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

Summary of the Doctoral Thesis  
for obtaining the degree of a Doctor of Pharmacy  
Speciality – Pharmaceutical Pharmacology

Rīga, 2017

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The Doctoral Thesis is available in the RSU library of and at RSU webpage: [www.rsu.lv](http://www.rsu.lv).

Secretary of the Doctoral Council:

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## ABBREVIATIONS

ANOVA – analysis of variance

ARRIVE – Animal Research: Reporting of In Vivo Experiments

AUC – area under curve

BDK – bradykinin

BIC – (+)-bicuculline

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

CGRP – calcitonin gene-related peptide

CNS – central nervous system

DTG – 1,3-di(2-tolyl)guanidine

ED<sub>50</sub> – 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

HEK293 – human embryonic kidney cell line

IC<sub>50</sub> – half maximal inhibitory concentration

i.p. – intraperitoneal

i.v. – intravenous

K<sub>i</sub> – equilibrium dissociation constant

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

NG108-15 – neuroblastoma-glioma hybrid cell line

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

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

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

TNF – tumor necrosis factor

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

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 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 (Gouliarov 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, a novel derivative of piracetam, 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 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. METHODS

## 1.1. 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.

## 1.2. *In vitro* methods

### 1.2.1. 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.

The method for [<sup>3</sup>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 [<sup>3</sup>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).

### **1.3.2. 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 neuroblastoma-glioma hybrid (NG108-15) cells were pre-incubated with 10  $\mu$ M E1R, 2  $\mu$ M 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  $\mu$ M 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  $\mu$ M NE-100 was used as the positive control and was pre-incubated with the cells for 20 min before the measurements were taken.

### **1.3.3. 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 °C and bubbled with 95% CO<sub>2</sub> and 5% O<sub>2</sub> (Pubill et al., 1998). The passive tension was fixed at 1 g. 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  $\mu$ M) of the Sig1R agonist PRE-084 were added. E1R was added to each isolated *vas deferens* at a concentration of 10  $\mu$ M. 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  $\mu$ M) was used in a similar experimental set-up.

## **1.4. *In vivo* methods: behavioural experiments**

### **1.4.1. 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 subcutaneously (s.c.) at a dose of 0.3 mg/kg.

### **1.4.2. 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).

### **1.4.3. 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.

### **1.4.4. 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.

#### **1.4.5. Chemoconvulsant-induced seizures**

Chemoconvulsant-induced clonic and tonic seizures were initiated by inserting a 27-gauge needle into the tail veins and infusing 1% pentylenetetrazol (PTZ) (Mandhane et al., 2007; Zvejniece et al., 2010) or 0.01% (+)-bicuculline (BIC) (Meldrum, 1975) at a constant rate of 20  $\mu$ l/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. Animals received i.p. injection of saline for control or Sig1R ligand 60 min before PTZ or BIC i.v. infusion.

#### **1.4.6. 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 high definition 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.

## 1.5. 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<sub>50</sub> values of the tested compounds in competitive radioligand binding assay. 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 data for the BDK-induced increase in [Ca<sup>2+</sup>]<sub>i</sub> 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<sub>50</sub> 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).

## 2. RESULTS

### 2.1. *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  $\mu$ M concentration had little to no activity in 77 radioligand displacement assays that included numerous ion channel, G protein-coupled receptor and central nervous system transporter targets (Table 2.1).

Table 2.1.

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

Receptor/ Ion Channel	Inhibition by 10 $\mu$ M E1R, %	Reference Compound	IC <sub>50</sub> , nM
Adenosine A <sub>1</sub>	2	DPCPX	0.87
Adenosine A <sub>2A</sub>	-16	NECA	10
Adrenergic $\alpha_1$	-5	prazosin	0.17
Adrenergic $\alpha_2$	-4	yohimbine	36
Adrenergic $\alpha_1$	-5	atenolol	230
Adrenergic $\alpha_2$	-2	ICI-118551	0.5
Angiotensin AT <sub>1</sub>	-14	saralasin	0.6
Angiotensin AT <sub>2</sub>	-5	angiotensin	0.13
Benzodiazepine (central)	-18	diazepam	110
Benzodiazepine (peripheral)	3	PK-11195	1.1
Bombesin BB	-5	bombesin	0.22
BDK B <sub>2</sub>	3	NPC-567	10
Calcitonin gene-related peptide CGRP	1	hCGRP	0.04
Cannabinoid CB <sub>1</sub>	-3	CP-55940	0.21
Cholecystokinin CCK <sub>1</sub>	9	CCK-8s	0.2

Table 2.1. (continued)

<b>Receptor/ Ion Channel</b>	<b>Inhibition by 10 <math>\mu</math>M E1R, %</b>	<b>Reference Compound</b>	<b>IC<sub>50</sub>, nM</b>
Cholecystokinin CCK <sub>2</sub>	8	CCK-8s	0.26
Dopamine D <sub>1</sub>	-6	SCH-23390	0.22
Dopamine D <sub>2S</sub>	5	(+)-butaclamol	1.4
Dopamine D <sub>3</sub>	7	(+)-butaclamol	1.2
Dopamine D <sub>4</sub>	-3	clozapine	32
Dopamine D <sub>5</sub>	-3	SCH-23390	0.15
Endothelin ET <sub>A</sub>	-2	endothelin	0.11
Endothelin ET <sub>B</sub>	2	endothelin	0.04
GABA (non-selective)	13	GABA	73
Galanin GAL <sub>1</sub>	-2	galanin	0.16
Galanin GAL <sub>2</sub>	-4	galanin	0.8
Platelet-derived growth factor	-6	Glycoprotein PDGF-BB	0.05
Chemokines CXCR2	2	IL-8	0.04
Chemokines CCR1	-5	MIP-1	0.08
TNF $\alpha$	-3	TNF $\alpha$	0.26
Histamine H <sub>1</sub>	-7	pyrilamine	0.82
Histamine H <sub>2</sub>	-12	cimetidine	270
Melanocortin MC <sub>4</sub>	4	NDP- $\alpha$ -MSH	0.2
Melatonin MT <sub>1</sub> (ML <sub>1A</sub> )	-6	melatonin	0.53
Muscarinic M <sub>1</sub>	-4	pirenzepine	27
Muscarinic M <sub>3</sub>	-7	4-DAMP	0.43
Muscarinic M <sub>4</sub>	-3	4-DAMP	0.83
Muscarinic M <sub>5</sub>	-12	4-DAMP	0.80
Neurokinin NK <sub>1</sub>	-11	[Sar <sup>9</sup> ,Met <sup>(O<sub>2</sub>)11</sup> ]-SP	0.24
Neurokinin NK <sub>2</sub>	-3	[Nleu <sup>10</sup> ]-NKA (4-10)	2.5
Neurokinin NK <sub>3</sub>	-7	SB-222200	8.8
NPY Y <sub>1</sub>	-15	NPY	0.07
NPY Y <sub>2</sub>	-18	NPY	0.09
Neurotensin NTS <sub>1</sub>	-3	neurotensin	0.24

Table 2.1. (continued)

Receptor/ Ion Channel	Inhibition by 10 $\mu$ M E1R, %	Reference Compound	IC <sub>50</sub> , nM
Opioid and opioid-like $\delta$ (DOP)	-4	DPDPE	1.9
Opioid and opioid-like $\kappa$ (KOP)	-5	U-50488	0.84
Opioid and opioid-like $\mu$ (MOP)	-7	DAMGO	0.74
Opioid and opioid-like NOP(ORL1)	4	nociceptin	0.29
Vasoactive intestinal peptide PAC <sub>1</sub> (PACAP)	-2	PACAP1-38	0.05
Peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ )	-9	rosiglitazone	12
Phencyclidine	6	MK-801	6.0
Prostanoid EP <sub>2</sub>	-13	PGE2	2.2
Prostanoid IP (PGI <sub>2</sub> )	-19	iloprost	13
Purinergic P2X	6	$\alpha$ , $\beta$ -MeATP	3.5
Purinergic P2Y	-4	dATP $\alpha$ S	25
Serotonin 5-HT <sub>1A</sub>	-4	8-OH-DPAT	0.51
Serotonin 5-HT <sub>1B</sub>	0	serotonin	12
Serotonin 5-HT <sub>2A</sub>	5	ketanserin	0.77
Serotonin 5-HT <sub>2B</sub>	-11	DOI	3.2
Serotonin 5-HT <sub>2C</sub>	-2	RS-102221	5.5
Serotonin 5-HT <sub>3</sub>	-1	MDL-72222	4.9
Serotonin 5-HT <sub>5a</sub>	-11	serotonin	280
Serotonin 5-HT <sub>6</sub>	-10	serotonin	180
Serotonin 5-HT <sub>7</sub>	-4	serotonin	0.49
<b>Sigma (non-selective)</b>	<b>-38</b>	<b>haloperidol</b>	<b>43</b>
Somatostatin sst	-5	somatostatin-14	0.14
Glucocorticoid	-11	dexamethasone	2.5
Vasoactive intestinal polypeptide (VIP) receptor 1	-11	VIP	0.12
Vasopresin <sub>1a</sub>	2	[d(CH <sub>2</sub> ) <sub>5</sub> , Tyr(Me) <sub>2</sub> ]-AVP	1.9

Table 2.1. (continued)

Receptor/ Ion Channel	Inhibition by 10 $\mu$ M E1R, %	Reference Compound	IC <sub>50</sub> , nM
Ca <sup>2+</sup> channel (L, verapamil site) (phenylalkylamine)	-1	D-600	20
K <sub>V</sub>	-2	$\alpha$ -dendrotoxin	0.29
SK <sub>Ca</sub> channel	-18	Apamin	0.03
Na <sup>+</sup> channel (site2)	5	Veratridine	7700
Cl <sup>-</sup> 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).

The only target for E1R (inhibition or enhancement of radioligand binding exceeding 20%) was the SigR; 10  $\mu$ M E1R did not displace the radioligand, but instead increased the specific binding of a non-selective radioligand [<sup>3</sup>H]-1,3-di(2-tolyl)guanidine (DTG) by 38% in Jurkat cells. In the same assay, the SigR antagonist haloperidol inhibited the binding of the radioligand with an IC<sub>50</sub> = 43 nM.

## 2.2. Activity of E1R on [<sup>3</sup>H]-(+)-pentazocine binding (Publication II)

Based on screening results, the activity of E1R was evaluated in [<sup>3</sup>H]-(+)-pentazocine binding assay. Unlike the selective Sig1R agonist PRE-084 (IC<sub>50</sub> = 192 nM) or the non-selective SigR antagonist haloperidol (IC<sub>50</sub> = 0.5 nM), E1R did not displace [<sup>3</sup>H]-(+)-pentazocine from the Sig1R (Figure 2.1.). As seen in Figure 2.1. E1R did not modulate binding of [<sup>3</sup>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 2.1.).

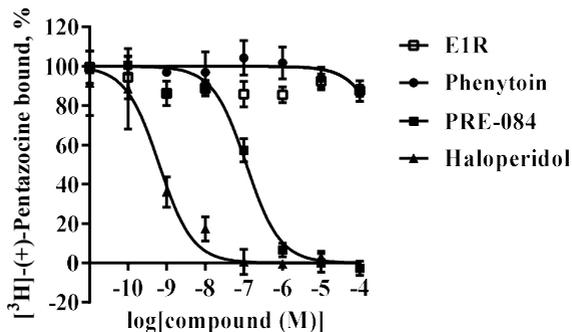


Figure 2.1. The effects of E1R and sigma receptor ligands on the binding of  $[^3\text{H}](+)\text{-pentazocine}$  to a Sig1R  
Synaptosomes from rat brains were incubated with 1.5 nM  $[^3\text{H}](+)\text{-pentazocine}$  at 30 °C for 150 min. Haloperidol (10  $\mu\text{M}$ ) was used to define nonspecific binding. The data represent at least three experiments performed in duplicate.

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

Selective Sig1R agonist PRE-084 at a dose of 2  $\mu\text{M}$  significantly enhanced the BDK-induced  $[\text{Ca}^{2+}]_i$  increase by  $34 \pm 4\%$  in NG108-15 cells. Moreover, E1R at a dose of 10  $\mu\text{M}$  significantly enhanced the  $[\text{Ca}^{2+}]_i$  increase by  $66 \pm 3\%$  (Figure 2.2.). In addition, PRE-084 effect on the  $[\text{Ca}^{2+}]_i$  changes was potentiated 3 times after pre-incubation with E1R, reaching  $212 \pm 6\%$  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  $\mu\text{M}$  (Figure 2.2.).

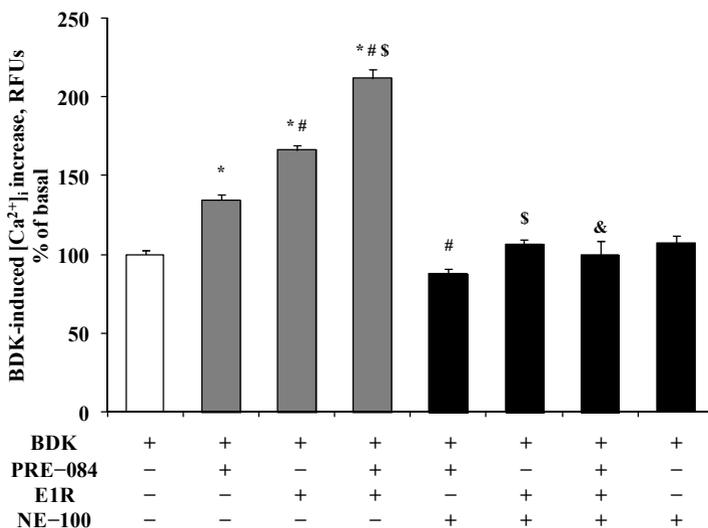


Figure 2.2. **The effects of Sig1R ligands and their combinations on 1  $\mu$ M BDK-induced  $[Ca^{2+}]_i$  increase in NG108-15 cells**

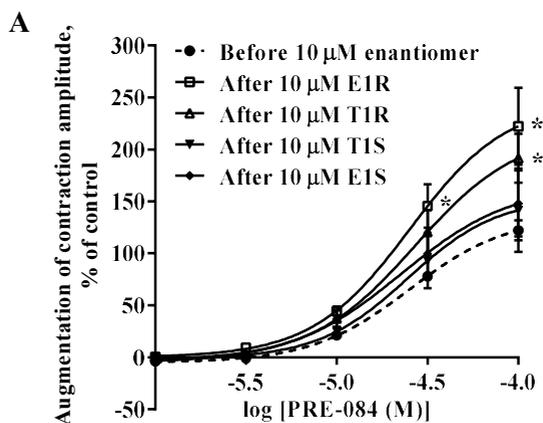
The cells were pre-incubated with 10  $\mu$ M E1R, 2  $\mu$ M PRE-084 or both in the dark at room temperature for 15 min. 40  $\mu$ M NE-100 was pre-incubated with the cells for 20 min before the measurements were taken. Changes in the  $[Ca^{2+}]_i$  were calculated as the percentage increase of the basal RFUs. Each column represents the mean  $\pm$  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).

## 2.4. 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  $\mu$ M) was  $122 \pm 11\%$  (Figure 2.3.A). Pre-incubation of *vasa deferentia* with a 10  $\mu$ M solution of each tested stereoisomer 10 min before the addition of PRE-084

significantly increased the intensity of the contractions (Figures 2.3.A and B). Comparison between the activities of all stereoisomers of methylphenylpiracetam showed that E1R is the most effective positive allosteric Sig1R modulator (Figures 2.3.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).



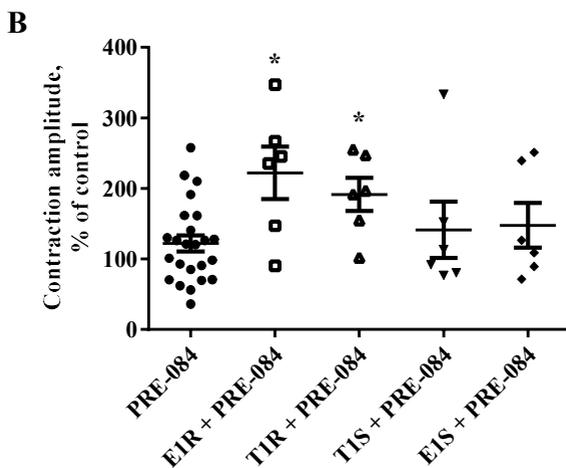


Figure 2.3. The sigma receptor activity assay in the electrically stimulated rat *vas deferens* model

(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*.

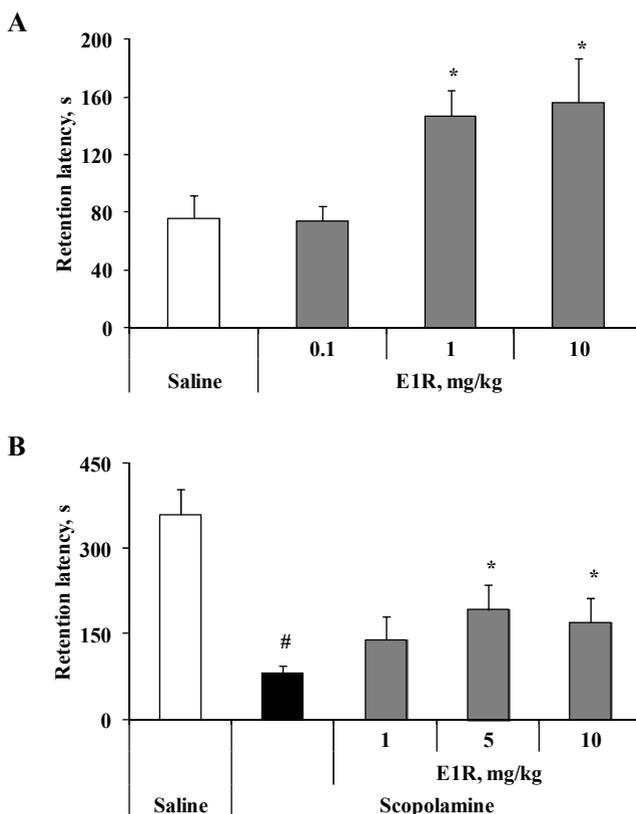
(B) Comparison between the activities of stereoisomers at the highest tested PRE-084 concentration. The results are expressed as the percentage of control contraction height and represent the means  $\pm$  S.E.M. (n = 6–24). \* $P < 0.05$  vs. PRE-084 treatment (two-way repeated ANOVA followed by the Bonferroni post-hoc test).

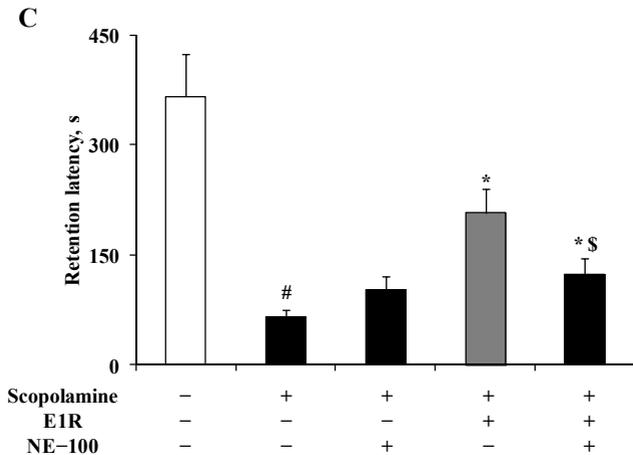
## 2.5. Effects of E1R on cognition in the PA test (Publication II)

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  $76 \pm 16$  s. Treatment with E1R significantly improved cognitive function in a dose-related manner. As shown in Figure 2.4.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  $360 \pm 45$  s, and pre-treatment with scopolamine significantly reduced the retention latency to  $81 \pm 13$  s (Figure 2.4.B). Figure 2.4.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 2.4.C).





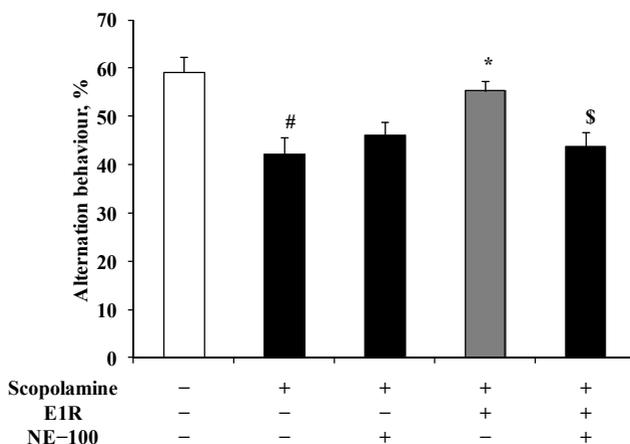
**Figure 2.4. Effects of E1R on memory and cognition in PA test**

(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  $\pm$  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 (5 mg/kg, i.p.) 60 min prior to the training session. Scopolamine (0.3 mg/kg, s.c.) was administered 40 min prior to the training session ( $n = 17-20$ ).  $\#P < 0.05$  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 ( $n = 20-25$ ).  $\#P < 0.05$  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).

## 2.6. 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 2.5.). The spontaneous alternation behaviour in the control animals was  $59 \pm 3\%$ ; pre-treatment with scopolamine significantly reduced the alternation behaviour to  $42 \pm 3\%$ . As shown in Figure 2.5., 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 2.5. 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  $\pm$  S.E.M. (n = 14–16). <sup>#</sup> $P < 0.05$  of the scopolamine-treated group vs. the saline group, <sup>\*</sup> $P < 0.05$  vs. the scopolamine-treated group, and <sup>\$</sup> $P < 0.05$  vs. the E1R-treated group (one-way ANOVA followed by the Newman-Keuls multiple comparison test).

## 2.7. 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 2.6.). In the

rota-rod, chimney and traction tests, the inhibitory activity of E1R on muscle function was observed at the following doses: ED<sub>50</sub> (ED<sub>16</sub>–ED<sub>84</sub>) mg/kg = 453 (398–516), 349 (199–611) and 595 (409–866), respectively.

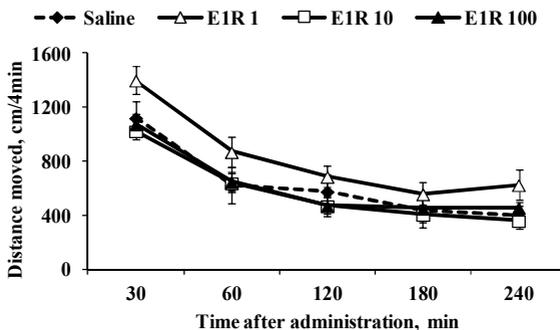
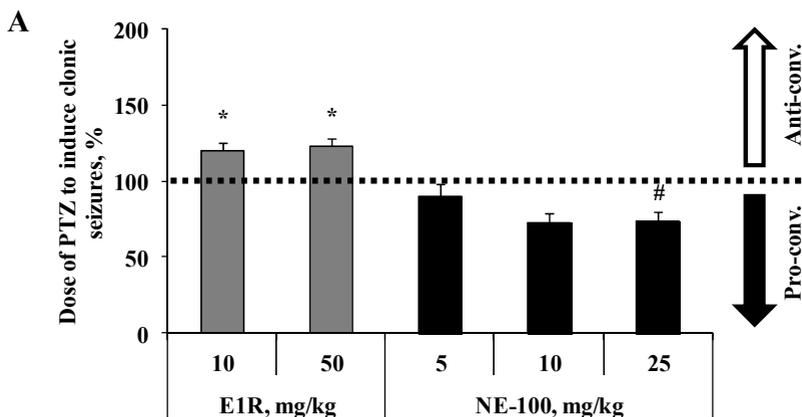


Figure 2.6. **The activity of E1R on moved distance in the open-field test**  
E1R was administered at doses of 1, 10 and 100 mg/kg (i.p.). The locomotor activities of mice were observed 30, 60, 120, 180 and 240 min after administration of E1R. The data are presented as the means  $\pm$  S.E.M. (n = 10).

## 2.8. 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  $24 \pm 1$  mg/kg and  $67 \pm 8$  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 2.7.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 2.7.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 2.7.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 2.7.A). NE-100 had no effect on tonic seizures (Figure 2.7.B). Selective Sig1R agonist PRE-084 did not change animal behaviour and the threshold for PTZ-induced seizures (data not shown; Publication IV).



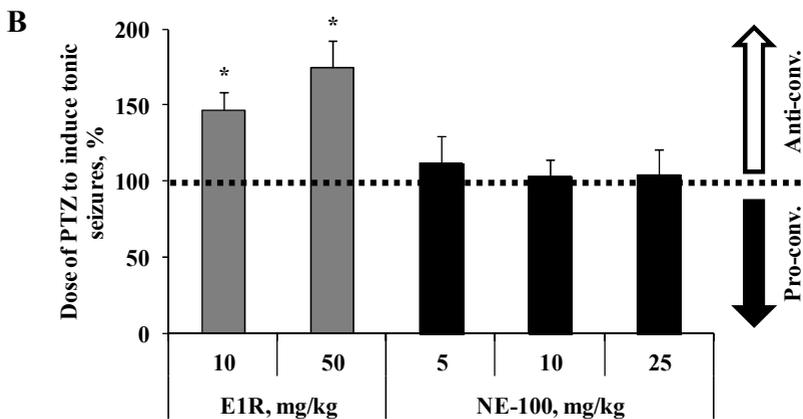
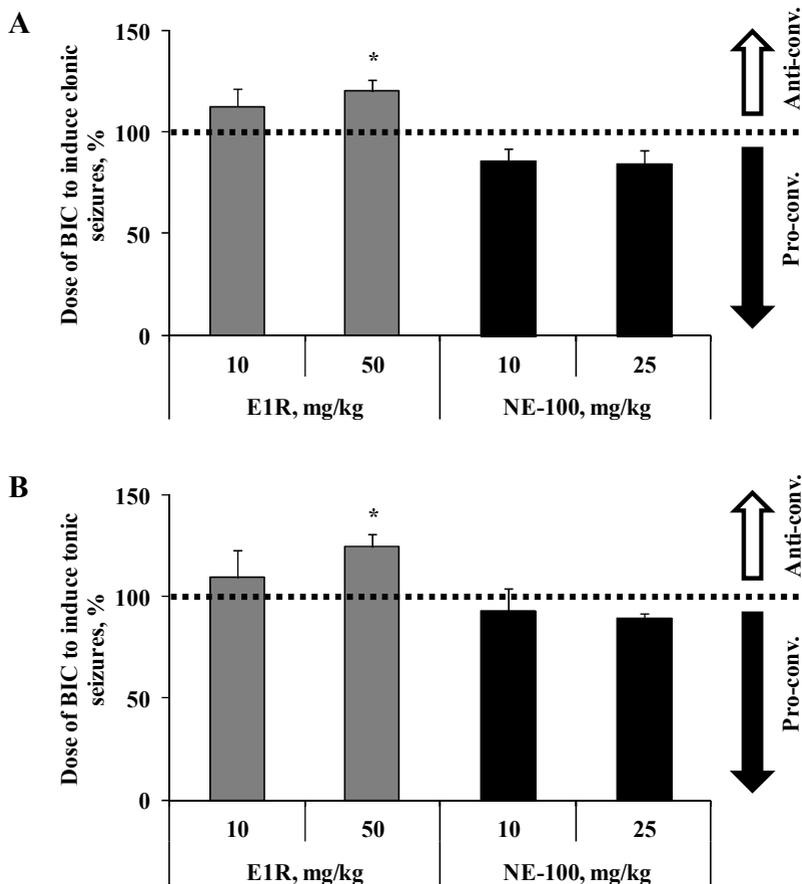


Figure 2.7. **Effects of E1R on PTZ-induced seizure thresholds**  
 Dotted line represents threshold for PTZ-induced seizures in control group (100%).  
 (A) Activity of Sig1R ligands on PTZ-induced clonic seizures. (B) Activity of Sig1R ligands on PTZ-induced tonic seizures. Compounds were administered i.p. 60 min before PTZ injection. Data are expressed as the means  $\pm$  S.E.M. (n = 8–10 in each group). \* $P < 0.05$  vs. control, # $P = 0.05$  vs. control (Student's t-test).

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  $0.49 \pm 0.06$  mg/kg of BIC. BIC-induced tonic seizures were induced at a dose of  $0.96 \pm 0.15$  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 2.8.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 2.8. 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  $\pm$  S.E.M. ( $n = 7-10$  in each group). \* $P < 0.05$  vs. control (Student's  $t$ -test).

## 2.9. 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 2.9.). E1R given at a dose of 10 mg/kg significantly increased the threshold on PTZ-induced tonic seizures by 39% (Figure 2.9.). The administration of NE-100 (5 mg/kg) before E1R (10 mg/kg) significantly restored the tonic seizure threshold to the basal level (Figure 2.9.) and therefore, showed that the anti-seizure effect of E1R was mediated through Sig1R activity.

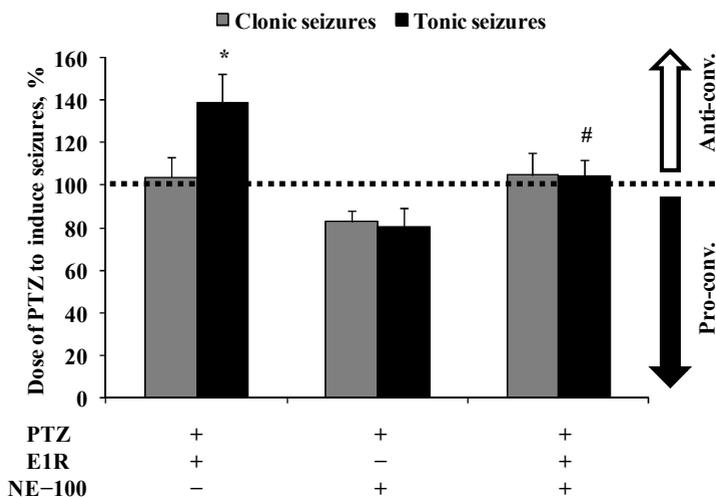
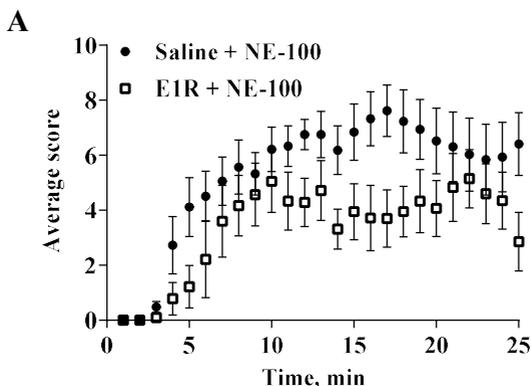


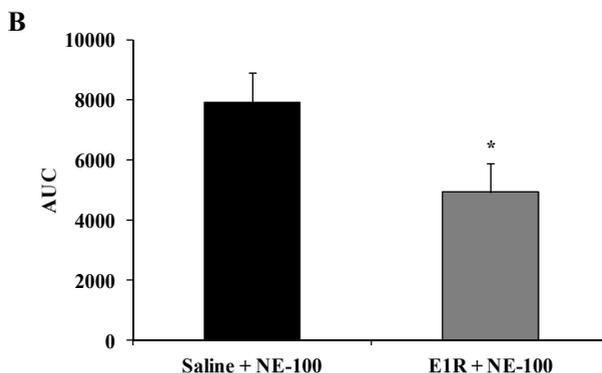
Figure 2.9. Effect of selective Sig1R antagonist on the anti-convulsive activity of E1R on PTZ-induced seizures

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  $\pm$  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).

## 2.10. 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 2.10.A and Table 2.2.). As demonstrated by the data expressed as areas under the curves (AUCs), E1R showed statistically significant effect (Figure 2.10.B).





**Figure 2.10. 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 ( $AUC_{0-1500s}$ ) was calculated from behavioural scoring curve. Data are expressed as the means  $\pm$  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 2.11.A). E1R also demonstrated higher latency times until the first occurrence of seizures induced by NE-100 (Table 2.2.) and increased the survival of animals (Figure 2.11.B). However, there was no significant difference when compared with the NE-100 treated animal group.

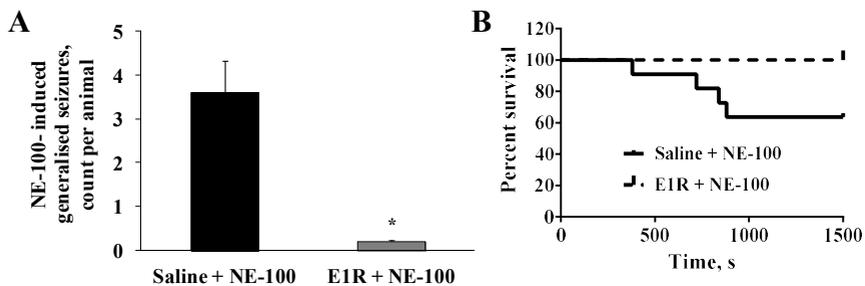


Figure 2.11. **The effects of E1R on NE-100-induced generalised seizures and survival**

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  $\pm$  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 2.2.

**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 $\pm$ 27	320 $\pm$ 34
Animals with generalised seizures, n/total n	11/11	1/5
Average maximal score (peak)	8.2 $\pm$ 0.8	5.8 $\pm$ 1.3
Average time to reach peak, s	569 $\pm$ 65	708 $\pm$ 166
Average behavioural score	5.3 $\pm$ 0.3	3.3 $\pm$ 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).

### 3. 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.

#### 3.1. 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, [<sup>3</sup>H]-1,3-di(2-tolyl)guanidine (DTG); however, we failed to demonstrate a Sig1R modulatory effect of E1R in a selective Sig1R radioligand [<sup>3</sup>H]-(+)-pentazocine binding assay using rat brain tissues (Publication II). At the same time, phenytoin also did not change the binding of [<sup>3</sup>H]-(+)-pentazocine, whereas PRE-084 and haloperidol dose-dependently competed with [<sup>3</sup>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 [<sup>3</sup>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 [<sup>3</sup>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 [<sup>3</sup>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 [<sup>3</sup>H]-(+)-pentazocine in HEK293 cells was also observed for phenytoin (Guo et al., 2013). Although [<sup>3</sup>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 [<sup>3</sup>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 [ $\text{Ca}^{2+}$ ]<sub>i</sub> increase, thus confirming its Sig1R positive allosteric modulatory effect *in vitro*.

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.

### **3.2. 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 dehydroepiandrosterone are known to bind to Sig1R under physiological conditions, and Sig1R constitutes one of the key targets in their neuromodulatory and behavioural effects (Monnet and Maurice, 2006; Su et al., 1988). Pregnenolone, dehydroepiandrosterone and other nonsteroidal Sig1R

agonists, influence the learning and memory processes in cholinergic and N-methyl-D-aspartic acid 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; Goulliaev 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 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.

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.

### **3.3. Sig1R ligands and seizures**

Positive allosteric Sig1R modulators demonstrate anti-seizure activity, thus showing an apparent correlation between the allosteric modulatory activity

and pharmacological effect. However, the anti-seizure activity is not associated directly with Sig1R for all allosteric Sig1R modulators. 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<sub>1</sub> dopamine receptor dependent (Bourne et al., 2001). This suggests that the pharmacological activity of Sig1R PAM could be a sum of different molecular mechanisms.

The seizure modulating activity is not unique to positive allosteric Sig1R modulators. For example, the 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 (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 (Skuzza, 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.

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 synaptic vesicle glycoprotein 2A with high affinity (Malykh and Sadaie, 2010). Piracetam itself demonstrates poor anti-convulsive activity and has lower affinity for synaptic vesicle glycoprotein 2A 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.

### 3.4. 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 [<sup>3</sup>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 3.1.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 3.1.) providing an increase in the dimeric (Figure 3.1.B) and/or monomeric (Figure 3.1.C) protein forms.

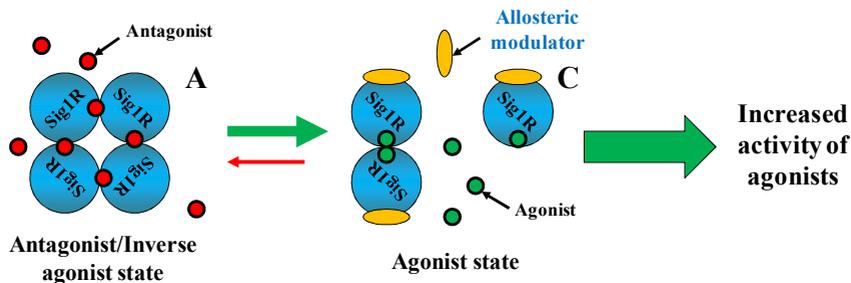


Figure 3.1. **Stabilization of the agonist state of Sig1R by PAMs**

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 3.1. 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. However, ANAVEX™ 2-73 is not only a Sig1R agonist but also M<sub>1</sub> muscarinic acetylcholine receptor agonist and an M<sub>2</sub>/M<sub>3</sub> receptor antagonist. ANAVEX™ 2-73 administered i.p. at doses of 0.3–1 mg/kg reversed alternation deficits and PA 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.

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<sub>1</sub> 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.

## 4. 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 profile of E1R may be of particular relevance to the search for and development of new therapies for the treatment of cognitive disorders, including those that are associated with neurodegenerative diseases, and epilepsy.

## 5. APPROBATION OF THE STUDY – PUBLICATIONS AND THESIS

Doctoral thesis is based on following SCI publications:

1. Veinberg G, Vorona M, Zvejniece L, Vilskersts R, Vavers E, Liepinsh E, Kazoka H, Belyakov S, Mishnev A, Kuznecovs J, Vikainis S, Orlova N, Lebedev A, Ponomaryov Y, Dambrova M, 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, *Bioorganic and Medicinal Chemistry*, **2013**, 21(10): 2764–71, **(Publication I)**.
2. Zvejniece L, Vavers E, Svalbe B, Vilskersts R, Domracheva I, Vorona M, Veinberg G, Misane I, Stonans I, Kalvinsh I, Dambrova M. The cognition-enhancing activity of E1R, a novel positive allosteric modulator of sigma-1 receptors. *British Journal of Pharmacology*, **2014**, 171(3): 761–771, **(Publication II)**.
3. Veinberg G, Vavers E, Orlova N, Kuznecovs J, Domracheva I, Vorona M, Zvejniece L, Dambrova M. Stereochemistry and its methyl derivative: improvement of the pharmacological profile. *Chemistry of Heterocyclic Compounds*, **2015**; 51: 601–606, **(Publication III)**.
4. Vavers E, Svalbe B, Lauberte L, Stonans I, Misane I, Dambrova M, Zvejniece L. The activity of selective sigma-1 receptor ligands in seizure models in vivo. *Behavioural Brain Research* **2017**; 328: 13–18, **(Publication IV)**.

Patent:

Zvejniece L, Dambrova M, Svalbe B, Vavers E, Kalvins I, Veinbergs G, Stonans I, Misane I. Use of 2-(5S-methyl-2-oxo-4R-phenyl-pyrrolidin-1-yl)-

acetamide in the treatment of seizures. **2017** (International filing date: 02.08.2016; Priority data: 03.08.2015); WO2017021881 (A1).

Results are reported in the following international conferences:

1. Vavers E, Svalbe B, Lauberte L, Veinberg G, Dambrova M, Zvejniece L. Sigma-1 receptor ligand activities in chemoconvulsant-induced seizure models in mice. *European Symposium: Physiopathology of sigma-1 receptors*, Barcelona, Spain, May 29–30, **2017**, Book of Abstracts, P.14.
2. Zvejniece L, Vavers E, Svalbe B, Veinberg G, Dambrova M. Allosteric modulators of Sigma-1 receptor. *European Symposium: Physiopathology of sigma-1 receptors*, Barcelona, Spain, May 29–30, **2017**, Book of Abstracts, P.11.
3. Vavers E, Zvejniece L, Svalbe B, Domracheva I, Vilskersts R, Makrecka-Kuka M, Veinberg G, Dambrova M. Allosteric Sigma-1 receptor modulators: a novel approach to treat memory disorders. *FEBS/IUBMB "Molecular basis of human diseases: 50 years anniversary of Spetses summer schools"*, Spetses island, Greece, May 27–June 1, **2016**, Book of Abstracts, P.72.
4. Vavers E, Zvejniece L, Dambrova M. Ligands of sigma-1 receptor: agonists, antagonists, allosteric modulators. *Drug Discovery conference*, Riga, Latvia, August 27–29, **2015**. Book of Abstracts, P.63.
5. Vavers E, Zvejniece L, Veinberg G, Svalbe B, Domracheva I, Vilskersts R, Dambrova M. Stereoselective pharmacological activity of 4,5-disubstituted piracetam derivatives, positive allosteric modulators of sigma-1 receptor. *Drug Discovery conference*, Riga, Latvia, August 27–29, **2015**. Book of Abstracts, P.156.
6. Vavers E, Zvejniece L, Veinberg G, Svalbe B, Domracheva I, Vilskersts R, Dambrova M. Novel positive allosteric modulators of sigma-1 receptor.

- European Society for Neurochemistry's Conference, "Molecular Mechanisms of Regulation in the Nervous System"*. Tartu, Estonia, June 14–17, **2015**. SpringerPlus 2015; 4 (Suppl 1):P51.
7. Zvejniece L, Vavers E, Svalbe B, Domracheva I, Vorona M, Veinberg G, Kalvinsh I, Dambrova M. Allosteric modulator of Sigma-1 receptors enhance cognition and memory. *The 17th World Congress of Basic and Clinical Pharmacology (WCP2014)*, Cape Town, South Africa, 13–18 July, **2014**. Basic & Clinical Pharmacology & Toxicology, 115 (Suppl. 1), p.177.
  8. Vavers E. Ligands of sigma-1 receptor: agonists, antagonists, allosteric modulators. *InnovaBalt, pre-Bos Roundtable, New therapeutic targets: Chemistry and Biology*, Malpils, Latvija, July 3–5, **2014**.
  9. Dambrova M, Vavers E, Zvejniece L. The cognition-enhancing activity of E1R, a novel positive allosteric modulator of sigma-1 receptors. *The 2nd GlowBrain project Workshop, Application of biomaterials and in vivo imaging in stem cell research*, Zagreb, Croatia, March 27–29, **2014**. Book of Abstracts, p. 43.
  10. Zvejniece L, Vilskersts R, Svalbe B, Vavers E, Misane I, Stonans I, Veinbergs G, Dambrova M. Stereoselective pharmacological activity of novel 4,5-disubstituted piracetam derivatives, positive allosteric modulators of sigma 1 receptor. *The 11th International Conference On Alzheimer's and Parkinson's Diseases, Mechanisms, clinical strategies and promising treatments of neurodegenerative diseases*, Florence, Italy, March 6–10, **2013**.
  11. Vorona M, Orlova N, Kuznecovs J, Veinberg G, Vavers E, Zvejniece L, Dambrova M. The Preparation and Biological Investigation of Stereoisomeric 2-(5-methyl-4-phenyl-2-oxopyrrolidin-1-yl)-acetamides.

Results are reported in the following local conferences:

1. Zvejniece L, Vāvers E. Sigma-1 receptors kā jauns zāļu mērķis Alcheimera saslimšanas ārstēšanai. *Ķīmijas, bioloģijas un medicīnas zinātņu nodaļas sēde "Novecošana: ar to saistītās slimības, problēmas, risinājumi"*, Rīga, Latvija, 15. jūnijs, **2017**.
2. Vāvers E. Sigma-1 receptora aktivitātes modulēšana: jauna pieeja atmiņas procesu uzlabošanai. *Ķīmijas, bioloģijas un medicīnas zinātņu nodaļas sēde "Farmācijas nozares jauno zinātnieku preklīniskie pētījumi: no dabas vielām līdz jaunām zālēm"*, Rīga, Latvija, 6. marts, **2015**.
3. Vāvers E, Zvejniece L, Švalbe B, Domračeva I, Vilšķērsts R, Dambrova M. Sigma-1 receptoru pozitīva allostēriska modulēšana uzlabo kognitīvo funkciju. *RSU 2014. gada zinātniskā konference*, Latvijas iedzīvotāju veselību apdraudošo eksogēno un endogēno faktoru izpēte, Rīga, Latvija, 10.–11. aprīlis, **2014**, Tēzes, 89.lpp.
4. Vāvers E, Zvejniece L, Ķīsis V, Švalbe B, Vilšķērsts R, Dambrova M. Jauns sigma-1 receptoru pozitīvs allostērisks modulators ar atmiņu uzlabojošu darbību. *Rīgas Stradiņa universitātes 2013. gada zinātniskā konference*, Latvijas iedzīvotāju veselību apdraudošo eksogēno un endogēno faktoru izpēte, Rīga, Latvija, 21.–22. marts, **2013**, Tēzes, 117. lpp.
5. Švalbe B, Zvejniece L, Vāvers E, Ķīsis V, Muceniece R, Dambrova M. Sigma-1 receptori kā jauns neiroprotektīvu zāļu mērķis. *Latvijas Universitātes 71. zinātniskā konference*, Medicīnas un bāzes zinātnes sekcija, Rīga, Latvija, 15. februāris, **2013**, Tēžu apkopojums, 24. lpp.

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