177.84; HRMS: calculated for $[C_{11}H_{13}NO+Na]^+$: 198.0895; found: 198.0900.

5.1.10. *threo*-(4*R*,5*R*)-5-Methyl-4-phenylpyrrolidin-2-one (4*R*,5*R*-9b)

The substitution of 3*R*,4*S*-**8a** with 3*R*,4*R*-**8b** in Section 5.1.9 afforded (4*R*,5*R*)-5-methyl-4-phenylpyrrolidin-2-one. Yield: 85% (375 mg). ^{1}H NMR ($CDCl_3$); δ = 1.20 (d, 3H, J = 6.5 Hz, 5-CH_3), 2.48–2.57 (m, 1H, 3-CH_2), 2.65–2.74 (m, 1H, 3-CH_2), 2.98–3.07 (m, 1H, 4-H) 3.65–3.75 (m, 1H, 5-H), 6.76 (br s, 1H, NH), 7.07–7.33 (m, 5H, C_6H_5); ^{13}C NMR ($CDCl_3$); δ = 20.39, 39.33, 49.66, 57.68, 127.23 (C-2, C-6 aromatic), 127.37, 128.82 (C-3, C-5 aromatic), 140.84, 176.48;. HRMS: calculated for $[C_{11}H_{13}NO+Na]^+$: 198.0895; found: 198.0888.

5.1.11. *erythro*-(4*S*,5*R*)-5-Methyl-4-phenylpyrrolidin-2-one (4*S*,5*R*-9c)

The substitution of 3*R*,4*S*-**8a** with 3*S*,4*R*-**8c** in Section 5.1.9 afforded (4*S*,5*R*)-5-methyl-4-phenylpyrrolidin-2-one. Yield: 84% (371 mg). ^{1}H NMR ($CDCl_3$); δ = 0.75 (d, 3H, J = 6.5 Hz, 5-CH_3), 2.55–2.69 (m, 2H, 3-CH_2), 3.64–3.72 (m, 1H, 4-H), 3.96–4.04 (m, 1H, 5-H), 6.78 (br s, 1H, NH), 7.07–7.33 (m, 5H, C_6H_5). ^{13}C NMR ($CDCl_3$); δ = 17.53, 35.21, 44.07, 53.72, 126.50, 127.13, 127.91, 128.51, 128.80, 138.83, 177.74; HRMS: calculated for $[C_{11}H_{13}NO+Na]^+$: 198.0895; found: 198.0901.

5.1.12. *threo*-(4*S*,5*S*)-5-Methyl-4-phenylpyrrolidin-2-one (4*S*,5*S*-9d)

The substitution of 3*R*,4*S*-**8a** with 3*S*,4*S*-**8d** in Section 5.1.9 afforded (4*S*,5*S*)-5-methyl-4-phenylpyrrolidin-2-one. Yield: 84% (371 mg). ^{1}H NMR ($CDCl_3$); δ = 1.20 (d, 3H, J = 6.5 Hz, 5-CH_3), 2.48–2.57 (m, 1H, 3-CH_2), 2.65–2.74 (m, 1H, 3-CH_2), 2.98–3.07 (m, 1H, 4-H) 3.65–3.75 (m, 1H, 5-H), 6.76 (br s, 1H, NH), 7.07–7.33 (m, 5H, C_6H_5); ^{13}C NMR ($CDCl_3$); δ = 20.38, 39.36, 49.64, 57.71, 127.22 (C-2, C-6 aromatic), 127.37, 128.82 (C-3, C-5 aromatic), 140.85, 176.59; HRMS: calculated for $[C_{11}H_{13}NO+Na]^+$: 198.0895; found: 198.0890.

5.1.13. Ethyl *erythro*-(4*R*,5*S*)-2-(5-methyl-4-phenylpyrrolidin-1-yl)-acetate (4*R*,5*S*-10a)

A solution of *erythro*-(4*R*,5*S*)-5-methyl-4-phenylpyrrolidin-2-one (4*R*,5*S*-**9a**) (351 mg, 2.00 mM) in toluene (30 ml) was added to a suspension of sodium hydride (56 mg, 2.35 mM) in toluene (30 ml). The stirred mixture was heated at 80–90 °C for 30 min and then cooled to room temperature. Ethyl bromoacetate (368 mg, 2.20 mM) was added to the reaction mixture, which was heated to 110–120 °C for 6 h and concentrated under reduced pressure. The residue was dissolved in toluene (30 ml). The obtained solution was washed with 5% aqueous HCl (2 × 50 ml), brine (2 × 50 ml), dried over anhydrous Na_2SO_4. The drying reagent was removed by filtration, and the solution was concentrated under reduced pressure. The residue was purified by silica column chromatography using CH_2Cl_2/MeOH (20:1). The fractions with R_f 0.48 were collected and evaporated under reduced pressure, affording ethyl (4*R*,5*S*)-2-(5-methyl-4-phenylpyrrolidin-1-yl)-acetate (367 mg, 70%) as a colourless oil. ^{1}H NMR ($CDCl_3$); δ = 0.72 (d, 3H, J = 6.6 Hz, 5-CH_3), 1.23 (t, 3H, J = 7.0 Hz, CH_2CH_3), 2.60–2.91 (d, 2H, J = 8.5 Hz, 3-CH_2), 3.65–3.74 (m, 1H, 4-H), 3.66 (d, 2H, J = 17.7 Hz, NCH_2COO), 4.01–4.10 (m, 1H, 5-H), 4.10–4.20 (m, 2H, CH_2CH_3), 4.38 (d, 1H, J = 17.7 Hz, NCH_2COO), 7.09–7.31 (m, 5H, C_6H_5); ^{13}C NMR ($CDCl_3$); δ = 14.14, 14.62, 35.21, 42.07, 42.29, 57.62, 61.34, 127.14 (C-2, C-6 aromatic), 128.03, 128.51 (C-3, C-5 aromatic), 138.88, 168.89, 174.69; HRMS: calculated for $[C_{15}H_{29}NO_3+H]^+$: 262.1443; found: 262.1433.

5.1.14. Ethyl *threo*-(4*R*,5*R*)-2-(5-methyl-4-phenylpyrrolidin-1-yl)-acetate (4*R*,5*R*-10b)

The substitution of 4*R*,5*S*-**9a** with 4*R*,5*R*-**9b** in Section 5.1.13 afforded (4*R*,5*R*)-2-(5-methyl-4-phenylpyrrolidin-1-yl)-acetate. Yield: 72% (377 mg). ^{1}H NMR ($CDCl_3$ δ = 1.16 (d, 3H, J = 6.3 Hz, 5-CH_3), 1.23 (t, 3H, J = 7.0 Hz, CH_2CH_3), 2.53–2.63 (m, 1H, CH_2), 2.76–2.86 (m, 1H, CH_2), 2.92–3.01 (m, 1H, 4-H), 3.71 (d, 1H, J = 17.7 Hz, NCH_2COO), 3.74–3.83 (m, 1H, 5-H), 4.10–4.20 (m, 3H, CH_2CH_3), 4.38 (d, 1H, J = 17.8 Hz, NCH_2COO), 7.18–7.33 (m, 5H, C_6H_5); ^{13}C NMR ($CDCl_3$), δ = 14.14, 17.97, 38.89, 41.88, 47.22, 61.22, 61.35, 127.25 (C-2, C-6 aromatic), 127.54, 128.33 (C-3, C-5 aromatic), 141.07, 168.75, 174.23; HRMS: calculated for $[C_{15}H_{29}NO_3+H]^+$: 262.1443; found: 262.1423.

5.1.15. Ethyl *erythro*-(4*S*,5*R*)-2-(5-methyl-4-phenylpyrrolidin-1-yl)-acetate (4*S*,5*R*-10c)

The substitution of 4*R*,5*S*-**9a** with 4*S*,5*R*-**9c** in Section 5.1.13 afforded (4*S*,5*R*)-2-(5-methyl-4-phenylpyrrolidin-1-yl)-acetate. Yield: 70% (367 mg). ^{1}H NMR ($CDCl_3$); δ = 0.72 (d, 3H, J = 6.6 Hz, 5-CH_3), 1.23 (t, 3H, J = 7.0 Hz, CH_2CH_3), 2.60–2.91 (d, 2H, J = 8.5 Hz, 3-CH_2), 3.65–3.74 (m, 1H, 4-H), 3.66 (d, 2H, J = 17.7 Hz, NCH_2COO), 4.01–4.10 (m, 1H, 5-H), 4.10–4.20 (m, 2H, CH_2CH_3), 4.38 (d, 1H, J = 17.7 Hz, NCH_2COO), 7.09–7.31 (m, 5H, C_6H_5); ^{13}C NMR ($CDCl_3$), δ = 14.13, 14.65, 35.21, 42.07, 42.29, 57.62, 61.34, 127.14 (C-2, C-6 aromatic), 128.03, 128.51 (C-3, C-5 aromatic), 138.88, 168.89, 174.68; HRMS: calculated for $[C_{15}H_{29}NO_3+H]^+$: 262.1443; found: 262.1448.

5.1.16. Ethyl *threo*-(4*S*,5*S*)-2-(5-methyl-4-phenylpyrrolidin-1-yl)-acetate (4*S*,5*S*-10d)

The substitution of 4*R*,5*S*-**9a** with 4*S*,5*S*-**9d** in Section 5.1.13 afforded (4*S*,5*S*)-2-(5-methyl-4-phenylpyrrolidin-1-yl)-acetate. Yield: 72% (377 mg). ^{1}H NMR ($CDCl_3$); δ = 1.16 (d, 3H, J = 6.3 Hz, 5-CH_3), 1.23 (t, 3H, J = 7.0 Hz, CH_2CH_3), 2.53–2.63 (m, 1H, CH_2),

2.76–2.86 (m, 1H, CH_2), 2.92–3.01 (m, 1H, 4-H), 3.71 (d, 1H, J = 17.7 Hz, NCH_2COO), 3.74–3.83 (m, 1H, 5-H), 4.10–4.20 (m, 3H, CH_2CH_3), 4.38 (d, 1H, J = 17.8 Hz, NCH_2COO), 7.18–7.33 (m, 5H, C_6H_5); ^{13}C NMR ($CDCl_3$), δ = 14.14, 17.97, 38.89, 41.87, 47.21, 61.22, 61.35, 127.25 (C-2, C-6 aromatic), 127.53, 128.33 (C-3, C-5 aromatic), 141.06, 168.75, 174.23; HRMS: calculated for $[C_{15}H_{29}NO_3+H]^+$: 262.1443; found: 262.1456.

5.1.17. *erythro*-(4*R*,5*S*)-2-(5-Methyl-2-oxo-4-phenyl-pyrrolidin-1-yl)-acetamide (4*R*,5*S*-2a)

A solution of ethyl (4*R*,5*S*)-2-(5-methyl-4-phenylpyrrolidin-1-yl)-acetate (4*R*,5*S*-**10a**) (350 mg, 1.34 mM) in methanol (30 ml) was treated with 25% aqueous ammonium (10 ml) for 12 h. The reaction mixture was concentrated under reduced pressure, and the residue was purified by column chromatography using CH_2Cl_2/EtOH (20:1). The fractions with R_f 0.32 were collected and evaporated under reduced pressure, affording *erythro*-(4*R*,5*S*)-2-(5-methyl-2-oxo-4-phenyl-pyrrolidin-1-yl)-acetamide (249 mg, 80%) as a white solid recrystallised from water. Mp 169–171 °C. $[\alpha]_D^{20}$ −96.7° (*c* 0.05, MeOH). Optical purity 99.3% according to chiral HPLC. ^1H NMR ($CDCl_3$); δ = 0.77 (d, 3H, J = 6.6 Hz, 5-CH_3), 2.62–2.81 (m, 2H, 3-CH_2), 3.66–3.75 (m, 1H, 4-H), 3.75 (d, 1H, J = 16 Hz, NCH_2COO), 3.98–4.08 (m, 1H, 5-H), 4.04 (d, 1H, J = 16 Hz, NCH_2COO), 5.48 and 6.29 (br s, br s, 2H, NH_2), 7.07–7.32 (m, 5H, C_6H_5); ^{13}C NMR ($CDCl_3$); δ = 14.72, 34.69, 42.40, 45.47, 59.03, 127.33 (C-2, C-6 aromatic), 127.87, 128.65 (C-3, C-5 aromatic), 138.19, 170.92, 175.10; HRMS: calculated for $[C_{13}H_{16}N_2O_2+Na]^+$: 255.1109; found: 255.1113. Anal. Calcd for $C_{13}H_{16}N_2O_2$ (232.28): C, 67.22; H, 6.94; N, 12.06. Found: C, 67.31; H, 6.99; N, 12.10.

5.1.18. *threo*-(4*R*,5*R*)-2-(5-Methyl-2-oxo-4-phenyl-pyrrolidin-1-yl)-acetamide (4*R*,5*R*-2b)

The substitution of 4*R*,5*S*-**10a** with 4*R*,5*R*-**10b** in Section 5.1.17 afforded (4*R*,5*R*)-2-(5-methyl-4-phenylpyrrolidin-1-yl)-acetamide. Yield: 72% (224 mg). Mp 117–118 °C. $[\alpha]_D^{20}$ +22.9 (*c* 0.05, MeOH). Optical purity 90.6% according to chiral HPLC. ^1H NMR ($CDCl_3$); δ = 1.23 (d, 3H, J = 6.2 Hz, 5-CH_3), 2.52–2.62 (m, 1H, 3-CH_2), 2.77–2.86 (m, 1H, 3-CH_2), 3.95–3.05 (m, 1H, 4-H), 3.67–3.85 (m, 1H, 5-H), 3.85 (d, 1H, J = 16 Hz, NCH_2COO), 3.97 (d, 1H, J = 16 Hz, NCH_2COO), 5.54 and 6.25 (br s, br s, 2H, NH_2), 7.16–7.33 (m, 5H, C_6H_5). ^{13}C NMR ($CDCl_3$); δ = 18.32, 38.54, 45.10, 47.15, 62.51, 127.33 (C-2, C-6 aromatic), 127.43, 128.95 (C-3, C-5 aromatic), 140.65, 170.80, 174.72; HRMS: calculated for $[C_{13}H_{16}N_2O_2+Na]^+$: 255.1109; found: 255.1115. Anal. Calcd for $C_{13}H_{16}N_2O_2$ (232.28): C, 67.22; H, 6.94; N, 12.06. Found: C, 67.25; H, 6.98; N, 12.08.

5.1.19. *erythro*-(4*S*,5*R*)-2-(5-methyl-2-oxo-4-phenyl-pyrrolidin-1-yl)-acetamide (4*S*,5*R*-2c)

The substitution of 4*R*,5*S*-**10a** with 4*S*,5*R*-**10c** in Section 5.1.17 afforded (4*S*,5*R*)-2-(5-methyl-2-oxo-4-phenyl-pyrrolidin-1-yl)-acetamide. Yield: 72% (224 mg). Mp 169–171 °C. $[\alpha]_D^{20}$ +96.7° (*c* 0.05, MeOH). Optical purity 99.0% according to chiral HPLC.

^1H NMR ($CDCl_3$); δ = 0.77 (d, 3H, J = 6.6 Hz, 5-CH_3), 2.62–2.81 (m, 2H, 3-CH_2), 3.66–3.75 (m, 1H, 4-H), 3.75 (d, 1H, J = 16 Hz, NCH_2COO), 3.98–4.08 (m, 1H, 5-H), 4.04 (d, 1H, J = 16 Hz, NCH_2COO), 5.48 and 6.29 (br s, br s, 2H, NH_2), 7.07–7.32 (m, 5H, C_6H_5). ^{13}C NMR ($CDCl_3$); δ = 14.72, 34.69, 42.40, 45.47, 59.03, 127.33 (C-2, C-6 aromatic), 127.87, 128.65 (C-3, C-5 aromatic), 138.19, 170.91, 175.10; HRMS: calculated for $[C_{13}H_{16}N_2O_2+Na]^+$: 255.1109; found: 255.1116. Anal. Calcd for $C_{13}H_{16}N_2O_2$ (232.28): C, 67.22; H, 6.94; N, 12.06. Found: C, 67.30; H, 6.95; N, 12.11.

5.1.20. *threo*-(4*S*,5*S*)-2-(5-Methyl-2-oxo-4-phenyl-pyrrolidin-1-yl)-acetamide (4*S*,5*S*-2d)

The substitution of 4*R*,5*S*-**10a** with 4*S*,5*S*-**10d** in Section 5.1.17 afforded (4*S*,5*S*)-2-(5-methyl-4-phenylpyrrolidin-1-yl)-acetamide. Yield: 72% (224 mg). Mp 117–118 °C. $[\alpha]_D^{20}$ −26.0 (*c* 0.05, MeOH). Optical purity 93.8% according to chiral HPLC.

^1H NMR ($CDCl_3$); δ = 1.23 (d,3H, J = 6.2 Hz, 5-CH_3), 2.52–2.62 (m, 1H, 3-CH_2), 2.77–2.86 (m, 1H, 3-CH_2), 3.95–3.05 (m, 1H, 4-H), 3.67–3.85 (m, 1H, 5-H), 3.85 (d, 1H, J = 16 Hz, NCH_2COO), 3.97 (d, 1H, J = 16 Hz, NCH_2COO), 5.54 and 6.25 (br s, br s, 2H, NH_2), 7.16–7.33 (m, 5H, C_6H_5). ^{13}C NMR ($CDCl_3$); δ = 18.33, 38.55, 45.10, 47.15, 62.51, 127.33 (C-2, C-6 aromatic), 127.43, 128.95 (C-3, C-5 aromatic), 140.65, 170.80, 174.71; HRMS: calculated for $[C_{13}H_{16}N_2O_2+Na]^+$: 255.1109; found: 255.1107. Anal. Calcd for $C_{13}H_{16}N_2O_2$ (232.28): C, 67.22; H, 6.94; N, 12.06. Found: C, 67.24; H, 6.96; N, 12.09.

5.2. Determination of optical purity by chiral HPLC

Chiral HPLC measurements were performed on a Waters Alliance (Waters Corporation, Milford, USA) LC system equipped with a 2695 separation module, quaternary pump, degasser, autosampler and column thermostat. Waters 2489 double-absorbed UV detector at 210 nm was used for the analysis. The output signal was monitored and processed using Waters Empower 2 software. The separation of *R*-**6** and *S*-**6** was performed on polysaccharide-based immobilised columns Chiralpak IA (250 × 4.6 mm I.D., with a particle size 5 μm) using the *i*-PrOH/*n*-hexane (1:9) mobile phase. The separation of 3*R*,4*S*-**8**, 3*R*,4*R*-**8**, 3*S*,4*S*-**8**, and 3*S*,4*R*-**8** was performed on coated Lux Cellulose-1 (150 × 4.6 mm I.D., with a particle size 5 μm) columns using EtOH/*n*-hexane (1:99) as the mobile phase. The separation of 4*R*,5*S*-**2**, 4*R*,5*R*-**2**, 4*S*,5*S*-**2**, and 4*S*,5*R*-**2** was performed on coated Lux Amylose-2 (150 × 4.6 mm I.D., with a particle size 5 μm) columns using EtOH/*n*-hexane (15:85) as the mobile phase. The chromatographic runs were performed at a flow rate of 1.0 ml/min and a column temperature of 25 °C. The injection volume was 10 μl and the analytical sample concentration was 0.5 mg/ml. The enantiomeric elution order was established by analysing racemic mixture and individual enantiomer samples.

5.3. X-ray crystallographic analysis

Diffraction data for the *erythro*-(4*R*,5*S*)-2-(5-methyl-2-oxo-4-phenyl-pyrrolidin-1-yl)-acetamide (4*R*,5*S*-**2a**) and *threo*-(4*R*,5*R*)-2-(5-methyl-2-oxo-4-phenyl-pyrrolidin-1-yl)-acetamide (4*R*,5*R*-**2b**) were collected on a Bruker-Nonius KappaCCD diffractometer using monochromic graphite Mo Kα radiation (λ = 0.71073 Å). The crystal structures were solved by a direct method and refined by a full-matrix least squares method.[12,13] Non-hydrogen atoms were refined using an anisotropic approximation, whereas H-atoms were refined by the riding model. Crystal data for 4*R*,5*S*-**2**: orthorhombic, a = 6.3800(2), b = 9.9220(3), c = 20.1510(6) Å, V = 1275.61(7) Å^3, Z = 4, and the space group is $P2_12_12_1$. A total of 2905 reflection intensities were collected up to $2\theta_{max}$ = 55°; for the structural refinement, 2367 reflections with $I > 2\sigma(I)$ were used. The final R-factor was 0.041. Crystal data for 4*R*,5*R*-**2**: monoclinic; a = 11.2168(5), b = 9.0714(4), c = 13.3621(7) Å, β = 109.849(2)°; V = 1278.9(1) Å^3, Z = 4, and the space group is $P2_1$. A total of 3571 reflection intensities were collected up to $2\theta_{max}$ = 58°; for the structure refinement, 1930 independent reflections with $I > 3\sigma(I)$ were used. The final R-factor was 0.054. Crystallographic data for the compounds were deposited into the Cambridge Crystallographic Data Centre as a Supplementary Publication Number CCDC 905537 for 4*R*,5*S*-**2a** and CCDC 904950 for 4*R*,5*R*-**2b**, respectively.

Copies of the data could be obtained, free of charge, by application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK.

5.4. The experimental model of vas deferens isolation and stimulation in vitro

Wistar rats were sacrificed by decapitation. Both vas deferens were excised, immersed in ice-cold Krebs–Henseleit (K–H) buffer solution (content in mmol/l: 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, EDTA 0.05) and cleaned from the surrounding tissues. The proximal portions of the ductus deferens (~15 mm) were mounted in 50 ml organ baths and incubated in K–H buffer solution bubbled with 95% CO_2 and 5% O_2 at 32 °C. The passive tension was fixed at 1.0 g and every 15 min, the buffer solution in the organ bath was changed. After a 60 min adaptation period, the isolated vas deferens were stimulated with an electrical current at the frequency of 0.1 Hz, pulse duration of 1 ms and at a voltage of 50 V. When the electrical current induced stable contraction amplitude, cumulative doses (from 1 to 100 μM) of selective sig-1R agonist PRE-084 were added. After reaching the plateau contraction amplitude at the highest studied agonist concentration (100 μM), the electrical stimulation was turned off and the isolated vas deferens was washed several times with the K–H buffer solution. After 30 min, electrical stimulation was resumed using the same parameters. When the electrical current induced stable contraction amplitude, the tested compounds were added to the isolated vas deferens at a concentration of 10 μM. After 10 min of electrical stimulation, a cumulative dose of PRE-084 was added. The response to the sig-1R agonist before and after the addition of tested compound was calculated as a percentage increase relative to the baseline contraction amplitude. All results are expressed as a mean ± SEM. The data were evaluated using an analysis of variance (ANOVA). Whenever ANOVA was significant, additional multiple comparisons were performed using a Newman–Keuls post-hoc test. *P*-values of less than 0.05 were considered to be significant.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2013.03.016. These data include MOL files and InChiKeys of the most important compounds described in this article.

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RESEARCH PAPER

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

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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

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-phenylpyrrolidin-1-yl)-acetamide; Figure 1), an enantiomer of 4,5-disubstituted piracetam (2-(5-methyl-2-oxo-4-phenylpyrrolidin-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.

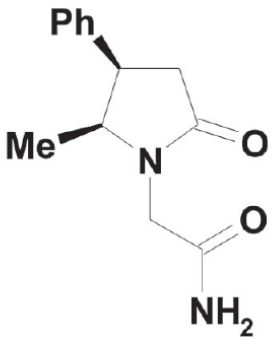

Figure 1
Structure of E1R.

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). [^{3}H](+)-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

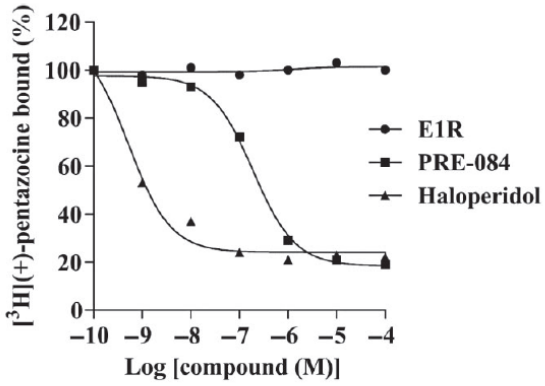

Figure 2

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.

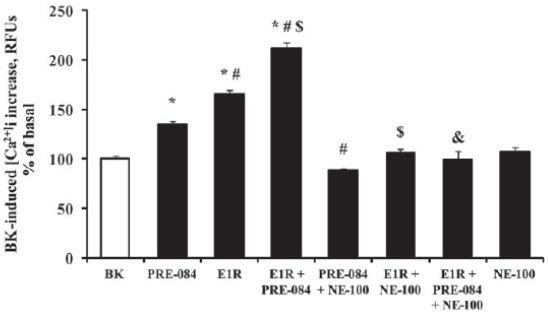

Figure 3

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.

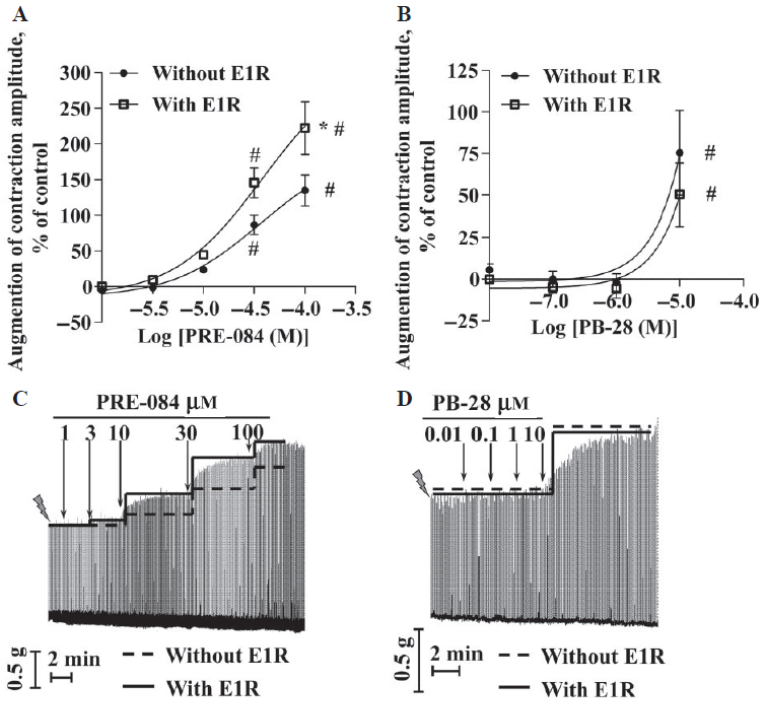

Figure 4

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

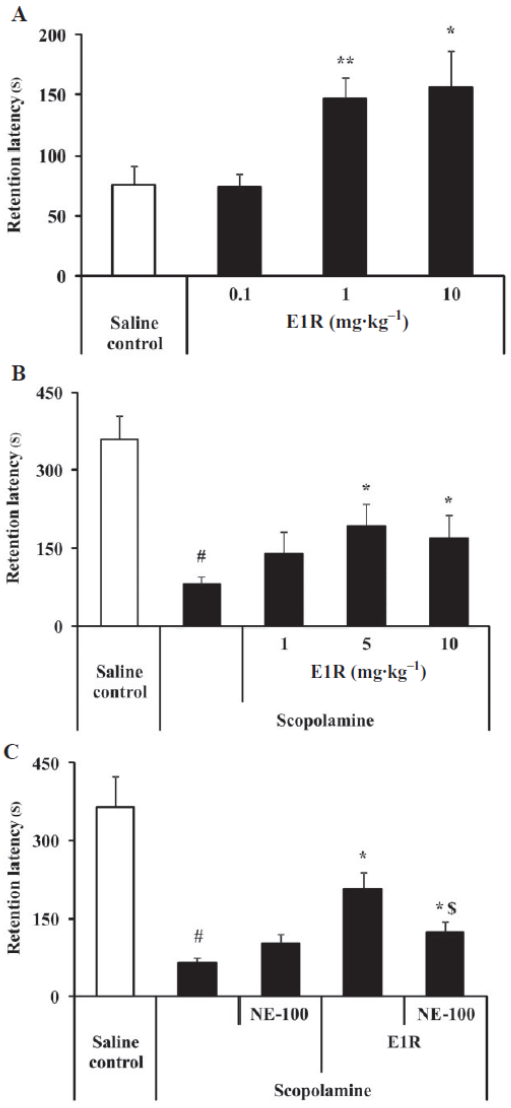

Figure 5

(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.

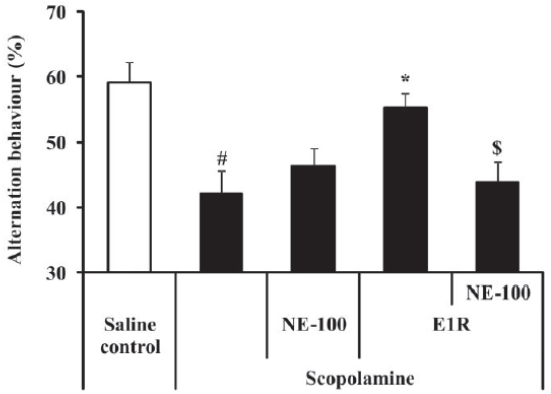

Figure 6

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:

http://dx.doi.org/10.1111/bph.12506

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

PUBLICATION III

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Химия гетероциклических соединений **2015**, *51*(7), 601–606

О Б З О Р Ы

Химия гетероциклических соединений

Stereochemistry of phenylpiracetam and its methyl derivative: improvement of the pharmacological profile

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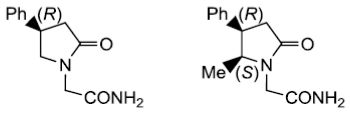


(*R*)-Phenylpiracetam E1R

Since the discovery of piracetam, its structural analogs based on the pyrrolidin-2-one pharmacophore have aroused great interest as a source of effective pharmacological central nervous system agents capable to facilitate memory processes and to attenuate the impairment of cognitive functions associated with head traumas, stroke, age, and age-related pathologies. The present review summarizes the data published during the last decade concerning the design, synthesis, and biological activity exploration of enantiomerically pure (4*R*)-2-oxo-4-phenylpyrrolidine-1-carboxamide ((*R*)-phenylpiracetam) and (4*R*,5*S*)-5-methyl-2-oxo-4-phenylpyrrolidine-1-carboxamide (E1R) and providing evidence for the direct relationship between the configuration of the stereocenters and biological properties of the respective enantiomers. The methodological approaches leading to the preparation of the single stereoisomers of molecules with one or two chiral centers are reviewed. The results of comparative pharmacological testing of individual enantiomers provides the evidence of their pharmacological advantages, justifying the choice of the most effective stereoisomer and the necessity for drug substance purification from the less active one(s).

Keywords: E1R, (4*R*,5*S*)-5-methyl-2-oxo-4-phenylpyrrolidine-1-carboxamide, (4*R*)-2-oxo-4-phenylpyrrolidine-1-carboxamide, (*R*)-phenylpiracetam, nootropic activity, stereoselective pharmacological activity, stereochemical resolution.

Since the 1960s, drugs from the so-called racetam family, differing from each other by the structure of the substituents around the pyrrolidin-2-one heterocycle, have maintained a well-deserved reputation as leading therapeutic agents for the improvement of cognitive functions, attention abilities, storage and retrieval of information, and mental conditions associated with head traumas, stroke, age, and age-related pathologies (Fig. 1).[1–4]

Despite the mentioned advances the discovery of more effective pharmaceuticals based on the pyrrolidin-2-one pharmacophore remains an active research field of medicinal chemistry and pharmacology. Promising studies in this area are associated with stereochemical resolution of racemic racetams aimed at the discovery of the individual enantiomers responsible for the biological effects.[5,6] Such an approach was successfully implemented in the case of racemic levetiracetam by stereoselective resolution, which resulted in the discovery of its more active (*R*)-enantiomer.[7] However, this methodology could not be considered

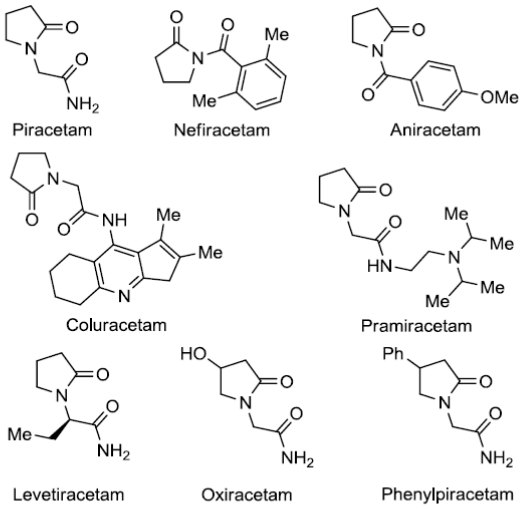


Figure 1. Nootropic racetam drugs.

* Здесь и далее в номере фамилия автора, с которым следует вести переписку, отмечена звездочкой.

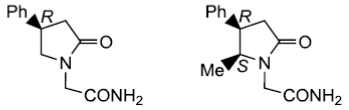


Figure 2. Structures of the biologically active enantiomers of 4-phenylpiracetam and 5-methyl-4-phenylpiracetam.

universal. In the case of oxiracetam, there were no differences in the therapeutic effects of the racemic drug and its (*R*)- and (*S*)-stereoisomers.[8]

In the case of racemic phenylpiracetam and its 5-methyl homolog, this approach was very fruitful. The preparation and comparative pharmacological investigation of all enantiomeric components of racemic molecules resulted in the discovery of the most active ones: (4*R*)-2-oxo-4-phenylpyrrolidine-1-carboxamide ((*R*)-phenylpiracetam) and (4*R*,5*S*)-5-methyl-2-oxo-4-phenylpyrrolidine-1-carboxamide (E1R) (Fig. 2).

The data published during the last decade concerning the preparation of these substances, as well as the discovery of the surprisingly high antidepressant activity of (*R*)-phenylpiracetam and memory improving effect of E1R, based on its positive sigma-1 receptor allosteric modulating properties, encouraged us to present them in a special review.

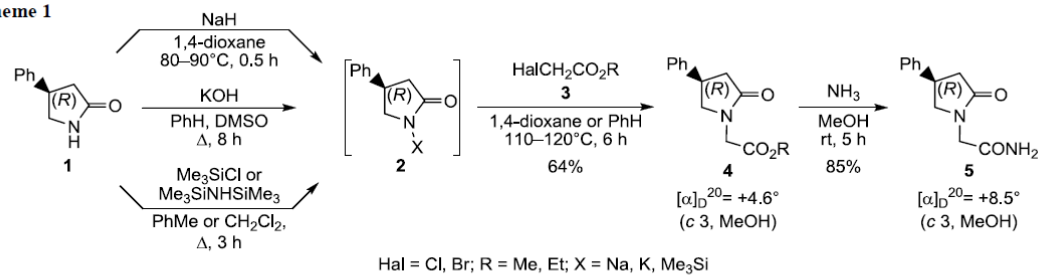
(*R*)-Phenylpiracetam – methods of preparation

The adaptation of already known approaches for the synthesis of racemic phenylpiracetam[9–12] by substituting the racemic 4-phenylpyrrolidin-2-one by its (4*R*)-enantiomer **1** as the source of chirality with the preservation of the configurational stability at the C-4 atom was successfully implemented for the preparation of (*R*)-phenylpiracetam (**5**) at the Latvian Institute of Organic Synthesis[13,14] and by Rezninkov and coworkers[15] (Scheme 1). Experimental procedures included the activation of amide group in (4*R*)-4-phenylpyrrolidin-2-one (**1**) by its *N*-metallation or *N*-silylation, the treatment of intermediate **2** with chloro- or bromoacetic acid ester **3**, and the carbamoylation of alkyl [(4*R*)-2-oxo-4-phenylpyrrolidin-1-yl]acetates **4** with gaseous or 25% aqueous ammonia.

n-Butyl (3*R*)-4-amino-3-phenylbutyrate (**6**)[16] as an alternative source of chirality was also successfully used for the preparation of (*R*)-phenylpiracetam (**5**) (Scheme 2).[17] Amino acid ester **6** was converted into its *N*-carbamoylmethyl derivative **8** by the treatment with chloro- or bromoacetamide (**7**) in DMF in the presence of $K_3PO_4 \cdot H_2O$. The following quantitative cyclization of compound **8** into the target product **5** was realized in refluxing toluene in the presence of a $K_3PO_4 \cdot H_2O$ and tetrabutylammonium bromide mixture. An alternative pathway included the alkylation of the amino acid ester **6** with chloroacetonitrile (**9**) and the conversion of *N*-cyanomethyl derivative **11** in 95% ethanol and a mixture of $K_3PO_4 \cdot H_2O$ with tetrabutylammonium bromide into **5** via its cyclization into unstable (4*R*)-*N*-cyanomethyl-4-phenyl-2-pyrrolidinone (**12**) and the transformation of its nitrile group into carbamoyl one.

On the basis of Lux Amylose-2 immobilized polysaccharide columns, a chiral HPLC analytical methodology was

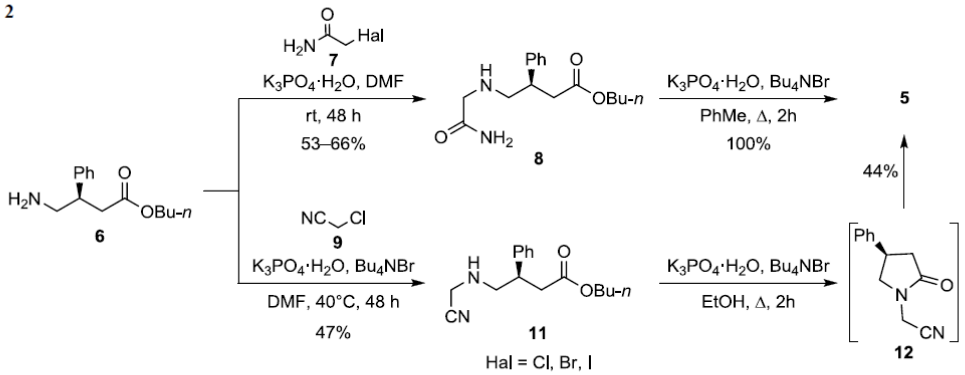
Scheme 1



Hal = Cl, Br; R = Me, Et; X = Na, K, Me_3Si

Preparation of (*R*)-phenylpiracetam (**5**)

Scheme 2



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Preparation of (*R*)-phenylpiracetam (**5**) starting from *n*-butyl (3*R*)-4-amino-3-phenylbutyrate (**6**)

developed for qualitative and quantitative determination of (*R*)-phenylpiracetam (**5**) and its (*S*)-antipode.[18]

(*R*)-Phenylpiracetam – molecular mechanism of action and pharmacological activity

Phenylpiracetam was originally designed as a nootropic drug for the sustenance and improvement of the physical condition and cognition abilities of Soviet space crews.[2] Later, especially during the last decade, phenylpiracetam was introduced into general clinical practice in Russia and in some Eastern European countries. The possible target receptors and mechanisms for the acute activity of this drug remained unclear, until very recently it was found that (*R*)-phenylpiracetam (**5**) (MRZ-9547) is a selective dopamine transporter inhibitor that moderately stimulates striatal dopamine release.[19] According to previous in vitro studies, racemic phenylpiracetam with a low micromolar affinity binds to nicotinic acetylcholine receptors, but has no affinity to dopamine (D1, D2 and D3) or serotonin (HT2) receptors.[20]

As a doping drug for the stimulation of physical activity, such as sports, phenylpiracetam was included in the list of banned substances issued by the World Anti-Doping Agency.[21,22] After chronic administration, racemic phenylpiracetam reduced the extent of neuralgic deficiency manifestations and retained the locomotion, research, and memory functions in gravitational cerebral ischaemia of Wistar rats.[23] Phenylpiracetam, especially (*R*)-phenylpiracetam (**5**), possesses antidepressant and stimulatory activity and also enhances memory processes, as shown in the passive avoidance response test.[23,24] Zvejniece and coworkers showed that the bioavailability of both phenylpiracetam enantiomers in the brain tissue after acute administration was similar.[24] Even though (*R*)-phenylpiracetam (**5**) is the most active enantiomer,[24] its (*S*)-antipode retains some activity in the majority of tests. Nevertheless, in the passive avoidance response test, the presence of (*S*)-phenylpiracetam in racemic phenylpiracetam weakened or suppressed the pharmacological activity of (*R*)-phenylpiracetam (**5**).[24]

E1R and its racemic analog – methods for the preparation of the individual stereoisomers

In the continuation of studies at the Latvian Institute of Organic Synthesis linked with the preparation of single stereoisomers of phenylpiracetam and its derivatives, similar explorations were undertaken in relation to 5-methyl-2-oxo-4-phenylpyrrolidine-1-carboxamide (**13**),[25] a close homolog of phenylpiracetam, containing two chiral centers in its molecule. The two pairs of enantiomers are shown in Figure 3.

The synthesis of all four stereoisomers of compound **13** could be envisioned by a common synthetic scheme involving the preparation of enantiomerically enriched diastereoisomeric mixtures (3*R*)-**16** and (3*S*)-**16**, their conversion into diastereomeric pairs of methyl 4-nitro-3-phenylpentanoates **17a**,**b** and **17c**,**d**, suitable for chromatographic separation into individual stereoisomers, and their following transformation into the target products (4*R*,5*S*)-**13a**, (4*R*,5*R*)-**13b**, (4*S*,5*R*)-**13c**, and (4*S*,5*S*)-**13d**, respectively (Scheme 3).[26,27]

According to the retrosynthesis presented in Scheme 3, the enantiomerically enriched (1*R*)- and (1*S*)-2-(2-nitro-1-phenylpropyl)malonates (3*R*)-**16** and (3*S*)-**16** with 87 and 94% chemical purity, respectively, as a source of chirality were

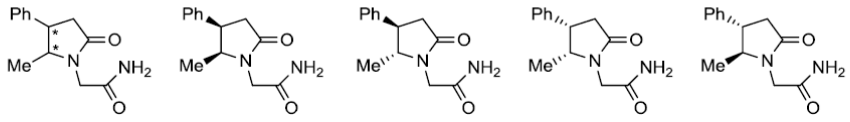
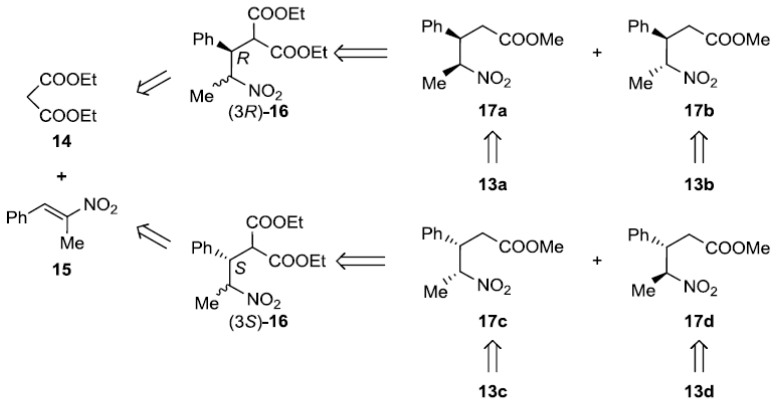


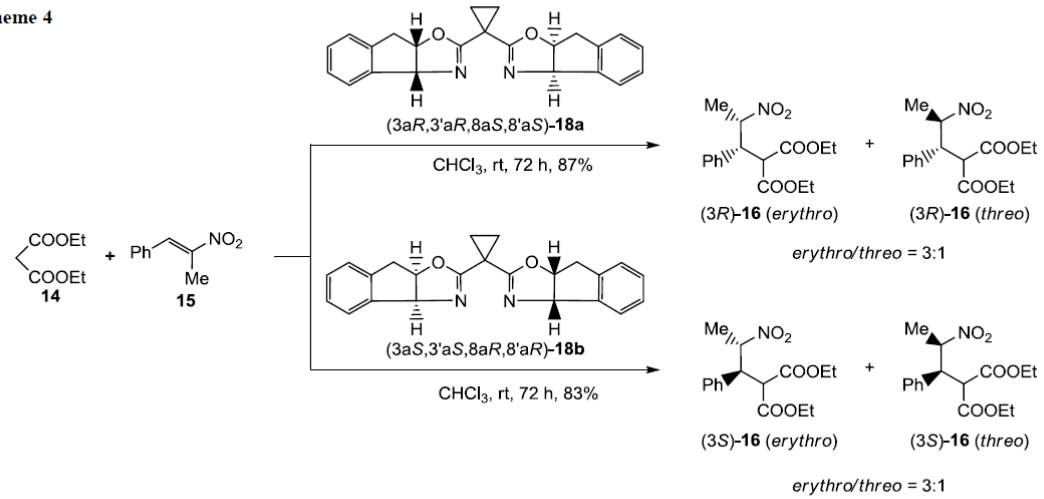
Figure 3. Chiral centers and stereoisomers of 5-methyl-2-oxo-4-phenylpyrrolidine-1-carboxamide (**13**).

Scheme 3



Retrosynthetic analysis of the preparation of diastereoisomeric 5-methyl-2-oxo-4-phenylpyrrolidine-1-carboxamides **13a–d**

Scheme 4



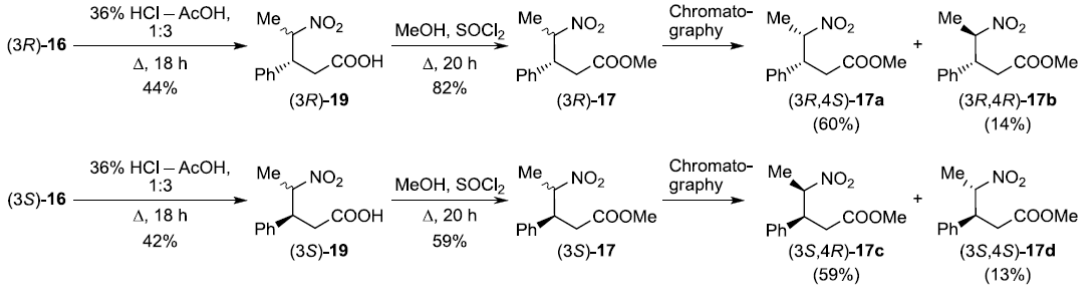
Asymmetric conjugated Michael addition of diethyl malonate (**14**) to β-methyl-β-nitrostyrene (**15**)

prepared by asymmetric conjugated Michael addition of diethyl malonate (**14**) to β-nitrostyrene (**15**) in the presence of chiral 2,2'-cyclopropane-1,1-diylbis(8,8a-dihydro-3a*H*-indeno[1,2-*d*][1,3]oxazole) Mg^{2+} complexes **18a**,**b** (Scheme 4).[26]

The conversion of compounds (3*R*)-**16** and (3*S*)-**16** into *erythro*- and *threo*-isomers of methyl 4-nitro-3-phenylpentanoate ((3*R*,4*S*)-**17a** and (3*R*,4*R*)-**17b**, respectively, and their enantiomers (3*S*,4*R*)-**17c** and (3*S*,4*S*)-**17d**, respectively) was realized by their acidic hydrolysis and decarboxylation, followed by esterification of carboxylic acids (3*R*)-**19** and (3*S*)-**19**. Conformational stability of compounds during mentioned transformations was in a good consistence with the yields of *erythro*- and *threo*-isomers **17a–d** after the chromatographic separation of diastereoisomeric methyl (3*R*)- and (3*S*)-4-nitro-3-phenylpentanoates (3*R*)-**17** and (3*S*)-**17** (Scheme 5).[26]

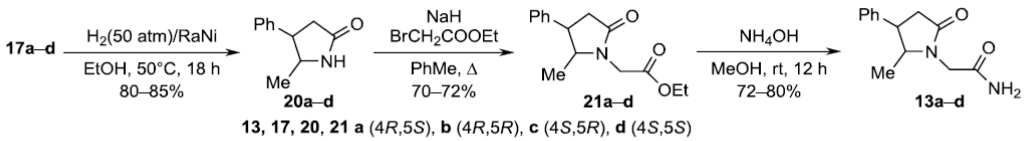
The individual methyl 4-nitro-3-phenylpentanoates **17a–d** thus obtained were converted into the target 5-methyl-substituted phenylpyrrolidin-2-ones (4*R*,5*S*)-**13a**, (4*R*,5*R*)-**13b**, (4*S*,5*R*)-**13c**, and (4*S*,5*S*)-**13d** by hydrogenation and cyclization of stereoisomers **17a–d** in the presence of Raney Ni catalyst into the appropriate 5-methyl-4-phenylpyrrolidin-2-ones **20a–d**, followed by the treatment of the latter with sodium hydride and ethyl bromoacetate and subsequent ammonolysis of ethyl 2-(5-methyl-2-oxo-4-phenylpyrrolidin-1-yl)acetates **21a–d** with ammonium hydroxide (Scheme 6).[26]

Scheme 5



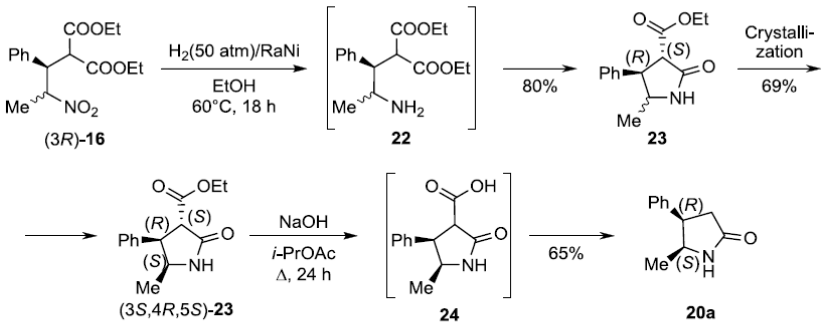
The preparation of two enantiomeric pairs of *erythro*- and *threo*-isomers of methyl 4-nitro-3-phenylpentanoate (**17**)

Scheme 6



The preparation of E1R (**13a**) and its stereoisomers **13b–d**

Scheme 7



A partial alternative route to E1R (**13a**)

Table 1. Optical rotation angles for the diastereoisomers of 2-(5-methyl-2-oxo-4-phenylpyrrolidin-1-yl)acetamide **13a–d**

Compound	Configuration	$[\alpha]_D^{20}$	Solvent, % concentration
13a	4*R*,5*S*	–96.7°	0.05, MeOH
13b	4*R*,5*R*	+22.9°	0.05, MeOH
13c	4*S*,5*R*	+94.1°	0.05, MeOH
13d	4*S*,5*S*	–26.0°	0.05, MeOH

An alternative method was also developed for the synthesis of (4*R*,5*S*)-5-methyl-4-phenylpyrrolidin-2-one (**20a**), the main chiral precursor in the preparation of E1R (**13a**) (Scheme 7).[28] In this case, the hydrogenation of the nitro group in malonate (3*R*)-**16** in the presence of Raney Ni catalyst was accompanied by intermolecular cyclization of the intermediate **22** into a diastereoisomeric mixture of ethyl (4*R*,3*S*)-5-methyl-2-oxo-4-phenylpyrrolidin-3-carboxylates (**23**). The isolation and purification of the individual enantiomer (3*S*,4*R*,5*S*)-**23** from the diastereoisomeric mixture was achieved by crystallization. The basic hydrolysis of the ethoxycarbonyl group and decarboxylation of the intermediate **24** lead to the formation of the target pyrrolidone **20a**, a precursor to E1R (**13a**).

The target diastereoisomers **13a–d** were recrystallized from water, and, according to the chiral chromatography data, their *ee* were in the range of 80–98%.[26] The angles of optical rotation for these compounds were in good agreement with the configuration of the phenyl and methyl groups (Table 1) and consistent with the X-ray analysis of the diastereoisomers **13a**,**b**.[26]

E1R – molecular mechanism of action and pharmacological activity

To profile the molecular mechanism of action, compound E1R (**13a**) was screened by the help of a commercially available assay panel (CEREP, Poitiers, France). In relation to 76 radioligand-binding assays, which included numerous ion channels, GPCR and CNS transporter targets, a very small activity was detected or it was absent.[29] The sigma-1 receptor was identified as the only target for this compound. The following detailed in vitro studies confirmed this screening data, providing solid evidence that E1R (**13a**) is a positive allosteric modulator of the sigma-1 receptor.[29]

E1R was found to be free of potential motor side effects due to the absence of effects on the locomotor activity, muscle tone or coordination at doses up to 200 mg/kg.[29]

The electrically stimulated rat *vas deferens* contractions induced by a selective sigma-1 receptor agonist PRE-084 were chosen for the comparative in vitro evaluation of the sigma-1 receptor positive allosteric modulatory effect for all of the individual stereoisomers **13a–d**. It was found that the diastereoisomers **13a**,**c** with the *erythro* configuration of the phenyl and methyl groups in the pyrrolidin-2-one heterocycle were more effective positive allosteric modulators than diastereoisomers with the *threo* configuration **13b**,**d**.[26] Thus, the pretreatment with E1R (**13a**) and compound **13b** enhanced the effect of PRE-084 approximately two times, whereas compounds **13c**,**d** exerted only some sigma-1 receptor positive modulatory activity. The administration of compounds **13a**,**b** in the passive avoidance test at a dose of 1 mg/kg increased the retention latency by approximately 3 times. The individual E1R (**13a**) was slightly more effective.[27,28] In addition, the memory-improving effects of E1R (**13a**) were also present in a scopolamine-induced cholinergic dysfunction model.[29] In conclusion, the obtained data demonstrated that E1R (**13a**) is the most active memory enhancing enantiomer of the 5-methyl-substituted phenylpiracetam homolog **13**, and its cognition enhancing activity is higher than that of (*R*)-phenylpiracetam. In addition, E1R (**13a**) did not affect the locomotor activity in mice. To our knowledge, E1R (**13a**) is currently the only piracetam derivative demonstrating sigma-1 receptor modulating activity and displaying substantial cognition enhancements linked to a novel drug target.

The development of stereochemical procedures for the preparation of stereoisomers of 2-oxo-4-phenylpyrrolidine-1-carboxamide (phenylpiracetam) and its close homolog 5-methyl-2-oxo-4-phenylpyrrolidine-1-carboxamide created prerequisites for their isolation as individual compounds. The comparative pharmacological testing provided evidence for high efficiency of (4*R*)-2-oxo-4-phenylpyrrolidine-1-carboxamide ((*R*)-phenylpiracetam) and (4*R*,5*S*)-5-methyl-2-oxo-4-phenylpyrrolidine-1-carboxamide (E1R).

(*R*)-Phenylpiracetam in contrast with its (*S*)-antipode and racemic phenylpiracetam is an excellent antidepressant. Its administration also causes comparably much more

potent stimulation of the locomotor activity in animals. E1R was characterized by enhanced cognition and alleviated scopolamine-induced cholinergic dysfunction without affecting the locomotor activity. These effects were related to the positive allosteric modulation of sigma-1 receptors. Therefore, E1R may be interesting as both a novel tool for studying sigma-1 receptor pharmacology and as a novel drug candidate for treating cognitive disorders.

The above-mentioned conclusions allow one to consider developing synthetic procedures for the stereocontrolled preparation of piracetam derivatives with one or two chiral centers in position 4 or postitions 4 and 5 in the pyrrolidin-2-one heterocycle as an effective tool for the elaboration of a novel mechanism-based nootropic drugs.

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Research report

The activity of selective sigma-1 receptor ligands in seizure models *in vivo*

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ABSTRACT

Sigma-1 receptor (Sig1R) is a ligand-regulated protein which, since its discovery, has been widely studied as a novel target to treat neurological disorders, including seizures. However, the roles and mechanisms of Sig1R in the regulation of seizures are not fully understood. The aim of the present study was to test and compare effects of often used selective Sig1R ligands in models of experimentally induced seizures. The anti-seizure activities and interactions of selective Sig1R agonist PRE-084, selective Sig1R antagonist NE-100 and novel positive allosteric Sig1R modulator E1R were evaluated in pentylenetetrazol (PTZ) and (+)-bicuculline (BIC)-induced seizure models in mice.

Sig1R antagonist NE-100 at a dose of 25 mg/kg demonstrated pro-convulsive activity on PTZ-induced seizures. Agonist PRE-084 did not change the thresholds of chemoconvulsant-induced seizures. Positive allosteric modulator E1R at a dose of 50 mg/kg showed anti-convulsive effects on PTZ- and BIC-induced clonic and tonic seizures. The anti-seizure activity of E1R was blocked by NE-100. Surprisingly, NE-100 at a dose of 50 mg/kg induced convulsions, but E1R significantly alleviated the convulsive behaviour induced by NE-100.

In conclusion, the selective Sig1R antagonist NE-100 induced seizures that could be partially attenuated by positive allosteric Sig1R modulator. Our results confirm that Sig1R could be a novel molecular target for new anti-convulsive drugs.

1. Introduction

Sigma-1 receptor (Sig1R) is a unique protein that regulates cellular protein functions, G-protein-coupled receptors and cell signalling molecules [1]. Sig1R has become increasingly studied as a target for medication development for neurological disorders, including seizures. This molecular chaperone protein can be regulated by several ligands. The classification of Sig1R ligands as agonists, antagonists and allosteric modulators is based on *in vivo* studies, which are necessary to fully describe and understand the pharmacological activity of Sig1R ligands.

The anti-convulsive activities of a number of selective Sig1R ligands have been studied mainly in glutamate- and opiate receptor-related chemoconvulsant induced seizure models *in vivo*. For example, high affinity Sig1R agonists, dextromethorphan (24 mg/kg, s.c.) and dimemorfan (24 mg/kg, s.c.), have prevented kainic acid-induced seizures in rats [2]. Similar activity has been shown for another Sig1R agonist, pentoxyverine, in rats on kainic acid-induced neurotoxicity [3]. Racemic (±)pentazocine co-administered with naloxone dose-dependently (20–100 mg/kg, s.c.) reduced tonic seizures induced by N-methyl-DL-aspartic acid in mice [4]. Antagonists of Sig1R, such as analogues of rimcazole (up to 60 mg/kg, i.p.), derivatives of BD-1008 (1–40 mg/kg, i.p.) and AC-927 (1–10 mg/kg, i.p.) protected against cocaine-induced seizures in mice [5–7].

Allosteric modulators of Sig1R have also demonstrated anti-convulsive activity. First described positive allosteric Sig1R modulator phenytoin [8] has been used in clinics against various types of epileptiform seizures for more than 70 years [9]. However, the antiepileptic activity of phenytoin is primarily related to the inhibition of voltage-gated sodium channels [10]. Recently the anti-convulsive effects of selective positive allosteric modulators of Sig1R SKF83959 (20–40 mg/kg, i.p.) and SOMCL-668 (40 mg/kg, i.p.) in models of pentylenetetrazol (PTZ)- and kainic acid-induced seizures were demonstrated to be mediated by modulating Sig1R [11]. Taken together, these data demonstrate that Sig1R ligands possess activity in models of experimentally induced seizures, but the roles and mechanisms of Sig1R in the regulation of seizures are not fully understood.

Selective Sig1R agonist PRE-084 and antagonist NE-100 have been widely used as pharmacological tools to study molecular mechanisms of

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Sig1R. PRE-084 is known for memory improving effects in pharmacological models of MK-801 and scopolamine-induced learning impairments [12], in the senescence-accelerated mouse model with age related cognitive deficits [13] and in animal models of Alzheimer's disease characterized by beta amyloid peptide-induced neurotoxicity [14]. PRE-084 has demonstrated also antidepressive activity [15] and neuroprotective properties in an ischemia-reperfusion-induced injury [16]. The *in vivo* activity of PRE-084 has been studied at a dose range from 0.1 to 60 mg/kg. In turn, NE-100 is used as a compound to confirm the selectivity of agonists on Sig1R sites. NE-100 has demonstrated anti-allodynic activity in capsaicin-induced mechanical hypersensitivity model [17]. In addition, a recently discovered Sig1R positive allosteric modulator E1R enhanced cognition and demonstrated efficacy against scopolamine-induced cholinergic dysfunction in mice [18]. It has been shown that E1R enhances the activity of PRE-084, while NE-100 blocks the *in vivo* effects of E1R [18]. Thus far the comparative anticonvulsive effects and interactions of Sig1R ligands have not been studied in the same experimental settings. Therefore, the aim of the present study was to test the seizure-modulating activity of selective Sig1R agonist PRE-084, selective Sig1R antagonist NE-100 and positive allosteric Sig1R modulator E1R in GABA-ergic signalling related chemoconvulsant PTZ and (+)-bicuculline (BIC)-induced seizure models in mice.

2. Material and methods

2.1. Animals

One hundred and forty-six, seventy-four, and thirty Swiss-Webster male mice were used in a PTZ-induced seizure model, a BIC-induced seizure model and an NE-100-induced seizure test, respectively. The mice weighed 25–40 g (Laboratory Animal Centre, University of Tartu, Tartu, Estonia). 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. All studies involving animals were conducted in accordance with ARRIVE guidelines [19,20]. Experimental procedures were performed in accordance with the guidelines reported in EU Directive 2010/63/EU and in accordance with local laws and policies. All procedures were approved by the Latvian Animal Protection Ethical Committee of Food and Veterinary Service in Riga, Latvia.

2.2. Chemoconvulsant-induced seizures

Chemoconvulsant-induced clonic and tonic seizures were initiated by inserting a 27-gauge needle into the tail veins and infusing 1% PTZ [21,22] or 0.01% BIC [23] at a constant rate of 20 µl/2 s to restrained animals. The infusion was halted when forelimb clonus followed by tonic seizures of the full body were observed. Minimal doses of PTZ or BIC (mg/kg of mouse weight) necessary to induce clonic and tonic seizures were considered as indices of seizure threshold.

The activity of Sig1R ligands on clonic and tonic seizure thresholds was studied in PTZ- and BIC-induced seizure models and each experimental set included a respective control group. The minimal dose of PTZ and BIC to induce clonic and tonic seizures in each experimental group is expressed as percentage from control, where 100% represents the seizure threshold for control group. Animals received i.p. injection of saline for control or Sig1R ligand 60 min before PTZ or BIC i.v. infusion. Each animal received a single dose of Sig1R ligand. In the PTZ-induced seizure model animals were divided in the following groups and experimental sets: saline (n = 8) and PRE-084 at a dose of 3 mg/kg (n = 8) for set number 1; saline (n = 8), NE-100 at doses of 5 mg/kg (n = 8) and 10 mg/kg (n = 8) for set number 2; saline (n = 10), PRE-084 at doses of 10 mg/kg (n = 10) and 50 mg/kg (n = 9), and NE-100 at doses of 25 mg/kg (n = 8) and 50 mg/kg (n = 7) for set number 3; saline (n = 10) and E1R at doses of 10 mg/kg (n = 9) and 50 mg/kg (n = 10) for set number 4; saline (n = 10), NE-100 at a dose of 5 mg/kg (n = 10), E1R at a dose of 10 mg/kg (n = 10) and group of combination of NE-100 at a dose of 5 mg/kg with E1R at a dose of 10 mg/kg (n = 10) for set number 5. In the set number 5 NE-100 was administered 80 min before PTZ infusion (*i.e.*, 20 min before E1R). In the set number 3 NE-100 at a dose of 50 mg/kg induced seizures before PTZ infusion, therefore the animals (n = 7) did not receive PTZ i.v. infusion and animal behaviour was observed and analysed separately (Material and methods Section 2.3.). In total 146 animals were used in PTZ-induced seizure model.

In the BIC-induced seizure model animals were divided in the following groups and experimental sets: saline (n = 10), PRE-084 at a dose of 50 mg/kg (n = 10) and E1R at a dose of 50 mg/kg (n = 10) for set number 1; saline (n = 10) and E1R at a dose of 10 mg/kg (n = 10) for set number 2; saline (n = 8) and NE-100 at doses of 10 mg/kg (n = 7) and 25 mg/kg (n = 9) for set number 3. In total 74 animals were used in this model.

2.3. NE-100-induced seizures

NE-100 at a dose of 50 mg/kg (n = 7) induced seizures before PTZ i.v. infusion, therefore animal behaviour was observed separately. The number of animals with seizures, the latency time until the first occurrence of seizures and duration time was analysed. Further, the NE-100-induced seizures were studied at a dose of 75 mg/kg, which induced seizures in 11 from total of 11 animals (n = 11). To compare the activities and interactions of the Sig1R ligands, the animals received selective Sig1R agonist PRE-084 (n = 6) and positive allosteric Sig1R modulator E1R (n = 6) at the same dosages as that of NE-100. In total 30 animals were used to observe, analyse and describe the seizures induced by NE-100. Compounds or saline were administered 30 min prior to NE-100. Mice were then placed immediately in observation chambers (40 × 25 × 15 cm) and video recorded for 25 min using a digital HD video camera recorder (Handycam HDR-CX11E, Sony Corporation, Tokyo, Japan). Scoring scale for observed behavioural responses of animals was adapted from previously published seizure rating scale [24]. Behavioural responses of animals were scored from the video files using the following scale: 0, no abnormality; 1, trembling or wobbly gait; 2, tail lifting; 3, clonic seizures while lying on belly; 4, tonic seizures while lying on belly; 5 clonic-tonic seizures while lying on belly; 6, motionless or sleeping; 7, clonic seizures while lying on side; 8, tonic seizures while lying on side; 9, clonic-tonic seizures while lying on side; 10, generalised seizures with wild jumping and running. If the animal died during the observation period the score of 11 was given for the rest of time points. Maximal score was given for each 20-s period. Latency time until the first occurrence of seizures induced by NE-100 was also determined from the video files.

2.4. Data and statistical analyses

The results are expressed as the means ± S.E.M. The data for the chemoconvulsant-induced seizures were analysed using Student's *t*-test and one-way ANOVA followed by Newman-Keuls multiple comparison test. The statistical calculations were performed using GraphPad Prism 3.0 (GraphPad Software, Inc., La Jolla, California, USA). P-values less than 0.05 were considered significant.

2.5. Chemicals

(4R,5S)-2-(5-Methyl-2-oxo-4-phenyl-pyrrolidin-1-yl)-acetamide (E1R) was obtained from JSC Grindeks (Riga, Latvia). 2-(4-Morpholinethyl) 1-phenylcyclo-hexanecarboxylate hydrochloride (PRE-084) and 4-methoxy-3-(2-phenylethoxy)-*N*,*N*-dipropylbenzeneethanamine hydrochloride (NE-100) were purchased from Tocris Bioscience (Bristol, UK). PTZ and BIC were procured from Sigma-Aldrich Co. (St. Louis, MO, USA). 0.9% physiological saline was

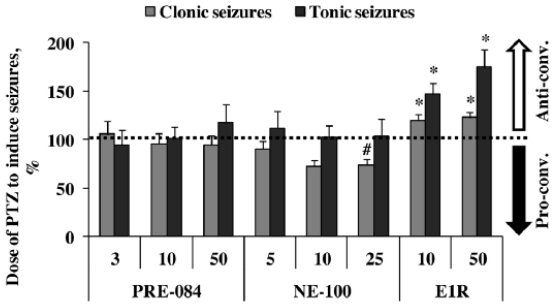

Fig. 1. Comparison of Sig1R ligands on PTZ-induced seizure thresholds. Dotted line represents threshold for BIC-induced seizures in control group (100%). Arrow up shows that compound increases seizure thresholds (> 100%) and possess anti-convulsive activity. Arrow down shows that compound decreases thresholds (< 100%) and possess pro-convulsive activity. Compounds were administered i.p. 60 min before PTZ injection. Data are expressed as the means ± S.E.M. (n = 8–10 in each group). $^{*}p < 0.05$ *vs.* control, $^{\#}p = 0.05$ *vs.* control (Student's *t*-test).

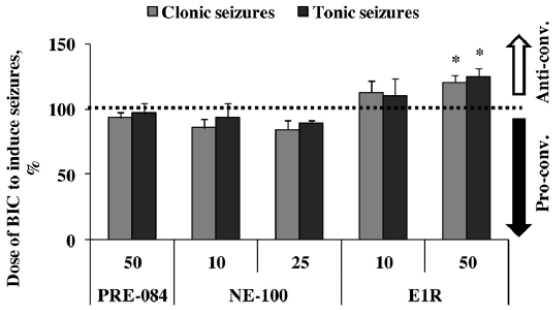

Fig. 2. Comparison of Sig1R ligands on BIC-induced seizure thresholds. Dotted line represents threshold for PTZ-induced seizures in control group (100%). Arrow up shows that compound increases seizure thresholds (> 100%) and possess anti-convulsive activity. Arrow down shows that compound decreases thresholds (< 100%) and possess pro-convulsive activity. Compounds were administered i.p. 60 min before BIC injection. Data are expressed as the means ± S.E.M. (n = 7–10 in each group). $^{*}p < 0.05$ *vs.* control (Student's *t*-test).

purchased from Fresenius Kabi (Warszawa, Poland).

PTZ was weighed and dissolved in 0.9% physiological saline to make a 1% PTZ solution. BIC was dissolved in DMSO to prepare a 1% stock solution, which was then diluted with saline to make a 0.01% BIC solution. PTZ and BIC solutions were freshly prepared before each experiment. Compounds were dissolved in saline before use.

3. Results

3.1. Activity of selective Sig1R ligands in the PTZ-induced seizure model

The 1% PTZ i.v. infusion induced clonic and tonic seizures in control animals at a dose of 24 ± 1 mg/kg and 67 ± 8 mg/kg, respectively. To test activities of Sig1R ligands, compounds were administered 60 min before PTZ. Selective Sig1R agonist PRE-084 tested at doses of 3, 10 and 50 mg/kg did not change animal behaviour and the threshold for PTZ-induced seizures (Fig. 1). Surprisingly, we discovered that the i.p. administration of selective Sig1R antagonist NE-100 at a dose of 50 mg/kg induced convulsions in mice before PTZ infusion. Therefore, animals from these group were excluded from the experiment, and the behaviours of these mice were further observed (the results are described in Section 3.4.). At a dose of 5 mg/kg NE-100 had no effect on seizure thresholds (Figs. 1 and 3). The administration of NE-100 at a dose of 10 mg/kg showed a tendency for pro-convulsive activity on PTZ-induced clonic seizures. NE-100 at a dose of 25 mg/kg demonstrated significant pro-convulsive activity on PTZ-induced clonic seizures (Fig. 1). NE-100 had no effect on tonic seizures (Fig. 1). Positive allosteric Sig1R modulator E1R demonstrated dose-dependent anti-convulsive effects on PTZ-induced tonic seizures (Fig. 1). E1R given i.p. at a dose of 10 mg/kg significantly increased the thresholds for clonic seizures by 20% and for tonic seizures by 47% (Fig. 1). The thresholds on PTZ-induced clonic and tonic seizures increased by 23% and 75%, respectively, after the administration of E1R at a dose of 50 mg/kg (Fig. 1).

3.2. Activity of selective Sig1R ligands in the BIC-induced seizure model

The selective Sig1R ligands were also tested on BIC-induced seizures. Clonic seizures in control animals were induced at a dose of 0.49 ± 0.06 mg/kg of BIC. BIC-induced tonic seizures were induced at a dose of 0.96 ± 0.15 mg/kg. PRE-084 administered at a dose of 50 mg/kg demonstrated no differences compared with the control group on the BIC-induced seizure thresholds (Fig. 2). The administration of NE-100 at a dose of 10 mg/kg had no effect on the seizure thresholds. NE-100 at a dose of 25 mg/kg showed a slight tendency for pro-convulsive activity in the model of BIC-induced clonic seizures. E1R given at a dose of 50 mg/kg significantly elevated the thresholds on BIC-induced clonic and tonic seizures by 21 and 25%, respectively (Fig. 2).

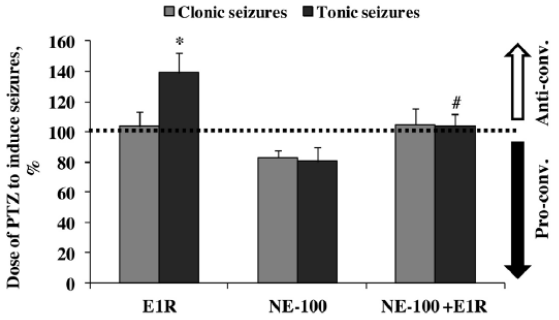

Fig. 3. Effect of selective Sig1R antagonist on the anticonvulsive activity of E1R on PTZ-induced seizures. Dotted line represents threshold for PTZ-induced seizures in control group (100%). Arrow up shows that compound increases seizure thresholds (> 100%) and possesses anti-convulsive activity. Arrow down shows that compound decreases thresholds (< 100%) and possesses pro-convulsive activity. E1R was administered i.p. at a dose of 10 mg/kg 60 min before PTZ injection. NE-100 was given i.p. at a dose of 5 mg/kg 80 min before PTZ injection. Data are expressed as the means ± S.E.M. (n = 10 in each group). $^{*}p < 0.05$ *vs.* control, $^{\#}p < 0.05$ *vs.* E1R (One-way ANOVA followed by Newman-Keuls multiple comparison test).

3.3. Anti-seizure effects of E1R are Sig1R dependent

To verify that Sig1R was involved in the anti-convulsive activity of E1R, we used selective Sig1R antagonist NE-100. For PTZ-induced seizures, pre-treatment with NE-100 alone at a dose of 5 mg/kg had no significant effect on seizure thresholds (Fig. 3). E1R given at a dose of 10 mg/kg significantly increased the threshold on PTZ-induced tonic seizures by 39% (Fig. 3). The administration of NE-100 (5 mg/kg) before E1R (10 mg/kg) significantly restored the tonic seizure threshold to the basal level (Fig. 3) and therefore, showed that the anti-seizure effect of E1R was mediated through Sig1R activity.

3.4. NE-100 per se induces seizures in mice after systemic administration

I.p. administration of NE-100 at doses of 50 and 75 mg/kg induced convulsions in mice. Convulsive activity after the administration of NE-100 at a dose of 50 mg/kg was observed for 5 from total of 7 animals. The convulsive behaviour in mice started approximately 9 min after

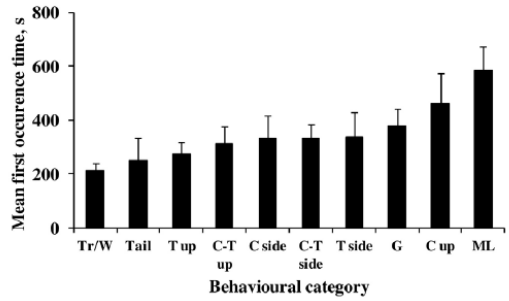

Fig. 4. Onset latencies of the different behavioural categories after NE-100 administration: trembling or wobbly gait (TR/W), tail lifting (Tail), clonic, tonic and clonic-tonic seizures while lying on belly (C, T and C-T up, respectively), clonic, tonic and clonic-tonic seizures while lying on side (C, T and C-T side, respectively), generalised seizures with wild jumping and running (G), motionless (ML). Data are expressed as the means ± S.E.M. (n = 11).

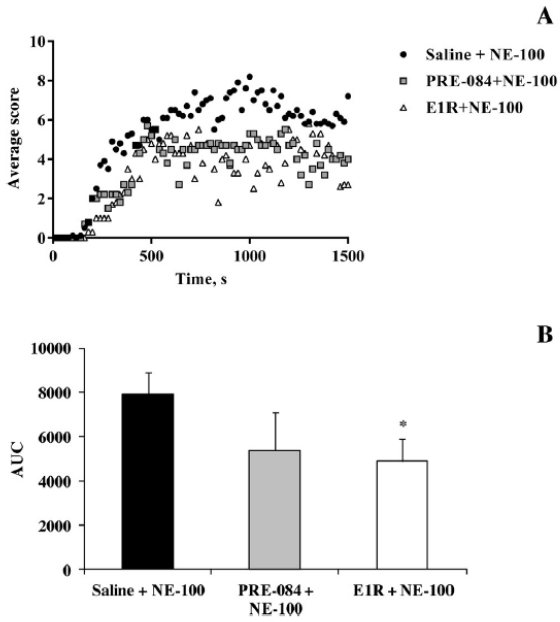

Fig. 5. The activity of PRE-084 and E1R on NE-100-induced convulsive behaviour. PRE-084 (75 mg/kg, n = 6), E1R (75 mg/kg, n = 6) or saline (n = 11) were administered i.p. 30 min prior to i.p. injection of NE-100 (75 mg/kg). (A) Average behavioural scores for each group during 25-min observation period. Data are expressed as the means for each 20 s period. (B) The area under curve ($AUC_{0\text{-}1500s}$) was calculated from behavioural scoring curve. Data are expressed as the means ± S.E.M. (n = 6–11). $^{*}p < 0.05$ *vs.* the saline group (One-way ANOVA followed by Newman-Keuls multiple comparison test).

NE-100 (50 mg/kg, i.p.) administration and lasted until 20 min (data not shown). NE-100 at a dose of 75 mg/kg induced generalised, tonic and clonic seizures for 11 from total of 11 animals. Convulsions started approximately 4 min after NE-100 (75 mg/kg, i.p.) injection (Fig. 5A). During the 25-min observation time, seizures appeared periodically, and 10 different behavioural categories were observed in the mice (Fig. 4). First observed behavioural response after administration of NE-100 at a dose of 75 mg/kg was trembling together with wobbly gait (animals were looking dizzy). NE-100-induced seizures started with tonic seizures followed by clonic-tonic seizures. After that seizure intensity increased and clonic, tonic and clonic-tonic seizures were observed while animal lied on side. This quite rapidly resulted in generalised seizures (approximately 6 min after NE-100 injection). The

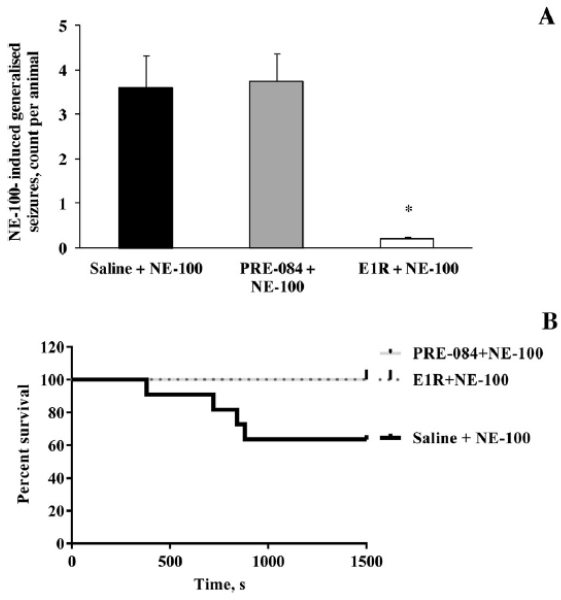

Fig. 6. The effect of Sig1R ligands on NE-100-induced generalised seizures and survival. PRE-084 (75 mg/kg, n = 6), E1R (75 mg/kg, n = 6) or saline (n = 11) were administered i.p. 30 min prior to i.p. injection of NE-100 (75 mg/kg). (A) Effects on NE-100-induced generalised seizures. Data are expressed as the means ± S.E.M. $^{*}p < 0.05$ *vs.* the saline group (One-way ANOVA followed by Newman-Keuls multiple comparison test). (B) Survival curve during 25-min observation period.

average time of the first occurrence (onset latency) for each behavioural category is shown in Fig. 4. During the later periods (after 25-min observation time), the animals remained motionless and slowly regained their normal behaviour. After administration of NE-100 at a dose of 75 mg/kg four animals died during the first 15 observation min (Fig. 6B).

3.5. The activity of selective Sig1R ligands on NE-100-induced seizures

PRE-084 and E1R at a dose of 75 mg/kg partially prevented NE-100 induced seizures and showed lower average behavioural score (Fig. 5A, Table 1). However, as demonstrated by the data expressed as areas under the curve (AUCs), only E1R effect was statistically significant (Fig. 5B). In addition, 2 and 1 animals in the PRE-084 and E1R, respectively, did not have any seizures after the administration of NE-100. E1R significantly reduced also the number of animals with generalised seizures induced by NE-100 and reduced the generalised

Table 1
The activity of Sig1R compounds on NE-100-induced convulsive behaviour.

	Saline + NE-100	PRE-084 + NE-100	E1R + NE-100
Animals with seizures, n/total n	11/11	4/6	5/6
Seizure onset time, s	238 ± 27	239 ± 35	320 ± 34
Animals with generalised seizures, n/total n	11/11	4/4	1/5
Average maximal score (peak), points	8.2 ± 0.8	5.7 ± 1.9	5.8 ± 1.3
Average time to reach peak, s	569 ± 65	330 ± 59	708 ± 166
Average behavioural score, points	5.3 ± 0.3	3.6 ± 0.2	3.3 ± 0.2

PRE-084 (75 mg/kg; n = 6), E1R (75 mg/kg; n = 6) or saline (n = 11) were administered i.p. 30 min prior to i.p. injection of NE-100 (75 mg/kg).

seizure count per animal, whereas PRE-084 had no effect (Fig. 6A). E1R also demonstrated higher latency times until the first occurrence of seizures induced by NE-100 (Table 1). However, there was no significant difference when compared with the NE-100 treated animal group (Table 1).

4. Discussion

The seizure-modulating activity of selective Sig1R agonist PRE-084, antagonist NE-100 and E1R, a positive allosteric Sig1R modulator, has never been described previously. In this study, we report for the first time the pro-convulsive and convulsive activity of selective Sig1R antagonist NE-100, whereas we did not observe any activity on PTZ- and BIC-induced seizures for Sig1R agonist PRE-084. In turn, Sig1R positive allosteric modulator E1R showed anti-convulsive effects both on PTZ- and BIC-induced clonic and tonic seizures. Interestingly, the effect of E1R was blocked by Sig1R antagonist NE-100, and the NE-100-induced convulsive behaviour was partially attenuated by E1R.

NE-100 is a selective Sig1R antagonist (K_i = 0.86 nM) displaying more than 55-fold selectivity over Sig2R and more than 6000-fold selectivity over dopamine, serotonin and phencyclidine receptors [25]. We found that NE-100 induced convulsions already after a single injection in mice. Previously only Sig1R agonist-induced dose-dependent convulsive behaviour has been described for (±)-pentazocine [26] and (+)-3-PPP [27]. (±)Pentazocine-induced seizures started with forelimb and head jerks followed by minor vibrissae twitching and facial jerks, and continuous forelimb and head jerks for first 20 min, but during the later period, animals remained motionless and slowly regained normal behaviour [26]. (+)-3-PPP elicited behavioural clonic or tonic-clonic convulsions within 3–6 min from the administration of the drug [27]. These behavioural seizures lasted for 3–5 min and were followed by death of some animals [27]. In our study we found that Sig1R antagonist NE-100 during 25 min observation period induced clonic, tonic, clonic-tonic and generalised seizures and also resulted in death of some mice. Similarly as for (±)-pentazocine, after the 25-min observation time NE-100-treated animals became motionless and later regained their normal behaviour. Overall, it can be concluded that both Sig1R agonists (±)-pentazocine and (+)-3-PPP and Sig1R antagonist NE-100 demonstrate similar convulsive activity and animal behaviour in experimental studies, which raises questions about activities of Sig1R ligands in the light of classic models of receptor agonism and antagonism.

In addition to *in vivo* induced seizures after single administration of NE-100, we found that NE-100 presents a dose-dependent pro-convulsive activity in PTZ- and BIC-induced seizure models. Previously, the pro-convulsive activity has been shown for Sig1R agonist (+)-pentazocine, which dose dependently (12.5–50 mg/kg, s.c.) reduced seizure threshold in flurothyl-induced seizure model [28]. However, in the same study structurally related Sig1R agonist (+)-cyclazocine demonstrated dose-dependent (2.5–10 mg/kg, s.c.) anti-convulsive activity [28]. In another study racemic (±)-pentazocine has demonstrated dose-dependent (10–50 mg/kg, i.p.) anti-convulsive activity on maximal electroshock-induced seizures [29]. Additionally, (±)-pentazocine dose-dependently (20–100 mg/kg, s.c.) reduced tonic seizures induced by N-methyl-DL-aspartic acid [4]. The role of Sig1R in flurothyl-induced seizures remains controversial, since (+)-pentazocine and (+)-cyclazocine possess opposite effects, even though both are Sig1R agonists and have binding affinities in the nanomolar range (K_i = 3 nM for (+)-pentazocine; K_i = 17 nM for (+)-cyclazocine) [30]. The anti-convulsive activity has been shown for Sig1R agonists dimemorfan (24 mg/kg, s.c.) and carbetapentane (12.5 and 25 mg/kg, i.p.) in kainic acid-induced seizure model [2,3], dextorphan (20–40 mg/kg, i.p.) in maximal electroshock-induced seizure model [31], and dextromethorphan in models of kainic-acid- and maximal electroshock-induced seizures (24 mg/kg, s.c. and 20–40 mg/kg, i.p., respectively) [2,31]. In turn, it has been shown that Sig1R agonists SA-4503 and 1,3-di(2-tolyl)guanidine (DTG) cannot protect against cocaine-induced seizures, while in the same study Sig1R antagonist panamesine demonstrated anti-convulsive activity [7]. Similar activity has been shown for Sig1R antagonists AC-927 (1–10 mg/kg, i.p.), LR-172 (1–30 mg/kg, i.p.) and BD-1047 (1–40 mg/kg, i.p.) on cocaine-induced seizures [5,6]. However, there is limited information available about activity of Sig1R antagonists in other seizure models. For example, in kainic-acid-, maximal electroshock- and PTZ-induced seizure model Sig1R antagonist BD-1047 possess seizure modifying activity, but the compound was used only at low doses (1 and 2 mg/kg, i.p.) [2,3,11]. Our results for the first time show the pro-convulsive activity of a selective Sig1R antagonist in GABA-ergic system-related chemoconvulsant seizure models and provide evidence for the involvement of Sig1R in processes of PTZ- and BIC-induced seizures.

Many studies have suggested the potential therapeutic use of Sig1R antagonists for the management of neuropathic pain. Selective Sig1R antagonists, such as BD-1063, BD-1047, E-52862, S1RA and NE-100 have been shown to attenuate the development of hypersensitivity in models of neuropathic pain in mice and rats [17,32,33]. It should be noted that above listed Sig1R antagonists in the respective studies have been used at quite high doses. For example, NE-100 administered s.c. at doses of 32, 64 and 128 mg/kg significantly reduced capsaicin-induced mechanical hypersensitivity in mice [17]. Additionally, the effects of S1RA on mechanical hypersensitivity induced by capsaicin in mice were studied at a dose of 64 mg/kg after i.p. and at a dose of 128 mg/kg after per oral administration [34]. Thus, the effective doses of NE-100 used to treat neuropathies are very close or even higher than doses shown to induce convulsions in our study. Side effects, such as cognitive impairment, have already been shown at the highest doses (500–800 mg p.o.) of S1RA in phase I clinical studies [35]. These effects should be seriously considered when studying Sig1R antagonists as novel drug candidates.

Surprisingly, our study demonstrated that Sig1R agonist PRE-084 neither had anti-seizure nor pro-convulsive nor convulsive activity up to a dose of 75 mg/kg. PRE-084 has a high affinity to Sig1R (K_i = 2.2 nM) [36]. DTG, a Sig1R and Sig2R agonist, has demonstrated anti-convulsive properties on BIC-induced seizures. However, the effects of DTG were not blocked by non-selective Sig1R antagonist haloperidol [37]. It seems that anti-seizure activity of Sig1R agonist compounds does not involve effects on GABA-ergic signalling.

Previously we showed that E1R, a phenylpiracetam derivative, is selective positive allosteric Sig1R modulator [18,38]. In this study E1R showed anti-convulsive effects both in PTZ- and BIC-induced seizure models. Some other known positive allosteric modulators of Sig1R, such as phenytoin and ropizine [8], SKF83959 [39], and SOMCL-668 [11] also have demonstrated anti-convulsive activity. Similarly as in the present study, the anti-convulsive activity of SKF83959 and SOMCL-668 in the PTZ-induced seizure model was blocked by a selective Sig1R antagonist BD-1047 [11]. This allows us to confirm the anti-convulsive properties of positive allosteric Sig1R modulators and the role of Sig1R in the mode of action of these compounds. Among all positive allosteric Sig1R modulators, E1R is the only one known modulator that possesses memory-improving effects [18]. Phenytoin treatment on epilepsy has been shown to induce learning and memory deficit. Add-on approaches for the management of memory deficits associated with conventional anti-epileptic drugs are needed [40]. The effects of E1R show that it might be a promising novel anti-seizure drug with no negative influences on memory typically encountered for many anti-epileptic drugs.

In conclusion, for the first time, our study demonstrates the pro-convulsive and convulsive activities of selective Sig1R antagonist NE-100. NE-100-induced convulsive behaviour can be partially attenuated by positive allosteric modulation of Sig1R. Additionally, the obtained results suggest that Sig1R could be considered as a molecular target for new anti-convulsive drugs.

Acknowledgment

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