Univerzita Karlova v Praze

2. lékařská fakulta

Studijní program: Neurovědy

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Biologický význam neuroaktivních steroidů v animálních modelech onemocnění mozku

Biological significance of neuroactive steroids in animal models of brain diseases

Dizertační práce

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Praha, 2015

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Identifikační záznam:

RAMBOUSEK, Lukáš. *Biologický význam neuroaktivních steroidů v animálních modelech onemocnění mozku.* [*Biological significance of neuroactive steroids in animal models of brain diseases*]. Praha, 2015. 97 s. Disertační práce (Ph.D.). Univerzita Karlova v Praze, 2. lékařská fakulta; Fyziologický ústav AV ČR, v.v.i., 2015. Školitel: RNDr., Karel Valeš, PhD.

ACKNOWLEDGEMENTS

I would like to gratefully thank to all the people that contributed to creating this thesis. First of all, I would like to thank my supervisor Dr. Karel Valeš from the Institute of Physiology CAS for his thorough guidance and for creating mutually supportive environment and to Prof. Jean-Marc Fritschy and Dr. Irene Knüsel from the University of Zürich, Switzerland for their supervision during my internship at University of Zürich.

Great thanks belong to co-workers from the Institute of Organic Chemistry and Biochemistry, Dr. Hana Chodounská, Dr. Eva Kudová who conducted large amount of research on the synthesis and synthesized the tested drug. I would also like to thank Dr. Ladislav Vyklický Jr. and Assoc. Prof. Aleš Stuchlík from the Institute of Physiology CAS for their valuable suggestions and tips concerning this work. Furthermore, I thank to all students and staff from Dr. Stuchlik and Prof. Fritschy laboratories for providing me advice and help throughout my work. Finally, I would like to thank to Dr. Christopher Campos for proof reading of this thesis.

ABSTRACT

Neurosteroids play an important role in the brain physiology and pathophysiology. They control inhibitory and excitatory neurotransmission. The presented thesis aims to investigate the biological significance of neuroactive steroid 3α5β-pregnanolone glutamate (3α5βP-Glu). We investigate the effect of 3α5βP-Glu in naïve rats. Next, we evaluate the effects of 3α5βP-Glu in animal model of schizophrenia and excitotoxic lesion of hippocampus induced by N-methyl-D-aspartate (NMDA). Furthermore, we morphologically characterize the NMDA lesion model.

3α5βP-Glu did not induce significant psychotomimetic side effects such as hyperlocomotion, sensorimotor grating deficit or memory impairment. Next, 3α5βP-Glu showed dose dependent pro-cognitive effects in animal model of schizophrenia; however, it had no effect on hyperlocomotion in this model. 3α5βP-Glu also ameliorated spatial learning deficit of rats induces by NMDA lesion of hippocampi in the Carousel maze and had mild effect on NMDA induced damage of hippocampus when applied before. Additionally, the morphological analysis of hippocampal NMDA lesion revealed overexpression of NMDA receptor NR1 and NR2B and downregulation of $GABA_A$ receptor α5 subunits. The lesion was very conservative, did not spread to other structures and did not affect GABAergic interneurons. Furthermore, the lesion progression was accompanied with severe activation of microglia and astrogliosis.

Taken together, this thesis shows that neuroactive steroid 3α5βP-Glu does not induce psychotomimetic side-effects typical for NMDA channel blockers. Moreover, results show that 3α5βP-Glu may represent a potential neuroprotective and procognitive drug.

Keywords: 3α5β-pregnanolone glutamate, NMDA lesion, animal model of schizophrenia, $GABA_A$ receptor, NMDA receptor, neuroprotection.

ABSTRAKT

Neurosteroidy jsou významnými regulátory ve fyziologii a patofyziologii mozku, které řídí inhibici a excitaci nervového přenosu. Cílem předkládané práce bylo zhodnotit biologický význam neuroaktivního steroidu 3α5β-pregnanolonu glutamátu (3α5βP-Glu). Jeho účinek byl testován u intaktních zvířat a také v animálních modelech schizofrenie a hippokampální léze indukované N-methyl-D-aspartátem (NMDA). Navíc byla provedena morfologická charakterizace modelu NMDA léze.

3α5βP-Glu nevyvolal u intaktních zvířat významné psychotomimetické vedlejší účinky charakterizované zvýšenou lokomoční aktivitou, poruchami senzorickomotorických funkcí a paměti. 3α5βP-Glu zmírnil poruchu učení v animálním modelu schizofrenie, ale neměl vliv na zvýšenou lokomoční aktivitu. 3α5βP-Glu také zvrátil poruchu učení v prostorové úloze „Carousel maze“ v modelu excitotoxické léze hippokampu. Pokud byl aplikován před lézí, měl také mírný neuroprotektivní účinek na morfologické poškození hippokampu. Morfologická analýza použitého modelu, NMDA léze hippokampu, odhalila zvýšenou expresi NR1 a NR2 podjednotek NMDA receptoru a sníženou expresi α5 podjednotky $GABA_A$ receptoru. Léze se nerozšířila do dalších struktur a neměla vliv na GABAergní interneurony. Rozvoj poškození vyvolaného lézí byl doprovázen silnou aktivací mikroglie a astrogliózou.

Výsledky této práce prokázaly, že neuroaktivní steorid 3α5βP-Glu nemá psychotomimetické účinky typické pro blokátory NMDA receptoru a představuje potenciální neuroprotektivní a prokognitivní látku.

Klíčová slova: 3α5β-pregnanolon glutamát, NMDA léze, animální model schizofrenie, $GABA_A$ receptor, NMDA receptor, neuroprotekce.

LIST OF SYMBOLS AND ABBREVIATIONS

β-CD	β-Cyclodextrine
17βHSD	17-hydroxysteroid dehydrogenase
3α5βP-Glu	3α5βP-pregnanolone glutamate
3α5βP-HS	3α5βP-pregnanolone hemisuccinate
3α5βP-S	3α5βP-pregnanolone sulfate
3αHOR	3α-hydroxy steroid oxidoreducatese
ACSF	artificial cerebrospinal fluid
AD	Alzheimer's disease
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	analysis of variance
AP	anteroposterior
Asn	asparagine
AU	arbitrary unit
BBB	blood-brain barrier
BDNF	brain-derived neurotrophic factor
C	carbon
CA	c*ornu ammonis*
cAMP	cyclic adenosine monophosphate
c_{max}	maximum (or peak) concentration
Contra	contralateral
CoA	coenzyme A
CNS	central nervous system
DAB	3,3-diaminobenzidine
DG	dentate gyrus
dH_2O	distilled water
DOC	11-deoxycorticosterone
Dpi	days post injection
DHEA	dehydroepiandrosterone
DHEAS	dehydroepiandrosterone sulfate
DHP	dihydroprogestrone
ER	receptor for estrogen
FDA	Food and drug administration

FJB	Fluoro-Jade B
GABA	γ-aminobutiric acid
GABAR	receptor for γ-aminobutiric acid
GFAP	glial fibrillary acidic protein
Gln	glutamine
Glu	glutamic acid
HSD	hydroxy steroid dehydrogenase
i.p.	intraperitoneal
i.v.	intravenous
IC_{50}	half maximal inhibitory concentration
Ipsi	ipsilateral
IR	immunoreactivity
k_e	elimination rate constant
LED	light-emitting diode
MK-801	dizocilpine
ML	mediolaterar
mRNA	messenger ribonucleic acid
NGS	normal goat serum
NMDA	N-methyl-D-aspartate
NMDAR	receptor for N-methyl-D-aspartate
NMR	nuclear magnetic resonance
NO	nitric oxide
NPY	neuropeptide Y
NS	not significant
OATP	organic anion transport protein
OD	optical density
p.o.	per os
P450	cytochrome P450
P450c17	17α hydroxylase
P450c11β	11β hydroxylase
PAT	pathologically activated therapeutic
PBS	phosphate buffered solution
PCP	phencyclidine
PFA	paraformaldehyde

pH	negative log of the activity of the hydrogen ion in an aqueous solution
pKa	acid dissociation constant
pp	prepulse
PPI	prepulse inhibition
PREG	pregnanolone
PREGS	pregnanolone sulfate
PROG	progesterone
PS	pregnenolone sulfate
RNA	ribonucleic acid
SEM	standard error mean
SRM	selected reaction monitoring
StAR	steroidogenic acute regulatory protein
$t_{1/2}$	half-life
THDOC	tetrahydrodeoxycorticosterone
THP	tetrahydroprogestrone
Thr	threonine
TM	trans-membrane
t_{max}	the amount of time that a drug is present at the maximum concentration
TRPM3	transient receptor potential melastatin 3
TSPO	translocator protein
Tyr	tyrosine

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1. INTRODUCTION

1.1. Neurosteroids

Steroids synthetized *de novo* in the brain and nervous system are termed neurosteroids, steroids that are not synthetized in classical steroidogenic tissues such as gonads, adrenals and placentae. The idea that brain could synthetize steroids originated in 1981 from experiments of French endocrinologist and biochemist Étienne-Émile Baulieu (Corpechot C. et al., 1981, Baulieu E.E., 1997). His group found high concentrations of dehydroepiandrosterone sulfate (DHEAS) in the brain compare to plasma and discovered that its concentration is dependent neither on adrenal secretion nor stress stimuli (Corpechot C. et al., 1981). Neurosteroids are endogenous regulators of brain excitability. They modulate glutamatergic and γ-aminobutiric acid (GABA) receptors as well as other neurotransmitters receptors. The most studied neurosteroids are dehydroepiandrosterone (DHEA), DHEAS, pregnanolone (PREG), pregnanolone sulfate (PREGS), pregnenolone, pregnenolone sulfate (PS) and allopregnanolone. Neurosteroids can be categorized based on their structure to pregnane (e.g. PREG, allopregnanolone, tetrahydrodeoxycorticosterone, dihydrodeoxycorticosterone) and androstane (e.g. androsterone) neurosteroids. Based on their effect on neuron excitability they can be divided into inhibitory and excitatory neurosteroids. However, many neurosteroids interact with both excitatory and inhibitory receptors for neurotransmitters (Agis-Balboa R.C. et al., 2006).

1.1.1. Structure

Neurosteroids are lipophilic compounds containing one cyclopentane and three cyclohexane rings. Three cyclohexane (A, B and C) rings each have the chair conformation and a cyclopentane (D) ring. A/B, B/C and C/D rings are linked together and thus ring flipping is not possible. Each center can be either cis-fused or trans-fused. Natural and synthetic steroids may have either *cis* or *trans* fused A/B rings. Whether the A/B ring is fused *cis* or *trans* may have essential effect in their bioactivity (e.g. neurosteroids). Other rings fusions (B/C and C/D) are usually *trans* with exception of cardiac glycosides. Substituents or hydrogens which are below the plane of the rings are designated as α position (*trans*) and those which are above the plane of the rings are designated as β position (*cis*) (Graff M.M., 1960). The methyl group at C-10 has been selected as a reference point. The numbering and stereochemistry is shown in Fig. 1 and 2.

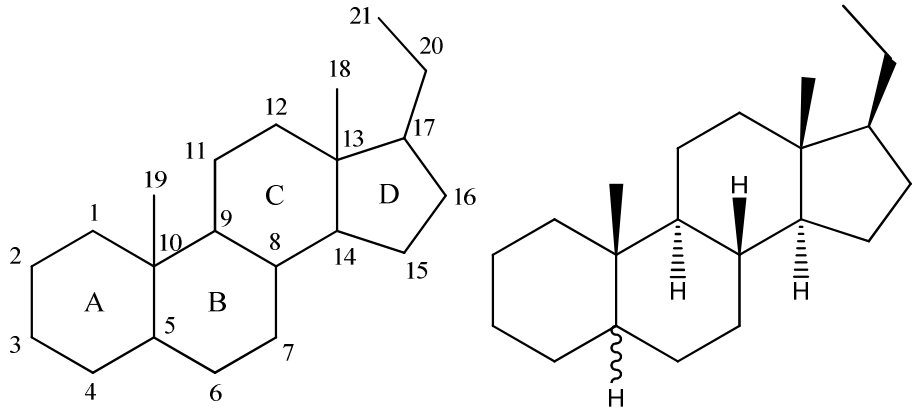


Figure 1: The numbering of carbon atoms and rings labeling are shown on an example pregnane.

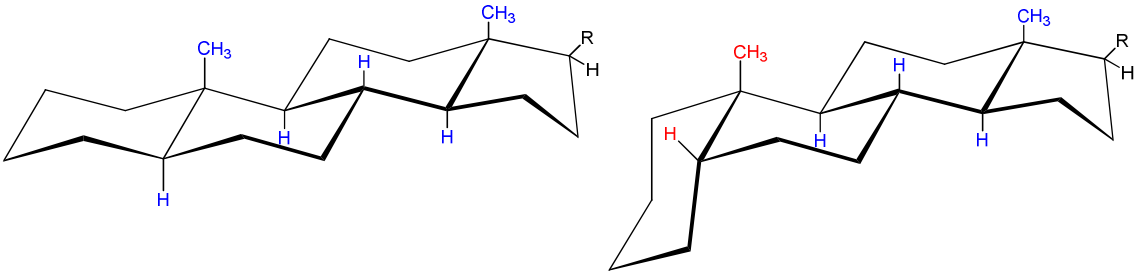


Figure 2: A/B *trans* steroid (5α), each chair is fused to the other by equatorial bonds leaving the angular hydrogens axial (on the left). A/B *cis* (5β) steroid has one bond equatorial and one axial (on the right).

1.1.2. Biosynthesis and metabolism

Neurosteroids are produced by oligodendrocytes, astrocytes as well as principal neurons (Jung-Testas I. et al., 1999; Hu Z.Y. et al., 1987; Tsutsui K. et al., 2000). These cell types express two main groups of enzymes involved in steroidogenesis: cytochrome P450 and non P450 group (Miller W.L., 1988; Nebert D.W. and Gonzalez F.J., 1987). The most important enzymes are summarized in table 1.

Table 1: Summary of enzymes involved in neurosteroid genesis and their abbreviation.

P450		**non P450**	
Cholesterol side-chain cleavage enzyme	P450scc	3β-hydroxy steroid dehydrogenase	3β-HSD
17α hydroxylase	P450c17	3α-hydroxy steroid oxidoreducatese	3α-HOR
21 hydroxylase	P450c21	17β-hydroxy steroid oxidoreducatese	17β-HOR
11β hydroxylase	P450c11β	11β-hydroxy steroid oxidoreducatese	11β-HOR
Aldosterone synthase	P450AS	Steroid sulfotransferase	STS
Aromatase	P450aro	5β -reductase	
7α hydroxylase	P450c7B	5α-reductase	

All steroids are produced from cholesterol (Do Rego J.L. et al, 2009). The first rate-limiting step in neurosteroid synthesis is conversion of cholesterol to pregnenolone (Zwain I.H. and Yen S.S.C., 1999). Cholesterol from mitochondrial membrane is cleaved by cholesterol side-chain cleavage enzyme P450scc (Patte-Mensah C. et al., 2003). The main precursor for neurosteroids, PREG is than converted to progesterone (PROG) by the enzyme 3β-hydroxysteroid dehydrogenase. PREG is further converted by P450c17 to DHEA. PROG is reduced by 5α reductase to 5α-dihydroprogestrone and further converted by 3αHOR to tetrahydroprogestrone (THP). There are α and β subtypes of enzymes 5-reductase and 3-hydroxysteroid oxidoreductase resulting in four products: allopregnanolone (THP-3α5α), isoallopregnanolone (THP-3β5α), isopregnanolone (THP-3β5β) and pregnanolone (THP-3α5β) (Compagnone N.A. and Mellon S.H., 2000; Kancheva R. et al., 2007).

PROG is also converted by 21 hydroxylating enzyme to 11-deoxycorticosterone (DOC). DOC is further reduced by either 5α reductase to 5α-dihydroDOC or by P450c11β to corticosterone. 5α-dihydroDOC is metabolized to two important neurosteroids, allotetrahydroDOC by 3αHOR and to tetrahydroDOC by 3αHSD (Stoffel-Wagner B., 2001).

Neurosteroids containing -OH group at the C-3 position such as DHEA, PREG, allopregnanolone and PREG are sulfated by the enzyme steroid sulfatase into conjugated sulfate esters. This step is very important since free and sulfated forms have different biological activity on receptors for neurotransmitters (Hobkirk R., 1985). The biosynthesis of neurosteroids from the precursor molecule cholesterol is schematically shown in the Fig. 3. The biosynthesis of neurosteroids and particular role of enzymes in both rodents and human is reviewed in the book “Neurosteroids, A new regulatory function in the nervous system” edited by Baulieu (Baulieu E.E. et al., 1999).

In the last decade, research has focused on anatomical and subcellular localization of steroidogenesis related enzymes as well as into regulation of this process by neurotransmitters and neuropeptides. For example, mRNAs for 5α-reductase and 3αHSD, important enzymes mediating neurosteroid biosynthesis, were found in principal excitatory neurons of cortex and hippocampus but were missing in GABAergic interneurons and glial cells in these areas. On the other hand, their mRNA levels were abundant in GABAergic medium spiny neurons of striatum and Purkinje neurons of cerebellum (Agis-Balboa R.C. et al., 2006). Additionally, it seems that biosynthesis of neurosteroids is regulated by neurotransmitters (Barbaccia M.L. et al., 1996; Guarneri P., 1998) as well as by peptide hormones produced by hypophysis and epiphysis (Liu T. et al., 2007).

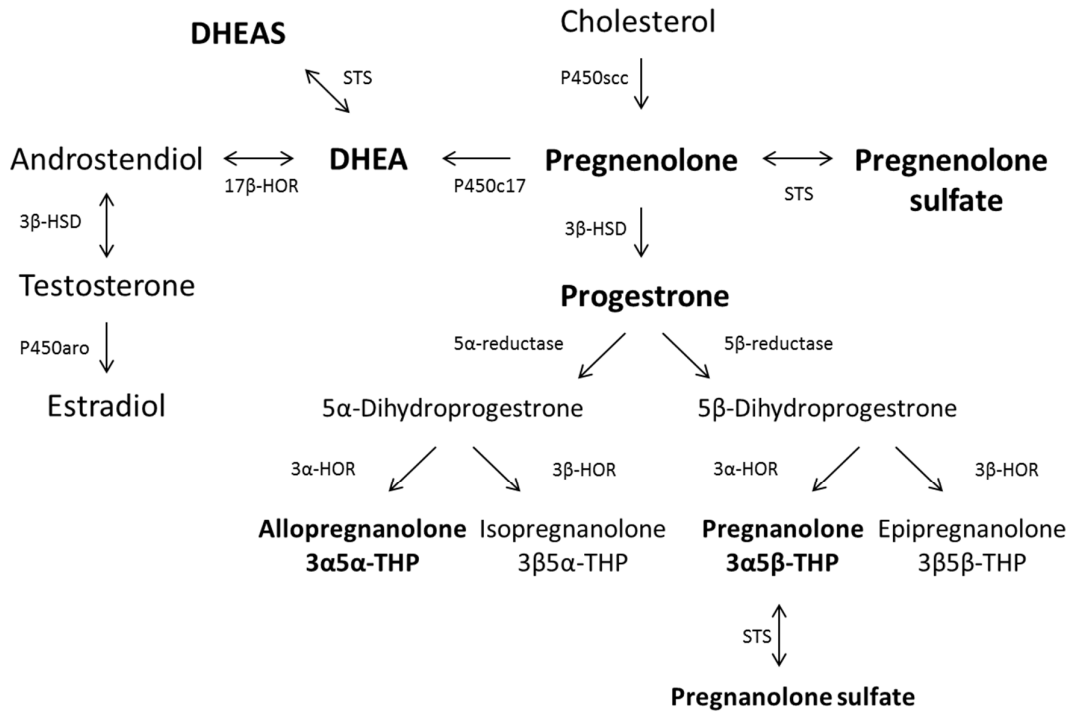


Figure 3: Schematic representation of selected neurosteroids biosynthesis from cholesterol in the brain. Biosynthesis of pregnenolone, progesterone, dehydroepiandrosterone as well as pregnanolone and its isomers is shown. Abbreviations are: dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulfate (DHEAS), tetrahydroprogestrone (THP), steroid sulfotransferase (STS), cholesterol side-chain cleavage enzyme (P450scc), hydroxy steroid oxidoreducatese (HOR), 3β-hydroxy steroid dehydrogenase (3β-HSD), aromatase (P450aro), 17α hydroxylase (P450c17).

1.1.3. Neurosteroids mechanism of action

Steroids such as PROG, testosterone or estradiol modulate nuclear steroid receptors and exert their effects via slow genomic mechanism. They bind to their intracellular receptors and regulate protein synthesis. These mechanisms include receptors for androgens, mineralocorticoids, glucocorticoids, estrogens and progesterones (McEwen B.S., 1991). The schematic representation of targets modulated by neurosteroids is shown in Fig. 4.

In 1941, Hans Hugo Bruno Selye discovered that progesterone metabolites have fast sedative and anesthetic effects on central nervous system (CNS) in rodents (Selye H., 1941). These effects were too fast to be accounted for by the genomic mechanism of action, indicating that they modulate receptors for neurotransmitters. Neurosteroids modulate directly a variety of transmembrane receptors for neurotransmitters including ionotropic glutamate, $GABA_A$, glycine and σ receptors and provide a fast non-genomic action (Rupprecht R., 2003; Baulieu E.E. and Robel P., 1995). Similarly to sex hormones, some neurosteroids can also modulate nuclear receptors. For example, allopregnanolone and pregnanolone bind to the nuclear pregnane X receptor at micromolar levels (Lamba V. et al., 2004).

Important structure-activity relationship factors include the geometry between A and B ring, the charge at C3 position and hydrogen bond acceptor at C20 position. Another important structural factor is lipophilicity of the molecule as well as flexible bond at C17 position (Purdy R. et al., 1990; Zorumski C. et al., 2000; Korinek M. et al., 2000).

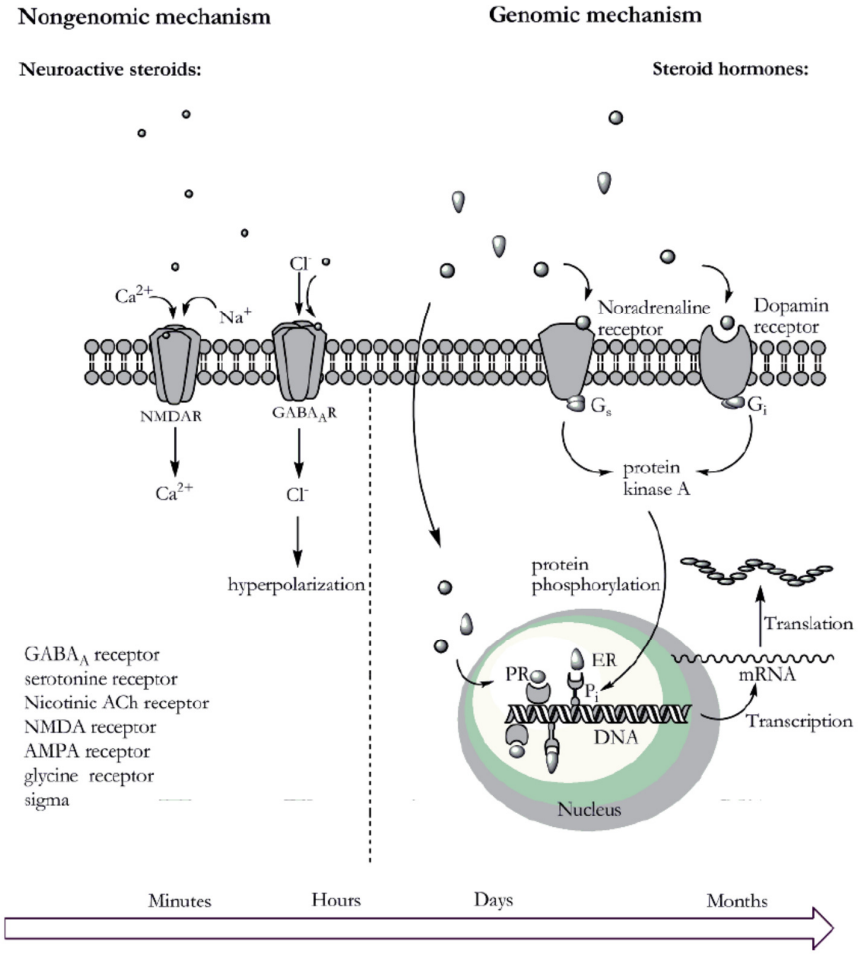


Figure 4: Schematic representation of genomic and non-genomic targets modulated by steroids. Neurosteroids such as pregnanolone sulfate or allopregnanolone can modulate receptors for neurotransmitters (e.g. NMDA and $GABA_A$ receptors) and mediate fast action within milliseconds to seconds. On the other hand, sex hormone progesterone penetrates into the cell and binds to receptor for progesterone. This leads to activation of mRNA transcription and consequent protein synthesis. Estrogen receptor can be also modulated by protein kinase A, which is activated by stimulation of transmembrane receptors for noradrenaline and dopamine. (Modified from Wang et al., 2008)

1.1.3.1. NMDA receptor

N-methyl D-aspartate (NMDA) receptors are glutamate-gated cation channels that are blocked by Mg^{2+} ions at negative membrane potential. After the activation of NMDA receptor, Na^+ and Ca^{2+} ions are transported into the cell through the channel pore. For the activation of NMDA receptor, two glutamate and two glycine molecules are required (Dingledine R. et al.,

1999). The ion channel is composed of four subunits. Endogenous NMDA receptor consists of two GluN1 and either two GluN2 or GluN3. The NR1 subunit is necessary for the assembly of functional NMDA receptors (Zukin R. and Bennett M., 1995). Furthermore, the receptor can have many isoforms (GluN1 has 8 different splice variants, GluN2A, B, C, D; NR3A, B) with diverse brain localization and function (Sheng M. and Kim M.J., 2002). NMDA receptor subunits all utilize a common topology characterized by a large extracellular N-terminus, a membrane region made of three trans-membrane segments (TM1, 3 and 4) plus a re-entrant pore loop (M2), an extracellular loop between TM3 and TM4 and a cytoplasmic C-terminus, which varies in size depending on the subunit and provides multiple sites of interaction with numerous intracellular proteins (Furukawa H. et al., 2005). They are expressed nearly at all neurons as well as glia cells. At neurons they are expressed both at synapse and extrasynaptically (Dingledine R. et al., 1999). NMDA receptors are important in brain development, generation of rhythms, neural plasticity, memory, learning and neuroprotection (Paoletti P. and Neyton J., 2007; Cull-Candy S. et al., 2001).

NMDA receptor can be pharmacologically modulated by number of agonists as well as antagonists. Compounds can act on binding site for Mg+ (channel blockers such as ketamine and dizocilpine), glutamate, glycine or at allosteric sites (Paoletti P. and Neyton J., 2007). However, binding sites for neurosteroids have yet to be identified. The current work of Vyklicky Jr. suggests that inhibitory neuroactive steroids access the NMDA receptor from cell membrane through lateral membrane diffusion (Borovska J. et al., 2012). They observed slower kinetics of inhibition onset and offset that is not typical for drug-receptor interaction in aqueous solution. Furthermore, they found the IC_{50} assessed for a set of synthetic C3 pregnanolone derivatives positively correlated with their lipophilicity. Another group showed binding of PS and PREGS to the S1S2 domain of NMDAR GluN2B subunit and suggested that steroids that bind to S1S2 domain are responsible for glutamatergic receptors potentiation whereas steroids binding to amino terminal domains are responsible for their inhibition (Cameron K. et al., 2012).

Neurosteroids can modulate NMDA receptors by different manners. They can either potentiate or inhibit NMDA receptor responses. PS was the first neurosteroid discovered to allosterically potentiate NMDA receptors (Wu F. et al., 1991; Park-Chung M. et al., 1997). It seems that negative charge on C3 and unsaturated bond between C5 and C6 are crucial structural properties responsible for positive modulation. A number of synthetic analogues of PS including pregnenolone hemisuccinate and hemiglutarate were shown to potentiate NMDA receptors as well (Weaver C. et al., 2000). Interestingly, experiments on recombinant

NMDA receptors revealed that the action of PS on NMDA receptor is dependent on its subunit composition. PS preferably potentiate responses of receptors containing NR1/NR2A and NR1/NR2B while it inhibits the responses of receptors containing NR1/NR2C and NR1/NR2D. In addition, the pretreatment with PS is more effective in receptor modulation than if it is co-applied with agonist (Horak M. et al., 2004). The authors explain this finding by allosteric coupling of agonist and PS binding sites, resulting in a decreased affinity to PS after activation with agonist.

PREGS is potent inhibitor of NMDA receptor responses. This compound is almost identical to PS; however, it does not have the double bound between C5 and C6. This makes the molecule planar and gives it completely different pharmacological properties. PS is use-dependent, non-competitive inhibitor of NMDA receptors. The steroid is unable to bind to the receptor without presence of glutamate (Petrovic M. et al., 2005). Contrary to classical channel blockers such as dizocilpine (MK-801), ketamine or phencyclidine (PCP), which are also use-dependent antagonists, PS shows voltage-dependent blockade. This finding indicates that the binding site for PS is different from the one for channel blockers and is located outside (Sedlacek M. et al., 2008). PS inhibits receptor responses with higher potency if they are composed of NR2C or NR2D than if they are composed of NR2A and NR2B subunits (Petrovic M. et al., 2005).

1.1.3.2. $GABA_A$ receptor

$GABA_A$ receptors mediate inhibitory neurotransmission (Kuffler S. and Edwards C., 1958). After their activation, Cl^- ions are transported into the cell through the channel pore. They belong to Cis-loop superfamily of ligand gated ion channels. It is built of 5 subunits composed of at least 16 isoforms (α1- 6, β1- 3, γ1- 3, δ, ε, π and θ) (Nayeem N. et al., 1994). The particular composition of subunits defines their biophysical and pharmacological properties (Sieghart W. and Sperk G., 2002). Each subunit consists of a long N-terminal extracellular hydrophilic region, followed by four transmembrane α-helices (M) with a large intracellular loop between M3 and M4, and ends with a relatively short extracellular C-terminal domain, and M2 forms the lining of the ion channel. Recombinant receptor studies indicated that two α, two β and a single γ subunit have to co-assemble to form $GABA_AR$ with functional properties resembling endogenous receptors (Sieghart W., 1995). The subunit composition determines their subcellular localization as well as function. For example, $GABA_ARs$ composed of the α1- 3, β1- 3, and γ2 are predominantly expressed synaptically, while $GABA_ARs$ composed of α4- 6, β2- 3 and δ subunits are located extrasynaptically

(Belleli D. et al., 2009). $GABA_AR$ has number of binding sites including 2 sites for endogenous ligand GABA located between α and β subunits, site for benzodiazepines, barbiturates as well as site for neurosteroids (Burt D. and Kamatchi G., 1991).

Harrison and Simmonds in 1984 first reported that steroids act on $GABA_AR$. They found that synthetic alphaxalone, a neuroactive steroid with anesthetic properties, has similar mechanism of action as barbiturates (Harrison N.L. and Simmonds M.A., 1984). Neuroactive steroids exhibit a range of effects from potentiation of GABA responses and direct receptor activation, to inhibition (Beleli D. et al., 2006). Smart and his group from University College London identified two discrete binding sites for neurosteroids mediating the potentiating and direct activation effects (Hosie A. et al., 2006).

Neurosteroids potentiating responses to GABA bind to a cavity formed by the α-subunit transmembrane domains (αGln 241 and αAsn 407), while direct activation of receptor is mediated by interfacial residues between α and β subunits (αThr 236 and βTyr 284) and is enhanced by steroid binding to the potentiation site (Hosie A. et al., 2006). The binding site for inhibitory neurosteroids has not been identified yet. It seems that is different than the potentiating site. The best described candidate so far is the ion channel lining, around the 2^{nd} residue, deep within the $GABA_A$ ion channel (Seljeset S. et al., 2015).

The neurosteroids containing reduced A-ring such as metabolites of testosterone and 3α5α-adiol act as $GABA_AR$ agonists (Frye C. et al., 1996). For non-sulfated neurosteroids, α configuration at C3 is necessary to provide potentiating effect on the $GABA_AR$ (Park-Chung M. et al., 1999). Sulfated and 3β-OH steroids have antagonist action. Next, it has been shown that position at C5 is critical for neurosteroid potency. 5α reduced neurosteroid seem to be more potent than 5β isomers. For example, it has been found that 3α5α-THDOC is much more potent modulator than 3α5β-THDOC (Mennerick S. et al., 2004). Furthermore, PS (Majewska M.D. and Schwartz R.D., 1987) and DHEAS (Majewska M.D. et al, 1990) are potent $GABA_AR$ inhibitors whereas pregnenolone itself has no action on $GABA_AR$ (Ong J. et al., 1987). The effect of most studied neurosteroids on $GABA_A$ and NMDA receptors is summarized in table 2.

Table 2: Summary of the mechanisms of action of the most studied neurosteroids on $GABA_A$ and NMDA receptors. 0 no action, - weak inhibition, -- inhibition or antagonism, + weak potentiation, ++ potentiation. -/+ concentration dependent dual effect.

Neurosteroid	NMDA receptor		$GABA_A$ receptor	
Pregnanolone	**0/-**	Weaver C. et al., 2000	**+**	Park-Chung P. et al., 1999
Pregnanolone sulfate	**--**	Weaver C. et al., 2000	**++**	Park-Chung P. et al., 1999
Pregnenolone	**0**	Weaver C. et al., 2000	**-/+**	Ong J. et al., 1987
Pregnenolone sulfate	**++/-**	Horak M. et al., 2006	**--**	Majewska M.D., 1987
Allopregnanolone	**+**	Weaver C. et al., 2000	**++**	Hosie A.M. et al., 2006
DHEA	**+**	Compagnone N.A. et al., 2000	**-**	Demirgören S. et al., 1991
DHEAS	**+**	Yaghoubi N. et al., 1998	**--**	Majewska M.D. et al, 1986

1.1.3.3. Other receptors

Neurosteroids modulate in addition to NMDA receptors also non-NMDA receptors for glutamate. It has been reported that sulfated steroids PS and PREGS inhibit AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate) receptor via the binding site for glutamate located on GluR2 subunit (Spivak V. et al., 2004; Cameron K. et al., 2012). Moreover, these neurosteroids inhibited kainite induced currents as well (Wu F. et al., 1991, Park-Chung M. et al., 1997). 17β-Estradiol influences indirectly also metabotropic glutamate receptors I and II via their interaction with membrane localized receptors for estradiol in hippocampus (Boulware M. et al., 2013).

It has been shown that allopregnanolone and THDOC allosterically potentiate also $GABA_C$ receptor (Morris K.D. et al., 1999). Importantly, neurosteroids modulate also glycine receptors. PROG and PREG (Weir C.J. et al., 2004; Wu F.S. et al., 1990; Maksay G. et al., 2001) inhibit glycine channels activation whereas allopregnanolone enhances glycine induced currents (Jiang P. et al., 2006). Another interesting target for neurosteroids is an intracellular σ receptor family. For example, PS, DHEA and DHEAS stimulate σ1 receptor (Su T. et al., 1988). The function of these metabotropic receptors is not fully understood. However, these receptors transduce signals *via* other receptors such as ion channels. DHEA can induce NMDA induced excitation through this receptor (Bergon R. et al., 1996). Another example, PS can increase NMDA and AMPA receptors activation by σ1 receptor activation (Chen L. et al., 2005; Schiess A.R. and Partridge L.D., 2005).

1.1.4. Physiological significance

Naturally occurring neurosteroids regulate many functions of the central and peripheral nervous system ranging from the development to complex behavior. Brain is capable to synthetize neurosteroids already during the embryogenesis. During neonatal development, they regulate dendritic growth, spine generation and synaptogenesis in cerebellum (Sasahara K. et al., 2007). It has been shown that administration of estradiol promotes dendrite growth and spine formation in Purkinje cells of cerebellum (Sakamoto H. et al., 2003). Additionally, PREG induces microtubule assembly (Marukami K. et al., 2000). PS was found to enhance expression of neuronal cell adhesion molecules and stimulated proliferation in hippocampus (Mayo W. et al., 2005). It seems that these effects on hippocampus plasticity are mediated via $GABA_A$ receptors expressed on neuroblasts. The effect of neurosteroids during the development is mediated not only *via* receptors for neurotransmitters but also *via* nuclear receptors (ERα and β) as well as other targets. Recently, transient receptor potential melastatin 3 (TRPM3) channel was discovered to be modulated PS. Activation of TRPM3 by PS potentiates spontaneous glutamate release onto neonatal Purkinje cells during a period of active glutamatergic synapse formation (Zamudio-Bulcock P.A. et al., 2011).

Neurosteroids also modulate stress, aggression and anxiety behavior. It has been shown that levels of progesterone, PREG and allopregnanolone are increased after stress stimuli (Barbaccia M.L. et al., 1998). Several studies also reported anxiolytic action of allopregnanolone and 3α5α-THDOC (Bitran D. et al., 1997).

Finally, neurosteroids modulate also learning and memory functions (Vallée M. et al., 2001a). Studies in animals demonstrated that the neurosteroids PREGS and DHEAS display memory-enhancing properties in aged rodents. Moreover, it was recently shown that memory performance was correlated with PREGS levels in the hippocampus, the key brain area for memory functions, of 24-month-old rats (Vallée M. et al., 2001b). Interestingly, DHEA and DHEAS levels decrease markedly with age in humans, and levels in elderly populations are reduced (Labrie F. et al., 1997; Orentreich N. et al., 1992)

1.1.5. Pathophysiological significance

The synthesis of neuroprotective neurosteroids increases after brain injury, neurodegeneration and during aging (Sierra A. et al., 2003). Steroidogenic acute regulatory (StAR) protein levels are elevated after kainic acid or NMDA lesions (Sierra A. et al., 2003) as well as during neurodegeneration (Lavaque E. et al., 2006) and aging in rats (Kimoto T. et al., 2001; Sierra A. et al., 2003). Other studies showed that the cerebrospinal fluid (Young J. et al., 1996) and

brain levels (Billiards S. et al., 2006) of allopregnanolone changed under hypoxic conditions in sheep. The regional levels of neurosteroids are affected also in neurodegenerative diseases. Decrease in neurosteroid levels was found in Alzheimer's disease (AD) (Marx C.E. et al., 2006), Parkinson's disease (di Michele F. et al., 2003) and Niemann-Pick disease type C (Griffin, L.D. et al., 2004). In AD, decrease of allopregnanolone levels in prefrontal cortex (Marx C.E. et al., 2006), increase of DHEA (Brown R.C. et al., 2003) and decrease of DHEAS and PS in the striatum and cerebellum (Weill-Engerer S. et al., 2002; Näsman B. et al., 1991) was reported. Interestingly, women have a higher risk of the development of sporadic AD. This increase in hypothesized to be connected with the postmenopausal loss of estrogens (Pike C.J. et al., 2009). Similarly, age related drop in androgen levels in males is linked to cognitive decline and increased risk of AD (Rosario E.R. et al., 2010).

The neuroprotective action of endogenous neurosteroids is mediated most likely by suppression of inflammatory reaction (He J. et al., 2004), providing neurotrophic support by increasing of brain-derived neurotrophic factor (BDNF) levels (Stein D.G. and Wright D.W., 2010), reducing edema (Roof R.L. et al., 1992), reducing lipid peroxidation and oxidative stress (Roof R.L. et al., 1997), promoting remyelination (De Nicola A.F. et al., 2006) and by preventing excitotoxicity *via* NMDA receptor inhibition and $GABA_A$ receptor potentiation (Weaver C. et al., 1997).

The most prominent neurosteroids involved in endogenous neuroprotection are allopregnanolone and PROG. Allopregnanolone provides protection mainly *via* potentiation of $GABA_A$ receptor. It prevented neuronal death induced by picrotoxin and kainic acid *in vitro* (Ciriza I. et al., 2004; Leśkiewicz M. et al., 1997), promoted remyelination (Ibanez C. et al., 2004) and reduced ischemic damage (Ishrat T. et al., 2010). PROG showed neuroprotective properties in animal models of stroke (Jiang N. et al., 2006), spinal cord injury (Fee D.B. et al., 2007) and cortical injury (Gilmer L. et al., 2008). It has been also shown that PROG helps to maintain blood-brain barrier (BBB) integrity (Si D. et al., 2014) and improves cognitive outcome (Si D. et al., 2013) in an animal model of traumatic brain injury.

Neurosteroids might play important role also in neuropsychiatric disorders such as schizophrenia and depression. Altered levels of DHEA and DHEAS in schizophrenic patients compare to controls were reported (Yanase T. et al., 1996; Morrison M.F. et al., 2000). Interestingly, also antipsychotic drugs regulate the levels of neurosteroids in brain. For example, olanzapine increased allopregnanolone concentrations in rat cerebral cortex (Marx C.E. et al., 2000).

1.2. Neuroactive steroids

Neuroactive steroids are by definition steroidal compounds that mimic action of neurosteroids; however, they are not synthetized in the brain. It includes all synthetic compounds as well as neurosteroids applied systematically. Nevertheless, the nomenclature is not harmonized. Some authors use the term neurosteroid for synthetic steroids as well as for neurosteroids applied systematically.

1.2.1. Therapeutic potential of neuroactive steroids

Neuroactive steroids represent molecules with therapeutically interesting properties. They are allosteric modulators of $GABA_A$ and NMDA receptors, the receptors responsible for the excitation-inhibition balance of the brain. Furthermore, neurosteroids and neuroactive steroids readily cross the blood-brain barrier easily due to their lipophilic structure. Moreover, their structure is susceptible to a variety of synthetic modifications allowing fine tuning of their pharmacological properties. They might be also devoid of psychotomimetic as well as cognitive side effects and they are predicted to have reduced abuse potential (Morrow A.L. et al., 2006). Results from preclinical and clinical studies suggests that neuroactive steroids may be a novel class of drugs for the treatment of epilepsy, pain, depression, anxiety, schizophrenia, sleep disorders, drug dependence and neurodegeneration. The following paragraphs describe preclinical evidence as well as summarize ongoing clinical trials investigating neuroactive steroids efficacy.

Neuroactive steroids have been shown to have anticonvulsant properties in a variety of animal models. Administration of allopregnanolone and PREG had significant effect in animal models of epilepsy induced by application of pentylentetrazol (Frye C.A. et al., 2000), pilocarpine (Sandison C.R., 1977) as well as NMDA (Gasior M. et al., 1997) and kainic acid (Sandison C.R., 1977). Furthermore, neuroactive steroids potentiated anticonvulsive effects of diazepam without inducing sedation or ataxia in mice (Gasior M. et al., 1997). Ganaxolone (synthetic derivative of allopregnanolone) even prevented the development of kindled seizures in rodent models (Reddy D.S. and Rogawski M.A., 2010). Furthermore, allopregnanolone and PREG were efficient in animal models of anxiety and insomnia (Schüle C. et al., 2011 and 2014; Gerorge O. et al., 2006).

The role of neuroactive steroids has been also studied in hippocampal pathology of aging and aging-associated diseases. Estradiol treatment decreased amount of neuroinflammation, neurodegeneration and stimulated progenitor cells proliferation in subgranular zone of hippocampus in aging mice (de Nicola A.F. et al., 2012). Furthermore, allopregnanolone

restores hippocampal-dependent learning and memory and neural progenitor survival in aging mouse transgenic model of Alzheimer's pathology as well as in non-transgenic mice (Singh, C. et al., 2012). This steroid also reversed cognitive deficit (Wang J.M. et al., 2010) and was effective to promote regeneration and reduce β-amyloid burden in animal models of AD (Chen S. et al., 2012).

Neurosteroids were effective also in animal models of affective disorders. Sulfated neurosteroids such as PS and DHEAS have antidepressant effect in both animals and humans (Reddy D.S. et al., 1998; Urani A. et al., 2001; Wolkowitz O.M. et al., 1999). Furthermore, the treatment with antidepressant drugs such as selective serotonin reuptake inhibitors directly modulates activity of neurosteroidogenic enzymes (Griffin L.D. and Mellon S.H., 1999).

A number of independent research institutions, according to a public database of clinical trials (clinicaltrails.gov), are currently recruiting patients to test the efficacy of PREG. For example, the U.S. Department of Veterans Affairs is conducting trails investigating efficacy in the treatment of traumatic brain injury, fatigue, musculoskeletal pain and cognitive decline. Next, the U.S. National Institute on Alcohol Abuse and Alcoholism is testing the hypothesis that pre-treatment with PREG, compared to placebo, will antagonize the acute effects of alcohol on the behavioral measures. PREG is also investigated to treat chronic low back pain and schizophrenia by Durham VA Medical Center (Marx C.E. et al., 2009).

Even though endogenous neurosteroids represent promising therapeutics, they are not in the focus of big pharma companies. Their endogenous origin makes it difficult to license them, which possesses a high risk for the companies. Therefore there is rather a trend to develop new and original compounds exhibiting similar or even better pharmacological properties than neurosteroids.

Some of the synthetic steroids such as ganaxolone, alphaxalone, alphadolone, hydroxydione and minaxolone have entered clinical trials as potential sedatives and anesthetics. However, majority of these drugs were withdrawn from the human development with regard to their toxic and carcinogen effects (Winter L. et al., 2003). Currently, Marinus Pharmaceutical, Inc. is continuing with the development of ganaxolone to modulate overexcited neurons in the brain by targeting mainly $GABA_A$ receptor. Ganaxolone has the same chemical structure as allopregnanolone, with the addition of a methyl group designed to prevent conversion back to an active steroid, thereby eliminating the opportunity for unwanted hormonal effects while preserving its desired CNS activity (http://www.marinuspharma.com/). According to the public database of clinical trials (clinicaltrails.gov), Marinus Pharmaceuticals, Inc. has investigated the effect of ganaxolone in

the treatment of partial-onset seizures, infantile spasms, posttraumatic stress disorder, Fragile X syndrome, as well as investigated ganaxolone as a smoking cessation candidate.

Furthermore, SAGE therapeutics is developing a number of neuroactive steroids to treat brain diseases connected to $GABA_A$ and NMDA receptors dysfunction. Their lead structure, SAGE-447, acts as an allosteric modulator of both synaptic and extra-synaptic $GABA_A$ receptors. This drug is currently in Phase II to treat super-refractory status epilepticus and in Phase I to treat essential tremor and postpartum depression. However, this drug has to be given intravenously. They reported a development of "second generation neurosteroids" (e.g. SAGE-217) that are bioavailable after p.o. administration. Furthermore, the company has a number of neuroactive steroids in the exploratory phase, investigating their therapeutic potential in Rett syndrome, Dravet syndrome, Fragile X syndrome, anxiety, depression, sleep disorders, mania, tremor, tinnitus and post-traumatic stress disorder (http://www.sagerx.com/).

1.2.2. Therapeutic potential of NMDA receptor modulators

A number of NMDA antagonists have been developed as potentially neuroprotective agents. However, majority of them have side effects that may render them unsuitable for clinical use. For example, simple structural derivative of glutamate, D-2-Amino-5-phosphonopentanoic acid, shows neuroprotective properties *in vitro* (Qiu S. et al., 2006) but it does not have therapeutic potential because it does not cross the BBB (Stone T.W. and Addae J.I., 2002). A competitive NMDA antagonist selfotel (CGS-19755) showed some neuroprotective properties *in vivo* and crossed the BBB (Trescher W.H. et al., 1997) but failed to produce significant effect in third phase of clinical trials (Morris G.F. et al., 1999). The non-competitive antagonists of NMDA receptor such as MK-801 and ketamine are strongly neuroprotective and cross the BBB but have been shown to have profound behavioral effects in animals (increased locomotion, circling, head weaving, and ataxia) as well as in humans (Bubenikova-Valesova V. et al., 2008). The adverse effects induced by non-competitive NMDA antagonists occur because of their dwell time in the ion channel is so long and the blockade influences the NMDA receptors in healthy brain areas (Lipton S.A., 2007). It seems that therapeutically acceptable agents for a neuroprotection are antagonists targeting only trauma influenced and thus open NMDA receptors. Memantine, an un-competitive antagonist acts only at tonically activated NMDA receptors with rapid dwell time in ion channel and does not affect receptors in healthy brain areas. The un-competitive antagonists show no serious adverse effects ($\leq$1% of patient suffer for adverse events in case of memantine) and are used in treatment of AD

(Lipton S.A., 2007). The endogenous neurosteroid PS shows use-dependent mechanisms of action on NMDA receptors as memantine (Petrovic M. et al., 2005). In other words, the inhibition effects are more expressed on glutamate-activated receptors. Another strategy to maintain glutamate homeostasis during neurodegenerative processes may represent NMDA antagonists at the glycine site (ACEA 1021, GV150526), polyamine site (eliprodil), redox site (dithiothreitol) and other drugs that may interfere with glutamate release by Na^{2+} channel blockade (Shim S.S. et al., 2007).

1.2.3. Pregnanolone glutamate

3α5β-pregnanolone glutamate (3α5βP-Glu) is a synthetic neuroactive steroid developed and synthesized at the Institute of Organic Chemistry and Biochemistry, Academy of Science of the Czech Republic. The 3α5βP-Glu was prepared by esterification of commercially available 3α-hydroxy-5β-pregnan-20-one with a protected glutamic acid followed by deprotection of the carboxy and amino groups (Kapras V. et al., 2012). The structure is shown in Fig. 5. The 3α5βP-Glu is well soluble in dimethyl sulfoxide, methanol and in water mixtures with methanol or acetonitrile. Because of its poor solubility in water, 3α5βP-Glu was dissolved in water solution of seven sugars ring molecule β-cyclodextrine (β-CD) for *in vivo* experiments. Cyclodextrins are water soluble and contain hydrophobic cavity which may encapsulate various hydrophobic molecules *via* non-covalent interactions such as steroids (Liu F.Y. et al., 1990). They have received considerable attention in pharmaceutical research because of improved water solubility, chemical stability and bioavailability of various drug molecules.

The 3α5βP-Glu is an analogue of endogenous PS. The sulfate group is replaced by the glutamic acid. This drug has been selected for further preclinical development because of its high affinity to NMDA and $GABA_A$ receptors. 3α5βP-Glu is covered by the patent applications WO2012110010-A1 and WO2010136000-A2.

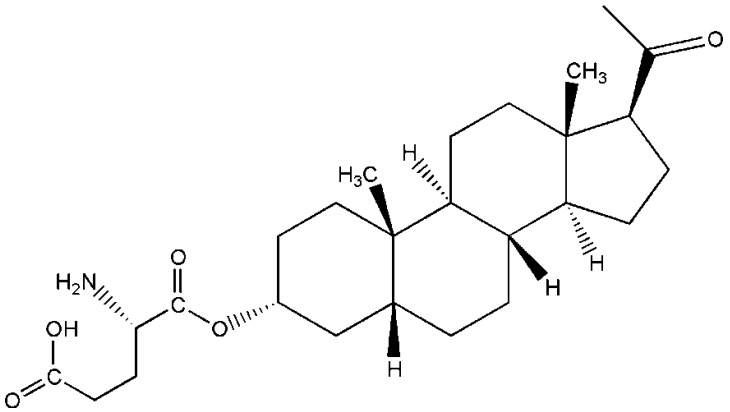


Figure 5: The structure of 3α5β-pregnanolone glutamate (3α5βP-Glu).

1.2.3.1. Pharmacokinetics

3α5βP-Glu is rapidly absorbed after intraperitoneal (i.p.) administration and penetrates through the BBB with a t_{max} of 60 min (Fig. 6). The drug is eliminated by a first-order process and it is not accumulated in the brain or other tissues. Furthermore, 3α5βP-Glu is bioavailable after per os (p.o.) administration (Rambousek L. et al., 2011). Pharmacokinetic properties of an analogous drug pregnanolone hemisuccinate (3α5βP-HS) showed similar good penetration through the blood brain barrier with a t_{max} of 10 min (Weaver C.E. et al., 1997). The slower t_{max} of 3α5βP-Glu may be related to the different pKa value and manner of dissolution. The β-cyclodextrine (FDA approved excipient) has been used for the dissolution of the steroid whereas in the other study dimethyl sulfoxide was used. The β-CD/3α5βP-Glu inclusion complex may interact with lipoproteins in the plasma and blood brain barrier as well and thus influence the final pharmacokinetic properties.

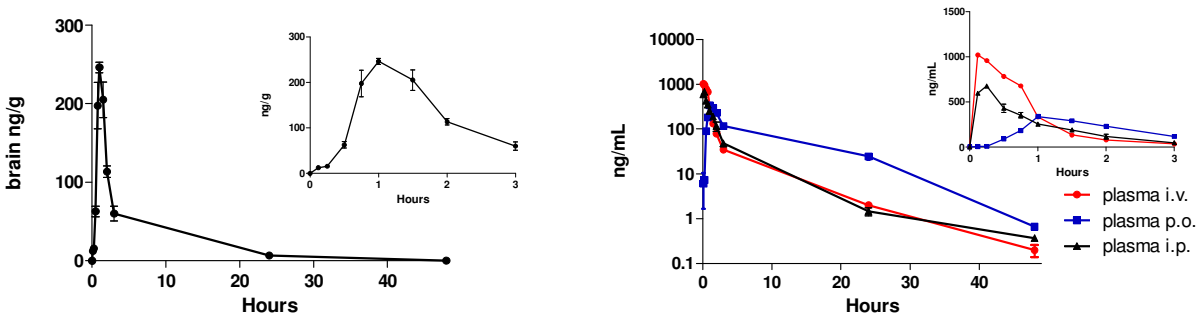


Figure 6: The left panel shows levels of 3α5βP-Glu in the brain after 1 mg/kg i.p. injection. The right panel demonstrates levels of 3α5βP-Glu in the plasma after 1 mg/kg i.v., i.p. and p.o. administration. The time course during the first 3 hours is for clarity shown at the smaller figure in the right upper corner. Data represent the mean ± SEM of four animals. (Rambousek L. et al., 2011)

1.2.3.2. Pharmacodynamics

Our previous studies showed that 3α5βP-Glu is a use-dependent inhibitor of NMDA receptor (Rambousek L. et al., 2011) as well as modulator of $GABA_A$ receptor (Vyklicky L., unpublished data).

The patch clamp techniques were used to study the effect of 3α5βP-Glu on NMDA receptors expressed in hippocampal neurons. Fig. 7A shows whole-cell currents induced by 100 μM NMDA applied in the presence of 10 μM glycine (no added Mg^{2+}) and reduced Ca^{2+} in cultured rat hippocampal neurons and the response to the presence of 100 μM 3α5βP-Glu. At this concentration of 3α5βP-Glu responses induced by NMDA application were inhibited by 54 ± 9 % (n= 7). This effect of 3α5βP-Glu was dose dependent (3- 300 μM) with a mean IC_{50} value of 69.1 ± 9.9 μM (n= 7) (Fig. 7B).

It has been previously shown that pregnanolone sulfate, an endogenous neurosteroid with presumably analogous mechanism of action, is the use-dependent and voltage-independent NMDA receptor inhibitor (Petrovic M. et al., 2005). Next experiments were aimed to test whether the inhibitory action of 3α5βP-Glu depends or is independent upon receptor activation. Two experimental protocols for 3α5βP-Glu and NMDA applications were used: (1) co-application of 3α5βP-Glu and NMDA after the onset of a response to NMDA and (2) sequential application of NMDA after pre-application of 3α5βP-Glu for 30 s. Fig. 8 shows responses induced by co-application of 100 mM NMDA and 50 μM 3α5βP-Glu on cultured hippocampal neurons. At concentrations close to the steroid IC_{50} value inhibition was 54%. After cessation of 3α5βP-Glu application, the response to NMDA recovered on a slow timescale (within seconds). Subsequent experiments were designed to test the effect of 3α5βP-Glu on the response to NMDA when 3α5βP-Glu is pre-applied in the absence of NMDA receptor agonist. As seen in Fig. 8 (right trace), the response to 100 μM NMDA observed after reapplication of 50 μM 3α5βP-Glu was significantly faster than that seen after co-application of 3α5β P-Glu and NMDA while onset kinetics were similar to that induced by control applications of 100 μM NMDA (without neurosteroid pre-application) (see overlaid responses in Fig. 8). Similar differences in NMDA receptor response onset kinetics were observed in five additional cells after either co- or pre-application of 3α5βP-Glu. These results show that the inhibitory action of 3α5βP-Glu depends upon receptor activation, and that the neurosteroids are use-dependent inhibitors of NMDA receptors (Rambousek L. et al., 2011).

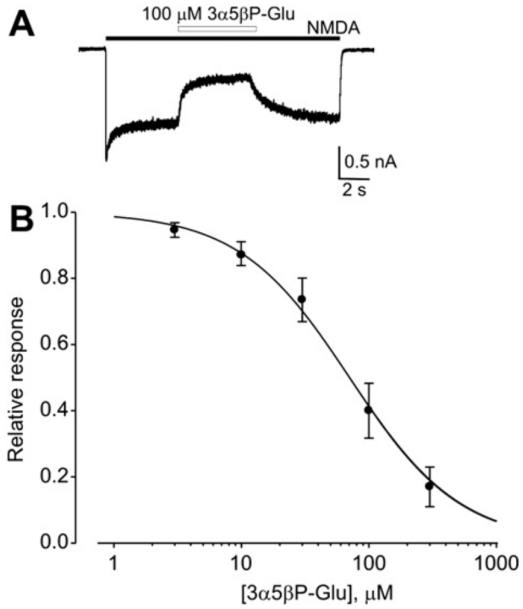


Figure 7: The effect of 3α5βP-Glu on native NMDA receptors. A. Whole-cells responses evoked in cultured hippocampal neurons by application of 100 µM NMDA (indicated by an open bar) and simultaneous application of 100 µM 3α5βP-Glu (indicated by a filled bar). Note the slow kinetics of the onset and offset of inhibition. B. Dose response relationship of the effect of 3α5βP-Glu on NMDA induced responses. The relative inhibition induced by 3α5βP-Glu (3, 30, 100 and 300 µM) was fit using nonlinear regression ($I = 1/1 + [3\alpha5\beta P\text{-Glu}]/IC_{50}$); where IC_{50} is the concentration of 3α5βP-Glu that produces 50% inhibition of the NMDA-evoked current and [3α5βP-Glu] is the neurosteroid concentration. Results from each cell were independently fit to the nonlinear regression and IC_{50} values were averaged ($IC_{50} = 69.1 \pm 9.9$; n = 7). (Rambousek L. et al., 2011)

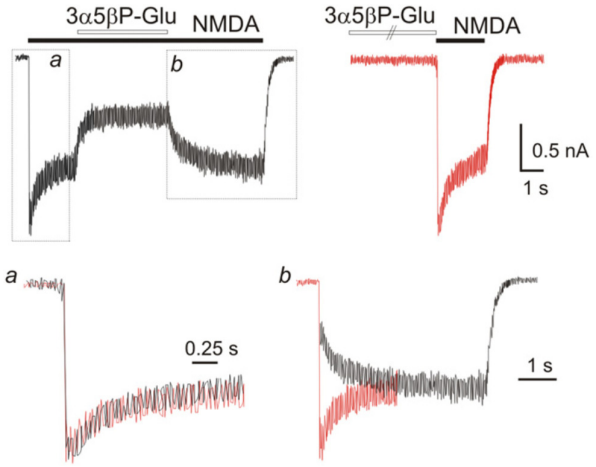


Figure 8: 3α5βP-Glu is a use-dependent inhibitor of NMDA receptor channels. Examples of recordings obtained from cultured hippocampal neurons. On the left, response to co-application of 50 µM 3α5βP-Glu and 100 µM NMDA after the onset of the response to the agonist was inhibited by 54% and recovered from the inhibition on a slow timescale. On the right, the onset of the response to the application of 100 µM NMDA made immediately after pre-application of 50 µM 3α5βP-Glu for 30 s was rapid, similar to the control NMDA response on the left. To illustrate the difference in the time course of responses to NMDA made after co-application of 3α5βP-Glu and NMDA (b; boxed area) and that made after pre-application of 3α5βP-Glu (indicated in red), both responses are shown superimposed on an expanded timescale. The rise time of responses to NMDA made after pre-application of 3α5βP-Glu (indicated in red) was similar to that of the control response to 100 µM NMDA (a; boxed area). (Rambousek L. et al., 2011)

Electrophysiology measurements also revealed that 3α5βP-Glu exhibits both potentiating and inhibitory effects on $GABA_A$ receptors (Vyklicky L., unpublished results). Potentiation is a dominant phenomenon at lower concentrations (below 30 μM) while inhibition prevails at higher concentrations of 3α5βP-Glu (Fig. 9A and B). It strongly indicates that there are two distinct binding sites at the ion channel which account for potentiation and inhibition by 3α5βP-Glu. Similar features were described in NMDA receptor ion channels where PS exhibits both potentiating and inhibitory effect due to binding to potentiating and inhibitory binding sites (Horak M. et al., 2004 and 2006). The dose response curve shown in Fig. 9C, whose formula is based on the two independent binding sites model, fits well the measured data yielding Ki = 52 μM, Kp = 0.2 μM and 49% potentiation of ion channel with 3α5βP-Glu bound to the potentiating binding site. Thus the $GABA_A$ receptors are significantly potentiated even at submicromolar concentrations.

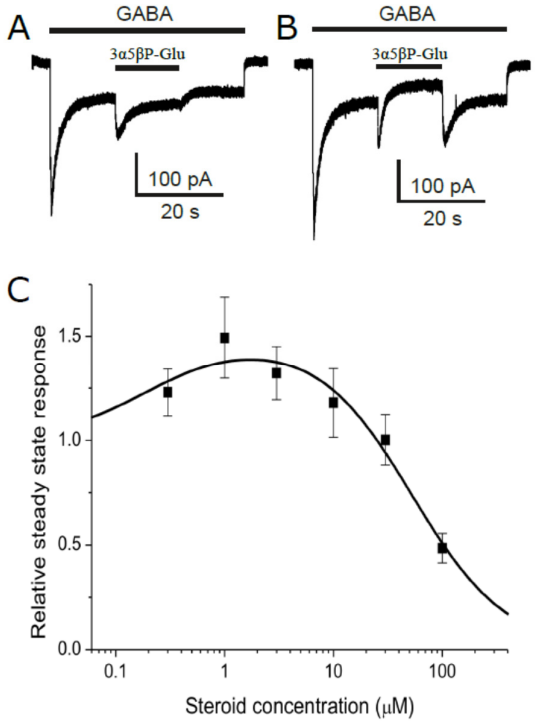


Figure 9: The effect of 3α5βP-Glu on the $GABA_A$ receptors. A, 10 μM 3α5βP-Glu induces 20% potentiation of the steady state $GABA_A$ receptor mediated current. B, 100 μM 3α5βP-Glu induces 51% steady state inhibition. The transient increase of the responses after the start of 3α5βP-Glu application is caused by faster binding of 3α5βP-Glu to the potentiating binding site compared to the inhibitory binding site. C, The concentration dependence of the relative response is well fitted with a dose response curve based on a model comprising independent potentiating and inhibitory binding site on the ion channel. (Vyklicky L., unpublished results)

Unfortunately, we do not have so far information about other possible targets such as the other receptors for neurotransmitters and nuclear receptors. It is also unclear whether it can

target receptors modulating neural growth, myelination or neuroinflammation. 3α5βP-Glu can presumably also integrate into cell membranes and influence its physical properties.

1.3. Behavioral phenotyping

1.3.1. Locomotor activity

Assessing an effect of drug on animal locomotor activity is one of the basic methods in behavioral pharmacology (Denenberg V.H., 1969). The examination of locomotion in rats and mice does not require active learning or conditioning as other behavioral tasks. It is often used as a first screen for pharmacological effects predictive of therapeutic efficacy of a drug class. It can be also used as a first test to indicate the onset of psychoactive drug with unknown pharmacokinetics. Locomotor activity in naïve animals after drug treatment can also predict potential psychotomimetic, sedative or extrapyramidal side effects of the drug. Psychostimulants (e.g. amphetamine) as well as NMDA-channel blockers (e.g. ketamine, PCP) induce hyperlocomotion (Newcomer J.W. et al., 1999). On the other hand, benzodiazepines such as $GABA_A$ agonist diazepam induce sedation (Liljequist S. and Engel J., 1982).

In an open-field test, the animal is placed into experimental box and its locomotor activity is tracked using automated monitoring systems. Parameters such as total distance traveled, inactivity, speed as well as thigmotaxis can be afterwards evaluated (Denenberg V.H., 1969). Locomotor activity can be assessed also in dynamic environment such as rotating arena (Stuchlik A. et al., 2014). In this thesis, the locomotor activity of naïve rats was assessed in the open-field test and the arena frame of Carousel maze.

1.3.2. Sensorimotor gating

Another important measure of information processing integrity is sensorimotor gating. Its deficit is phenomena of neuropsychiatric disorders and their animal models (Braff D.L. et al., 2001). It is also impaired after the administration of psychoactive drugs, especially non-competitive NMDA antagonists (Bubenikova-Valesova V. et al., 2008).

The acoustic startle reflex is a very basic response to intense external sensory stimuli. If a weak sound preceding the loud acoustic stimulus inhibits the startle reflex; this is called pre-pulse inhibition (PPI). It is widely used to assess sensorimotor gating in animals and humans, which is impaired in schizophrenia (Swerdlow N.R. et al., 2008), Alzheimer's disease (Ueki A. et al., 2006), bipolar disorder (Kohl S. et al., 2013), obsessive-compulsive disorder (Kohl S. et al., 2013) and attention deficit-hyperactivity disorder (Schulz-Juergensen S. et al., 2012).

When considering the usefulness of PPI in animal models of schizophrenia and other psychiatric disorders, one must consider several important points. The PPI paradigm is fully translational; i.e., it can be feasibly used both in laboratory rodents and humans (Braff D.L. et al., 1992), which facilitates direct comparison of gating deficits in human CNS disorders and their animal models. This is a significant advantage of the test, although it is not the only translatable task out of the plethora of tests used in animal models. The test is also quite simple and basic gating deficits can be expected to reflect complex impairments of informational processing, which may lead to cognitive deficit. However, when interpreting the results of PPI experiments, it should be emphasized that the test is perhaps too simple to reveal memory or cognitive impairments. In memory terms, PPI is essentially a modification of reflex behavior (startle). Despite this limitation, the prepulse inhibition of the startle reflex is abundantly used and a very informative behavioral paradigm for gating mechanisms studied in animal models of CNS disorders (Nekovarova T. et al., 2011).

1.3.3. Cognitive functions

Behavioral tests for the assessment of cognitive functions belong to powerful tools to evaluate behavioral deficits in animal models of neuropsychiatric disorders (Stuchlik A. et al., 2014). Spatial orientation or place navigation (i.e., navigation to directly imperceptible goals) belongs to central models for studying cognitive deficits in experimental animals (Stuchlik A. et al., 2013). Hippocampus dependent memory in rats and mice is often utilized as a model of human learning and memory, since it is enormously developed in these species. Spatial orientation of rats has been used as a model of human declarative memory (Eichenbaum H., 2003). Some argue that hippocampus supports declarative memory, our capacity to recall facts and events, whereas others view the hippocampus as part of a system dedicated to calculating routes through space, and these two contrasting views are pursued largely independently in current research (Eichenbaum H. and Cohen N.J., 2014).

In this thesis, passive avoidance task and active allothetic place avoidance task (the Carousel maze) were utilized to asses learning and memory in both naïve animals and animal models. The principle and experimental set-up for both tasks is described in detail in methods. These tasks allow as studying different aspects of memory. Depending on the experimental design, memory acquisition, consolidation as well as retention can be tested.

1.4. Animal model of schizophrenia induced by NMDA receptor hypofunction

Schizophrenia is a chronic and devastating mental illness that affects 1% of the world's population (Freedman R., 2003; Rössler W. et al., 2005). Three main types of symptoms characterize this disorder: positive symptoms (hallucinations, delusions), negative symptoms (social withdrawal, anhedonia, emotional blunting), and deficits in cognitive functions. Cognitive symptoms have negative impact on a patient's well-being, worsen functional manifestations of schizophrenia symptoms, and reduce patient compliance. Little success of various pharmacological strategies utilized to improve cognitive functions in schizophrenic patients underlines the difficulty of cognitive deficit remediation (Miyamoto S. et al., 2005).

Schizophrenia symptoms are induced in healthy volunteers (Javitt D. and Stephen R., 1991; Newcomer J.W. et al., 1999; Krystal J.H. et al., 1994) or exacerbated in schizophrenic patients (Malhotra A.K. et al., 1997; Lahti A.C. et al., 2001) by application of non-competitive NMDA receptor antagonists (e.g. PCP, ketamine or MK-801). In addition, their acute or repeated administrations have been established as a pharmacological tool to produce positive and cognitive schizophrenia-like intermediate phenotypes in rodents (Nilsson M. et al., 2001; Stuchlik A. et al., 2004; Stuchlik A. and Vales K., 2005). Despite no animal model fully mimics schizophrenia symptoms, administration of NMDA antagonists induce motor dysfunction, hyperlocomotion, stereotypic behaviors, PPI and latent inhibition deficits, complex impairments in cognitive functions, and social interactions, which are reversed by antipsychotics (Bubenikova-Valesova V. et al., 2008). Even low doses of MK-801 have been shown to mimic social withdrawal as a model of negative symptom of this disease (Bubenikova-Valesova V. et al., 2008).

These findings led to formulation of the NMDA receptor hypofunction hypothesis (Kim J.S. et al., 1980), which suggests that altered function of NMDA receptors results in excessive release of glutamate (Adams B. and Moghaddam B., 1998) and acetylcholine in the cerebral cortex (Kim S.H. et al., 1999) and, consequently, in the activation of the mesolimbic dopaminergic circuits. Since glutamate is the main excitatory neurotransmitter in the mammalian brain, its excessive release causes an increase in the activity of excitatory neurons. Glutamate also controls inhibitory tone by activating NMDA receptors on GABAergic and monoaminergic neurons (Olney J.W. et al., 1999). Consequently, the disinhibition of the major glutamatergic excitatory pathways causes secondary overstimulation of primary corticolimbic neurons (Zhang Y. et al., 2008), resulting in the manifestation of schizophrenia-like symptoms.

NMDA receptors are expressed in nearly all subtypes of neurons and direct pharmacological manipulation of this group of receptors may produce global disruption of brain functions and severe side-effects ranging from disruption of motor systems to impairment of attention and memory (Manahan-Vaughan D. et al., 2008a). Therefore, therapeutic strategies have focused on indirect modulation of NMDA receptor function. These strategies involve activation or positive modulation of glycine/d-serine site of NMDA receptors (Manahan-Vaughan D. et al., 2008b; Yang C.R. and Svensson K.A., 2008) and/or agonism and potentiation of metabotropic glutamate receptors (Vales K. et al., 2010; Yasuhara A. and Chaki S., 2010). Another promising strategy is NMDA receptor use-dependent modulation by neuroactive steroids.

We employed systemic application of MK-801 (a non-competitive NMDA receptor blocker) to induce schizophrenia-like behavior in Long-Evans rats (a procedure described in Stuchlik A. and Vales K., 2005). We tested spatial cognitive performance and locomotor activity in the Carousel maze (active allothetic place avoidance on a rotating arena), an aversive task with high demand on cognitive coordination of information from dissociated spatial frames. In addition, locomotor activity was tested in a stable environment, an open-field test.

1.5. Model of hippocampal excitotoxic damage induce by NMDA

Excessive stimulation of NMDA and AMPA receptors with glutamate is allowing high levels of Ca^{2+} to enter the neuron (Manev H. et al., 2009). Elevated Ca^{2+} levels are activating enzymes such as phospholipases, endonucleases and proteases leading to severe cell damage. This pathological phenomenon called excitotoxicity plays an important role in focal brain ischemia (Lai T.W. et al., 2014; Caccamo D. et al., 2004), traumatic brain injury as well as is present in neurodegenerative diseases (Mehta A. et al., 2013).

An interesting possibility how to test the efficacy of neuroprotective compounds to prevent or treat neuronal damage caused by the NMDA receptors over-activation is the permanent lesion of hippocampus induced by a microinjection of NMDA. Glutamic acid itself cannot be used for the excitotoxic lesions because it is rapidly metabolized and does not simulate long-term action. The lesion technique, the permanent removal or destruction of nervous tissue, has been the oldest and most widely used technique in the study of the brain function. Many techniques have been described, such as aspiration, resection, electrolytic lesion (Cassel J.C. et al., 1998) and chemical lesion. The most common chemical ablation method is injection of the neurotoxins (Pouzet B., 1999; Riedel G. et al., 1999; Clark R.E. et

al., 2000; Jarrard L.E., 1989). In these procedures, neurotoxin injections are made at one or multiple sites across the brain region of interest.

NMDA lesion of hippocampus has been used by many scientists as a model to induce hippocampal-dependent deficit in learning and memory (Ferbinteanu J. and McDonald R.J., 2001; Ito R. et al., 2005). It is well known that depending on the site of lesion, it induces impairment of cognitive functions including spatial memory (Quinn J.J. et al., 2002). In this work, we utilized bilateral lesion of hippocampi induced by NMDA infusion. Hippocampus is a paired structure, one of the main components of the mammalian brain. It belongs to a limbic system and plays major role in learning and memory.

2. AIMS AND HYPOTHESIS

Neuroactive steroids are potent modulators of $GABA_A$ and NMDA receptors. These two receptor systems are responsible for controlling inhibitory and excitatory neurotransmission in the brain. Their dysfunction is underlying pathology, progression and acute symptoms of many psychiatric disorders as well as neurological diseases. This includes schizophrenia, disorders of cognition and mood, depression, epilepsy, pain as well as ischemia, and neurodegeneration. However, therapeutics targeting these receptors often failed in the development, mainly because of the safety or efficacy limitations.

We hypothesize that targeting NMDA and $GABA_A$ receptors with neuroactive steroids may represent safe and efficient strategy to ameliorate disease symptoms resulting from misbalanced inhibitory and excitatory neurotransmission.

The aim of this thesis was to investigate the biological significance and safety profile of the neuroactive steroid, 3α5βP-Glu, in selected animal models.

We asked the following questions:

Experiment 1: Does 3α5βP-Glu induce psychotomimetic behavior (hyperlocomotion, sensorimotor gating deficit and impaired cognition) similarly to NMDA channel blockers?

Experiment 2: Is 3α5βP-Glu capable of reversing cognitive deficit and hyperlocomotion in the animal model of schizophrenia induced by MK-801?

Experiment 3: Is 3α5βP-Glu capable of reversing cognitive deficit induced by bilateral lesions of hippocampi?

Furthermore, the thesis aimed to morphologically characterize an animal model used in this thesis, the NMDA lesion of hippocampus. This lesion has been used to simulate excessive release of glutamate during traumatic events; however, the model has never been characterized in detail. Therefore we asked the following questions:

Experiment 4: How does NMDA lesion affect the hippocampus over 30 days? In particular, how is the expression of the major NMDA and $GABA_A$ receptor subunits affected? Is there neuroinflammation present? Which neurons are affected? Does the lesion also affect GABAergic interneurons?

3. METHODS

Animals

Adult male Long-Evans and Wistar rats (weighting from 250 to 350 g) from a breeding colony of the Institute of Physiology, Academy of Sciences, Czech Republic, Prague were used in the thesis. Rats were kept in transparent Plexiglas cages measuring 25x25x50cm located in an air-conditioned animal room with a 12:12 hour light-dark cycle with the lights turned on beginning at 7:00 a.m. Animals had free access to water and chow. All experiments were done in accordance with European Union regulations on animal care and protection, the Animal Protection Code of the Czech Republic and NIH guidelines. Number of animals used in the thesis is specified for each experiment bellow.

3.1. Experiment 1

Locomotor activity in open-field test

Long-Evans rats were treated with 3α5βP-Glu dissolved in hydroxypropyl-β-cyclodextrine (β-CD, 72 mM saline solution) adjusted pH 7.4 by 1 M NaOH at doses of 1 or 10 mg/kg i.p. 60 min prior to the test. Control animals received β-CD solution only. Locomotor activity, expressed as the total distance travelled during 30 min in a box (68 cm × 68 cm × 30 cm) located in a soundproof room, was measured using a video tracking system for automation of the behavioral experiments (Noldus, Netherlands, EthoVision Colour Pro-Version 3.1), as previously described (Bubenikova-Valesova V. et al., 2007). Data were tested for significant differences using the one-way ANOVA, followed by the Tukey's post hoc test. Results are represented as the mean ± S.E.M. Eight animals in each group were used (32 in total).

Prepulse inhibition of acoustic startle response (PPI)

Long-Evans rats were treated with 3α5βP-Glu dissolved in β-CD at doses of 1 or 10 mg/kg., 60 min before test session. Control animals received β-CD solution only. Day before test session rats were pre-trained by five minutes of acclimatization period and five single startle pulses (125 dB, 40 ms). The test session was composed of a mixture of the following types of trials that were presented against a constant 75 dB background noise: a) trials in which a prepulse stimulus has intensities 83 or 91 dB preceded a startle-eliciting pulse stimulus with 30, 60 or 120 milliseconds with duration 20 ms, b) trials in which no discrete stimulus other than the constant background noise was presented (ns [no-stimulus] trials), and c) trials in which startle pulse were presented alone 125 dB (40 ms) (startle pulse). All stimuli and background noise employed in the experiment consisted of broadband white noise. After a 5-

minute period of acclimatization to the background noise, 72 discrete trials were presented according to a variable inter-trial interval with a mean of 14 s (ranged from 4–20 s). The first (block 1) and last block (block 3) consisted of 6 consecutive startle pulse trials. The middle block (block 2) consisted of 60 trials, i.e., 12 trials of each of the 5 conditions, presented in a pseudorandomized order. The test session lasted approximately 20 min. PPI was calculated as the difference between the average values of the single pulse and prepulse-pulse trials and was expressed as a percentage of PPI [100 – (mean response for prepulse-pulse trials/startle response for single pulse trials) × 100]. Data from the four single pulse trials at the beginning of the test session were not included in the calculation of PPI and acoustic startle response values. Animals showing average startle amplitudes lower than 10 mV were removed from the calculation of PPI and were marked as non-responders (about 3% of the total number). The number of removed animals did not differ between treatment groups. Data were tested for significant differences using the one-way ANOVA, followed by the Tukey's post hoc test. Results are presented as the mean ± S.E.M. Eight animals in each group was used (32 in total).

Passive avoidance task

Passive avoidance task is a fear-aggravated behavioral task used to evaluate learning and memory in rodents. The experimental chamber is divided into lit and dark compartments (Fig. 10). Compartments are separated by computer controlled doors. The experiment was performed in 3 consecutive days (habituation, learning and test sessions). Wistar rats were allowed to explore both compartments on the first day for 10 min (habituation). On the following day (training), animals were placed into lit compartment and they immediately went into dark compartment. After they entered the dark compartment, the door automatically closed and they received mild footstock. They did not have possibility to escape to lit compartment. In order to test the effect of drugs on memory acquisition, animals were treated with drugs before placing into experimental apparatus. Memantine (Abcam, UK; 2.5, 5 and 10mg/kg) and MK-801 (Sigma Aldrich; 0.1 mg/kg and 1.5 mg/kg) were applied i.p. 20 min whereas 3α5βP-Glu dissolved in β-CD (1 and 10 mg/kg) 60 min before the training session. Control animals received β-CD solution only.

Control animals with normal learning and memory associate the foot shock with dark chamber and avoid entering this compartment following day. On the third day (test session), animals were placed into lit compartment and they can freely explore the experimental chamber. The latency when they first entered the dark compartment was measured as well as

the total time spent in dark compartment. The measurement was performed automatically using infrared beam system and recorded (Multiconditioning system, TSE, Germany). Experiment was terminated after 5 min. Data were tested for significant differences using the one-way ANOVA, followed by the Tukey's post hoc test. Seven to ten Wistar rats were used in this experiment (71 in total).

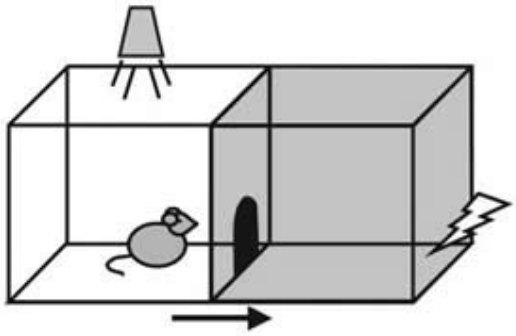


Figure 10: Schematic picture of passive avoidance apparatus. (From Wetsel R.M.R and William C., 2006)

Carousel Maze

The Carousel maze apparatus was described in detail in our previous study (Stuchlik A. et al., 2004 and 2013). Briefly, it consists of a smooth metallic circular arena (82 cm in diameter), enclosed with a 30-cm high transparent Plexiglas wall and elevated 1 m above the floor of a 4 m × 5 m room (Fig. 11). The room contained an abundance of extra-maze landmarks. The rats were initially placed in the arena rotating at 1 rpm in a place directly opposite to the shock sector. Animals had to avoid a directly imperceptible 60° sector, defined in the North of the four arbitrary cardinal compass directions. The sector was identifiable solely by its relationships to distal room cues. A latex harness was attached between the shoulders of the rats, which carried an infrared light-emitting diode (LED). A computer-based tracking system (iTrack; Biosignal Group, USA) was located in an adjacent room. The tracking system recorded the rat's position every 40 ms. Position time series were stored for off-line analyses (Track Analysis; Biosignal Group, USA). Whenever the rat entered the to-be-avoided sector for more than 0.5 s, the tracking system delivered a shock and counted an entrance. If the rat did not leave the sector, additional shocks were given every 1.4 s, but no more entrances were counted until the rat left the sector for more than 0.5 s. Mild shocks (50 Hz, 0.5 s, 0.4–0.7 mA) were administered from a computer-driven shock generator through the implanted low-impedance hypodermic needle implanted on the rats' backs and through the contact between the rats' keratinized paws and the grounded arena floor. Since the voltage drop is highest at the contact between rats' paws and the floor, the rats "feel" the shock most likely in their feet.

We avoided using a grid because in the Carousel maze it is necessary to allow accumulation of scent marks on the floor in order to generate a conflict between the arena and the room frames. The exact shock current, ranging between 0.4 and 0.7 mA, was adjusted for each rat to elicit a rapid escape response but not freezing.

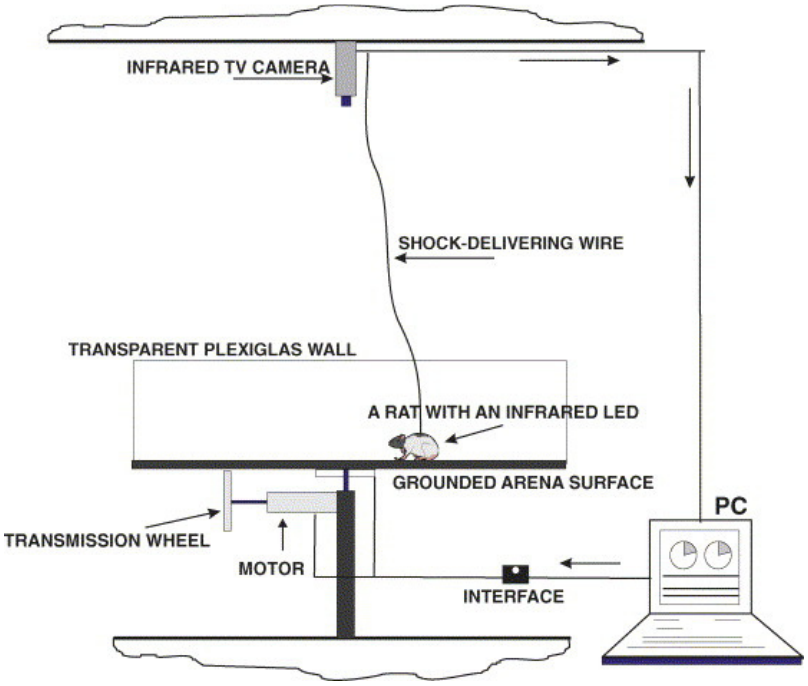


Figure 11: Schematic picture of Carousel maze apparatus. (From Stuchlik A. and Vales K., 2005)

The total distance traveled in a session (measured in the arena frame) reflected active locomotor activity without the contribution of the passive arena rotation. The distance was measured by the off-line tracking system by summing linear distances of points recorded each 1 s (the sampling frequency was 40 Hz). This sampling eliminated non-locomotor movements of the rat such as shivering. The number of entrances into the to-be-avoided sector (number of errors) measured the efficiency of avoidance in the Carousel maze. Another measure of the spatial performance within the session was the maximum time between the two entrances in a session (maximum time avoided). In healthy rats this training procedure was found to be long enough to induce optimal spatial avoidance of the punished sector and the data from the fourth session was close to asymptotic levels of performance (Stuchlik A. et al., 2008). Thus, only data from session four were analyzed.

Long-Evans rats were trained in four acquisition sessions of Carousel maze. Memantine (Abcam, UK; 5 mg/kg), ketamine (Vetoquinol S.A., France; 5 and 10 mg/kg) and MK-801 (Sigma Aldrich; 0.1 mg/kg, 0.2 mg/kg and 0.3 mg/kg) were applied i.p. 20 min and 3α5βP-

Glu dissolved in β-CD (1 and 10 mg/kg) 60 min before each session. Control animals received β-CD solution only. Six to eight animals were used in each group (62 rats in total). We evaluated total distance and a number of errors of animals in fourth session of Carousel maze. Results were analyzed using a two-way ANOVA (Groups × Sessions) with repeated measures on sessions, and Tukey's post hoc test was used when appropriate. Significance was accepted at $P < 0.05$.

3.2. Experiment 2

Carousel Maze

The apparatus was described above in the Experiment 1. Seventy seven Long-Evans rats were used for this experiment. We evaluated total distance and a number of errors of animals in the fourth session of Carousel maze.

MK-801 was utilized to induce schizophrenia-like behavior in rats. MK-801 was dissolved in a saline solution at a concentration of 0.1 mg/ml. MK-801 was injected i.p. 30 min prior to behavioral testing at a dose of 0.1 mg/kg of body weight. 3α5βP-Glu working solutions were prepared by dissolution of 3α5βP-Glu in β-CD, 72 mM saline solution, adjusted pH 7.4 by 1 M NaOH at concentrations of 0.0001 mg/ml, 0.001 mg/ml, 0.01 mg/ml, 0.1 mg/ml, 1 mg/ml and 10 mg/ml. Saline solution (0.9% NaCl, 1 ml/kg of body weight, i.p.) was injected as a control for MK-801 or 3α5βP-Glu application. Drugs and chemicals used in the study were purchased from Sigma Aldrich (Germany).

3α5βP-Glu was injected i.p. 30 min prior to testing in combination with MK-801 or saline at a volume of 1 ml/kg in all experiments. All animals received the same volume of liquid per 1 kg of body weight. In the Carousel maze, 3α5βP-Glu was injected at doses of 0.0001 mg/kg, 0.001 mg/kg, 0.01 mg/kg, 0.1 mg/kg, 1 mg/kg and 10 mg/kg before each session. Results were analyzed using a two-way ANOVA (Groups × Sessions) with repeated measures on sessions, and Tukey's post hoc test was used when appropriate. Significance was accepted at $P < 0.05$. Results are represented as the mean ± S.E.M. Five to eight animals in each group were used (77 in total).

Open-field test

In the open-field test, 3α5βP-Glu was applied at doses of 0.1 mg/kg and 1 mg/kg 30 min prior to testing. Animals were placed individually into an open-field arena (68×68×30 cm) located in a soundproof room. Locomotor activity, expressed as a total distance traveled, was

monitored for 30 min using a video tracking system for automation of the behavioral experiments (EthoVision-version 2.1., Noldus, The Netherlands). Data were tested for significant differences using the two-way ANOVA, followed by the Tukey's post hoc test. Results are represented as the mean ± S.E.M. Ten animals in each group were used (60 in total).

3.3. Experiment 3

NMDA lesion

Long-Evans rats were anaesthetized with isofluran. During the surgery animals were fixed utilizing a stereotactic apparatus and anaesthetized with isofluran (3.5 %) propelled by atmospheric air. Small openings in the skull were made using a micro-drill and subsequently 2 μL of NMDA (90 mM NMDA in phosphate buffer, #M3262, Sigma Aldrich, Germany) were bilaterally applied (1 μL each) at a flow rate of 0.2 mL/min into the dorsal hippocampi and 2 min after the end of the infusion the needle was retracted. Control animals (sham lesion) received sterile PBS. The application coordinates [-4 mm anteroposterior (AP) from bregma, ±2.5 mm mediolateral (ML) and 4 mm below the skull surface] were measured in relation to a stereotaxic atlas (Paxinos G. and Watson C., 1998).

Drug treatment

In the behavioral experiment, rats were randomly assigned into experimental groups according to the 3α5βP-Glu application protocol: 30 min, 3 hours and 24 hours after application of NMDA into the dorsal hippocampi. At each time point, 3α5βP-Glu was injected at 0.1 mg/kg, 1 mg/kg or 10 mg/kg i.p. of body weight dissolved in β-CD. Controls group of animals received buffered saline solution infusions into the hippocampus and β-CD solution i.p. Five to seven Long-Evans rats were used in each group (64 in total).

In the morphological experiment, animals were treated with memantine (5 mg/kg) or 3α5βP-Glu (1 mg/kg). Memantine was applied 15 min before the infusion of NMDA into hippocampus. 3α5βP-Glu was applied 30 min before. Three Long-Evans rats were used in each group (12 in total).

Carousel Maze

After 7 days of recovery from surgery (NMDA lesion), animals were trained in Carousel Maze (described above in Experiment 1) for 4 consecutive days. The interval between sessions was 24 h. The total distance traveled in a session (measured in the arena frame) reflected active locomotor activity without the contribution of the passive arena rotation. The distance was measured by the off-line tracking system by summing linear distances of points recorded each 1 s (the sampling frequency was 40 Hz). This sampling eliminated non-locomotor movements of the rat such as shivering. The number of entrances into the to-be-avoided sector (number of errors) measured the efficiency of avoidance in the Carousel maze. Another measure of the spatial performance within the session was the maximum time between the two entrances in a session (maximum time avoided). Results were analyzed using a two-way ANOVA (Groups × Sessions) with repeated measures on sessions, and Tukey's post hoc test was used when appropriate. Significance was accepted at $P < 0.05$.

Histology and immunohistochemistry

Animals were perfused 7 days after the experiment (NMDA lesion). The histology protocol as well as immuhistochemistry protocols for glial fibrillary acidic protein (GFAP) and Iba-1 stainings are described in Experiment 4.

Fluoro-Jade B staining

Fluoro-Jade B (FJB) stains selectively neurodegenerating neurons. For FJB (Millipore, USA) staining brains were rinsed five times in 0.1 M phosphate-buffered saline (pH= 7.4) and left overnight in 30 % sucrose. Brains were then frozen and embedded in medium for frozen tissue and cryo-sectioned into 40 µm slices. The sections were treated for 20 min by 0.01 % $KMnO_4$, washed three times in distilled water and incubated for 30 min in darkness using 0.0001 % solution of FJB (4 mL of stock solution added to 96 mL of 0.1 % acetic acid). Following further washing in distilled water the sections were mounted. Brain sections were imaged using microscope Zeiss AxioVision Imaginer Z1 (Zeiss, Germany) equipped with a digital camera. Alexa 448 filter was used.

Nissl staining

Nissl staining is a classical neuroanatomy technique staining basic nucleic acids. This technique is utilized to stain all cell bodies (neurons and glia). Sections for Nissl staining were mounted on gelatin-coated glass slides and let dry overnight. Second day, the slides were

dipped in the following solutions: dH_2O (5 min), Cresyl violet solution (5 min, Sigma Aldrich, Germany), dH_2O (30 s), 96% ethanol and 0.5% acetic acid (2.5 min), isopropanol (5 min), isopropanol:xylol 1:2 (5min), xylol (4 x 2 min). Afterwards, they were coversliped with Eukitt™ (Sigma Aldrich, Germany) and imaged with Zeiss AxioVision Imaginer Z1 (Zeiss, Germany) equipped with a digital camera.

3.4. Experiment 4

NMDA lesion

We utilized Long-Evans rats in this experiment. Five animals per group were used [SHAM and NMDA lesion group x 1, 3, 7 and 30 days post injection (dpi); 40 in total]. All animals were anaesthetized with isofluran 3.5% (Abbot Laboratories, UK) in a specialized initial chamber. During a surgery animals were fixed using a stereotactic apparatus (TSE systems, Germany) and anaesthetized with isofluran 1.5 - 2%, propelled by atmospheric air. Small opening in the skull was made using a micro-drill (Dremel, USA) and subsequently 1 µL of 25 mM NMDA (Sigma Aldrich, Germany) or 10 mM of sterile PBS (Sigma Aldrich, USA) was applied unilaterally at a flow rate of 0.2 µL/min into the dorsal hippocampus with pump (TSE, model 540310 plus) and 2 min after the end of infusion the needle (#7635-01, Hamilton) was retracted. To prevent the loss of tissue integrity, we utilized lower concentration of NDMA in this experiment. The application coordinates (−4 mm AP from bregma, ±2.5 mm ML and 4.8 mm below the skull surface) were measured in relation to a stereotaxic atlas (Paxinos G. and Watson C., 1998).

The NMDA (#M3262, Sigma Aldrich, USA) working solution was prepared in 10 mM PBS (Sigma Aldrich, USA), pH adjusted to 7.4, fractioned on a small volume aliquots (20 µl) and stored at -20 °C. A fresh aliquot from same the batch was used for all experiments. Each aliquot was used only once.

Histology

After competition of the experiment (1, 3, 7 or 30 dpi), animals were anaesthetized with and overdosed by mixture of ketamine (Vetoquinol) and xylasine (Alfasan) and transcardially perfused at a flow 50 mL/min with 250 ml, 4 °C, 4% paraformaldehyde (PFA) in 0.1 M PBS, preceded by a 5 min rinse with 0.01 M PBS. After postfixation in the same fixative overnight, the brains were stored in a 1% PFA solution at 4 °C, cryoprotected in 10% and consequently 30 % sucrose solution. Brain tissue was stored at -80°C. Free-floating sections (40 µm thick)

were cut coronally on a sliding microtome (Leica), and 18 random sampled serial sections were collected from bregma -1.5 to -6.6 mm and stored at - 20°C in cryoprotective solution.

Fluoro-Jade B staining

FJB staining protocol was identical as described in experiment 3. Extent of damage was evaluated separately in individual areas of the hippocampus, namely CA1, CA3, dentate gyrus, hilus and subiculum. The Range of damage was expressed as a percentage of hippocampal area with Fluoro-Jade B positive neurons. Evaluation scale: 0 – 0-5%, 1 – 6-25%, 2 – 26-50%, 3 – 51-75%, 4 > 75% Fluoro-Jade B positive neurons in individual hippocampal area. Maximal value of the damage for individual animal was 4.

Immunohistochemistry

Immunohistochemistry was performed with one series of brain sections per animal, allowing us to perform experiment under equal conditions for all animals. List of primary antibodies used in the study is shown in table 3. The free-floating sections were rinsed in Tris-Triton solution (0.05 M Tris, 0.015 NaCl, 0.05 % Triton, pH = 7.4) three times for 10 min and followed by overnight incubation in the primary antibodies diluted in Tris-Triton containing 2% normal goat serum (NGS), 0.2% TritonX-100 at 4°C on the shaker.

Sections used for NMDAR NR1 and NR2B stainings were additionally digested by pepsin before incubating in primary antibodies. This procedure significantly improves the detection of membrane proteins (Watanabe M. et al, 1998). Briefly, the sections were incubated at 37°C for 10 min in 0.2M HCl solution and consequently for additional 10 min in 0.2 M HCl solution containing 0.15 mg/mL pepsin (Dako, S3002, CA, USA). After washing in PBS for 5 min and twice in Tris-Triton for 10 min the sections were incubated in primary antibodies.

Second day, the sections were rinsed in three times for 10 min in Tris-Triton, then incubated with biotinylated secondary goat antibodies diluted at 1:300 in Tris-Triton containing 2% normal goat serum (NGS) and 2% rat serum for 30 min at room temperature under continuous agitation. After three washing cycles in PBS the sections were incubated in Elite ABC Kit (Vector Laboratories Inc., California, USA) diluted in PBS, 0.2% TritonX-100 for 1 h, and washed three times for 10 min in Tris-Triton. Secondary antibodies were visualized using 0.05% solution of 3,3-diaminobenzidine (DAB, Fluka, Switzerland) and 0.01 % H_2O_2 in Tris-Triton pH 7.7. A fresh aliquot from same batch was used for each experiment. The color reaction was terminated after 8 min by transferring the sections in ice-cold PBS. To

prevent intra-group variation, all sections for each marker were stained in one DAB experiment. After two additional washes in PBS the sections were mounted on gelatine-coated slides, dried overnight, dehydrated and cover slipped with Eukitt™ mounting media (Sigma Aldrich, Germany).

Table 3: List of primary antibodies, their manufactures and dilutions used.

Antibody	Manufacturer	Description/Nr.	Dilution
$GABA_AR$ α1	Fritschy J.M. and Möhler H., 1995	Guinea pig antiserum, 102	1:20000
$GABA_AR$ α2	Fritschy J.M. and Möhler H., 1995	Affinity purified guinea pig antiserum, 104	1:1000
$GABA_AR$ α5	Fritschy J.M. and Möhler H., 1995	Guinea pig antiserum, 110	1:3000
$GABA_AR$ γ2	Fritschy J.M. and Möhler H., 1995	Guinea pig antiserum, 15	1:15000
NMDAR NR1	NeuroMab, CA, USA	Mouse monoclonal, clone N308/48	1:2000
NMDAR NR2B	NeuroMab,CA, USA	Mouse monoclonal, clone N59/36	1:2000
Iba-1	Wako	Rabbit polyclonal, 019-19741	1:4000
GFAP	Dako Schweiz AG, Switzerland	Rabbit polyclonal, Z334	1:5000
Parvalbumin	SWant, Switzerland	Rabbit polyclonal, PV-25	1:5000
Calretinin	SWant, Switzerland	Rabbit serum, 7696	1:2000
Calbindin	SWant, Switzerland	Rabbit serum, CB-38	1:3000
NPY	Peninsula Laboratories, USA	Rabbit serum, T4069	1:1000

All antibodies were tested for their specificity using fluorescent secondary antibodies and consequent imaging with confocal microscope (Zeiss 710, Germany) before we performed the study. Additionally, different types of perfusions (PFA, PBS and ACSF) and post-fixation times were tested to ensure the optimal tissue conditions for the selected battery of antibodies.

Densitometry

Densitometry measurements of $GABA_A$ and NMDA receptor subunits immunoreactivities were measured using ImageJ software (NIH, Maryland, USA). Digital images were acquired at a magnification 20x (NA 0.8 NA air) using a 3-CCD digital color camera (Hitachi HV - F22, 1360 x 1024 pixels, pixel size 4.65 μm) mounted in automated upright slide scanning microscope, Zeiss Mirax mini slides-canner (Zeiss, Jena, Germany). Acquired images of hippocampus and neocortex were then exported at a 5x magnification using Panoramatic viewer (3DHISTECH, Hungary). The optical density (OD) for each hippocampus was

normalized to the optical density of neocortex. The optical density of neocortex did not differ significantly within the groups. Four to six consecutive coronal sections from each animal were analyzed. The optical densities for each hippocampus were averaged per animal using the following formula:

$$OD = \frac{\sum_{i=0}^{n}(OD_{neocortex} - OD_{hippocampus})}{n}$$

where *n* is number of sections and *OD* optical density measured as pixel intensity in a range of 0 – 255 using ImageJ software.

A densitometric segmentation analysis was performed for the anti-GFAP (astroglial marker), and anti-Iba1 (activated microglia marker) immunoperoxidase stainings to measure the relative percentage of the labeled cells covered by the immunoreactive signals in the hippocampal formation. The quantification was performed using a developed macro in ImageJ software (NIH, Maryland, USA). The pictures obtained with Zeiss Mirax mini slides-canner were converted to 8-bit images, Gaussian blur filter was applied and the background was subtracted. Next the threshold function was applied and relative percentage of the labeled cells covered by the immunoreactive signal was automatically calculated.

Data were analyzed and statistically compared between groups using two-way ANOVA. Ipsilateral and contralateral hippocampi were analyzed separately. Staining intensities, represented as normalized relative optical densities using 2 x 4 ANOVA design to detect effect of group (NMDA lesion or PBS infusion) and day post injection. Following the confirmation of main effects, Bonferroni's post hoc tests were performed. Statistical significance was set at $P < 0.05$.

4. RESULTS

4.1. Experiment 1: The effect of 3α5βP-Glu in naïve animals

4.1.1. The acute effect of 3α5βP-Glu on locomotor activity in open-field test

The administration of 3α5βP-Glu did not significantly affect locomotor activity in naïve rats at doses of 1 or 10 mg/kg in open-field test. One-way ANOVA test failed to confirm group effect ($F_{(2,27)} = 1.83$, $P > 0.05$). The distance traveled during 30 min is illustrated in Fig. 12.

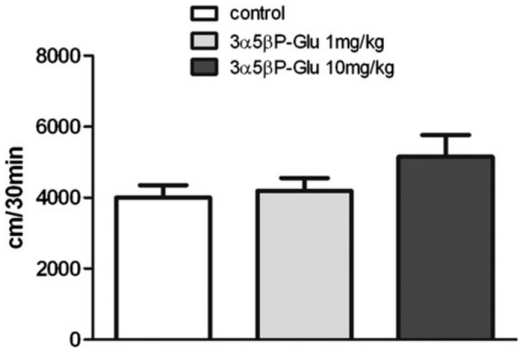


Figure 12: Acute effect of 3α5βP-Glu at doses 1 and 10 mg/kg i.p. administration on locomotion generated during 30 min in an open-field apparatus. There was no significant effect of 3α5βP-Glu on locomotor activity in a novel environment. Data are represented as mean ± SEM.

4.1.2. The acute effect of 3α5βP-Glu on sensorimotor gating in PPI test

The acute administration of 3α5βP-Glu at doses 1 and 10 mg/kg had no significant effect on prepulse inhibition of the acoustic startle response (Fig. 13). One-way ANOVA test failed to confirm any group effect after prepulse 78 dB ($F_{(2,27)} = 0.402$, $P > 0.05$) and after 86 dB prepulse ($F_{(2,27)} = 0.6424$, $P > 0.05$). Results showed that 3α5βP-Glu at highest dose which can be dissolved in β-CD does not induce either hyperlocomotion typical of other classes of NMDA receptor antagonist or sedation typical for $GABA_A$ receptor agonists.

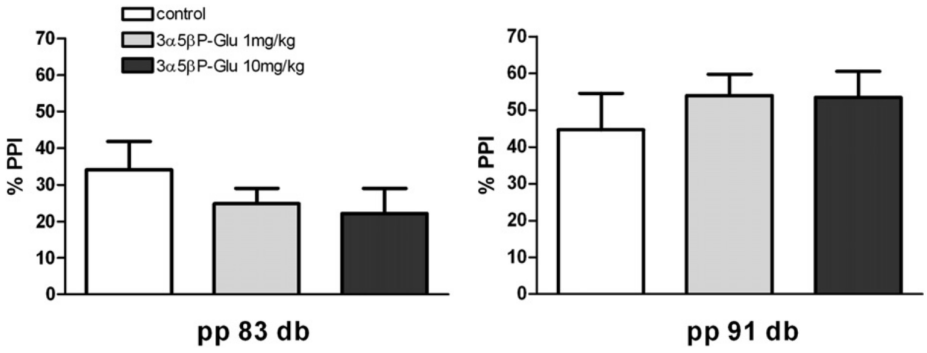
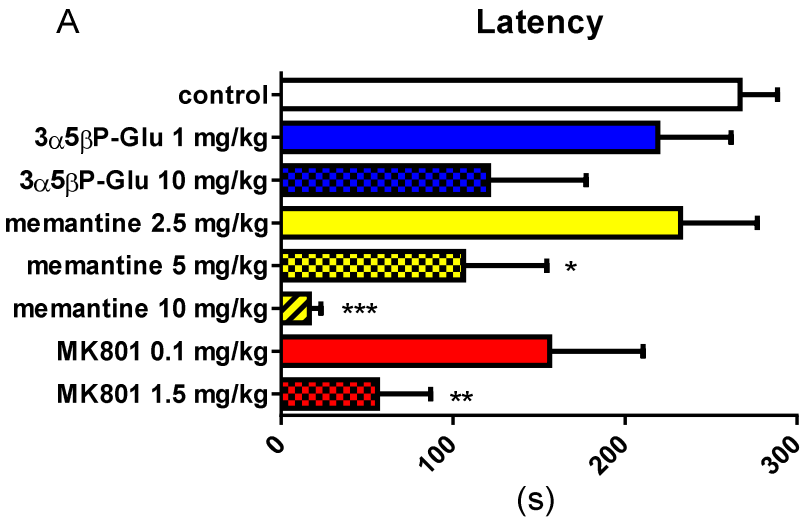


Figure 13: Effect of acute application of 3α5βP-Glu at dose 1 and 10 mg/kg i.p. on prepulse inhibition of the acoustic startle response [prepusle (pp) 83 and 93 dB]. There was no significant effect of 3α5βP-Glu on PPI. Data are represented as mean ± SEM.

4.1.3. The acute effect of 3α5βP-Glu on learning in a passive avoidance task. Comparison with other use-dependent NMDA antagonists.

In this test, we evaluated the latency when rats first entered the dark compartment (Latency, Fig. 14A) and time spent in dark compartment (Dark time, Fig. 14B). Control animals treated with PBS did not show any learning impairment in both parameters. One-way ANOVA revealed the effect of treatment for Latency parameter ($F_{(9,26)}$= 4.161, P = 0.0003) as well as for the Dark time parameter ($F_{(9,69)}$= 3.502, P = 0.0013). Consequent Bonferroni's post-hoc test revealed significant memory impairment measured as latency when rats entered the dark compartment after higher dose of MK-801 (1.5 mg/kg) and memantine at doses 5 and 10 mg/kg (P < 0.01). The time spent in dark compartment was significantly higher (P < 0.01) for the highest doses of MK-801 (1.5 mg/kg) and memantine (10 mg/kg). 3α5βP-Glu induced mild dose-dependent deficit in this task; however, it was not significant. We observed the same trend in parameters, the latency and time spent in dark compartment.



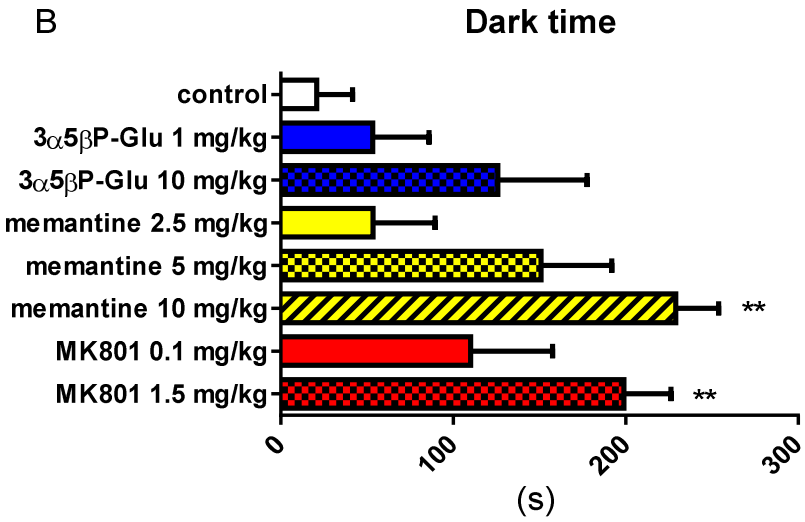


Figure 14: The latency when rats first entered the dark compartment (A, latency) and time spent in dark compartment (B, darktime) in the passive avoidance task after treatment with 3α5βP-Glu or other NMDA antagonists. Data are presented from the last day of training as mean ± SEM. *$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

4.1.4. The effect of 3α5βP-Glu on locomotor activity and spatial learning after sub-chronic treatment in the Carousel maze. Comparison with other use-dependent NMDA antagonists.

The one-way ANOVA revealed the effect of treatment for both total path ($F_{(8,53)}$= 14.96, P < 0.0001) and number of entrances ($F_{(8,53)}$= 23.69, P < 0.0001) parameters at fourth session of the Carousel maze. Sub-chronic treatment with 3α5βP-Glu at doses 1 and 10 mg/kg significantly affected neither locomotor activity nor spatial memory in the Carouse Maze. On the other hand, consequent Bonferroni's post-hoc test revealed that classical NMDA channel blockers MK-801 and ketamine induced significant hyperlocomotion as well as deficit in spatial learning. Memantine did not significantly affect learning and locomotor activity in Carousel Maze. Total path and number of entrances at 4th session of Carouse Maze are shown in Fig. 15.

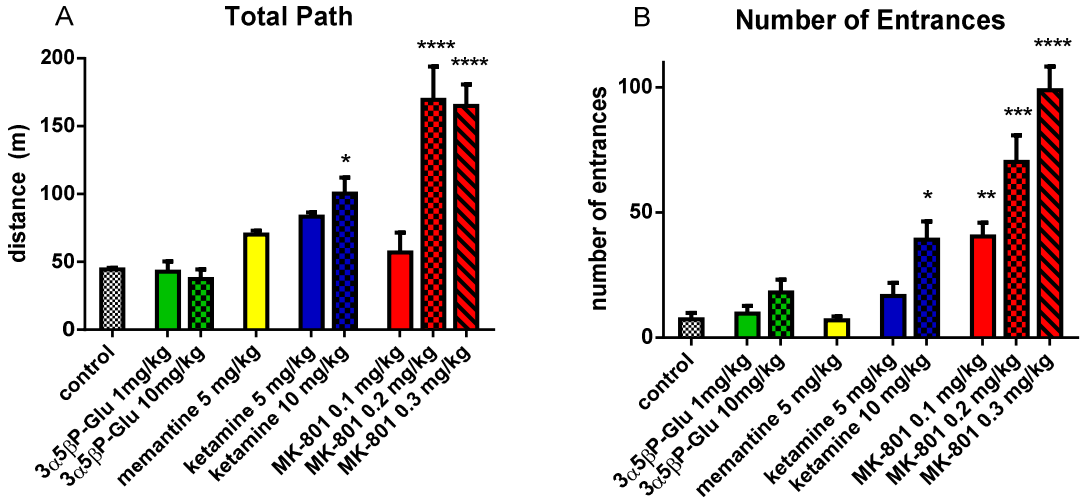


Figure 15: Locomotor activity (A, total path) and spatial learning performance (B, number of entrances) in the Carousel maze after sub-chronic treatment with 3α5βP-Glu or other NMDA antagonists. Data are shown from the last day of training as mean ± SEM. *$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

4.2. Experiment 2: The effect of 3α5βP-Glu in animal model of schizophrenia

4.2.1. Locomotor activity and cognitive performance in Carousel Maze

We assessed the effect of 3α5βP-Glu on spatial MK-801-induced cognitive deficit in a test of cognitive coordination and spatial learning, the Carousel maze. Two-way ANOVA revealed a significant interaction between the effects of 3α5βPGlu and MK-801 on the number of entrances into the punished sector ($F_{(6, 75)}$= 3.3, P < 0.01), reflecting U-shaped dose-dependent positive effect of 3α5βP-Glu on the number of entrances in rats with MK-801 injection. Furthermore, the analysis revealed significant main effect of 3α5βP-Glu ($F_{(6, 75)}$= 4.16, P < 0.01) but not MK-801 treatment ($F_{(1, 75)}$= 2.04, P = 0.15). Post-hoc tests showed that the MK-801 did induce cognitive deficit, but it was ameliorated by 3α5βP-Glu at doses 0.001, 0.01, 0.1, 1 and 10 mg/kg. Application of 3α5βP-Glu without MK-801 led to similar dose-dependent effect on the number of entrances that was not statistically significant (Fig. 16A).

Application of MK-801 did not affect locomotor activity in the Carousel maze ($F_{(1, 75)}$= 2.94, P = 0.09) (Fig. 16B). In addition, two-way ANOVA revealed main effect of 3α5βP-Glu treatment ($F_{(6, 75)}$= 3.05, p = 0.01). However, the source of these effects could not be determined by Newman-Keuls post-hoc test. Interactions between the two factors did not reach significance ($F_{(6, 75)}$= 1.1, p > 0.05).

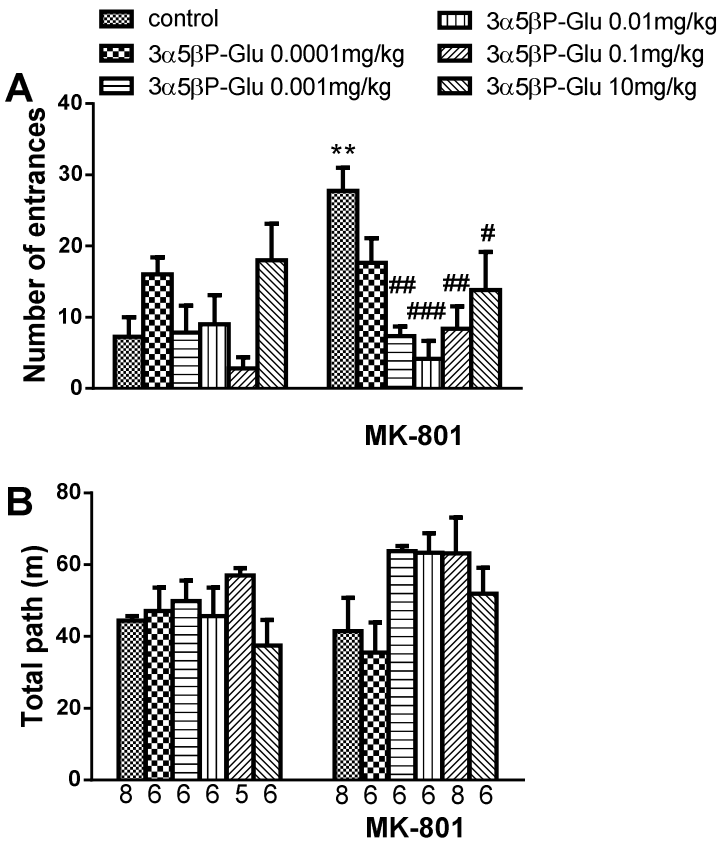


Figure 16: Effects of 3α5βP-Glu and MK-801 treatment on spatial learning and locomotor activity in the Carousel maze. Effect of MK-801 (0.1 mg/kg) and 3α5βP-Glu treatment was assessed by a measure of spatial learning (number of entrances), (A) and locomotor activity (B). Administration of 3α5βP-Glu did not alter spatial learning at any dose in naive rats. MK-801 induced spatial learning deficit, which was reversed by 3α5βP-Glu administration at doses of 0.001, 0.01, 0.1, 1 and 10 mg/kg. Locomotor activity was not significantly affected either by administration of MK-801 or any dose of 3α5βP-Glu. Numbers below the bars denote the numbers of animals in particular groups. Data are represented as mean ± SEM. **$p < 0.01$ compared to saline/saline group. #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ compared to MK-801/saline group.

4.2.2. Locomotor activity in open-field test

MK-801 significantly increased locomotor activity in the open-field test (Fig. 17). A two-way ANOVA found a significant main effect of MK-801 ($F_{(1, 54)} = 8.98$, $p < 0.01$), but not 3α5βP-Glu ($F_{(2, 54)} = 0.36$, $p > 0.05$) or interaction between the two drugs ($F_{(2,54)} = 0.49$, $p > 0.05$). Thus, 3α5βP-Glu does not affect MK-801-induced hyperactivity (Fig. 17). Importantly, it does not affect open-field locomotion when applied alone either.

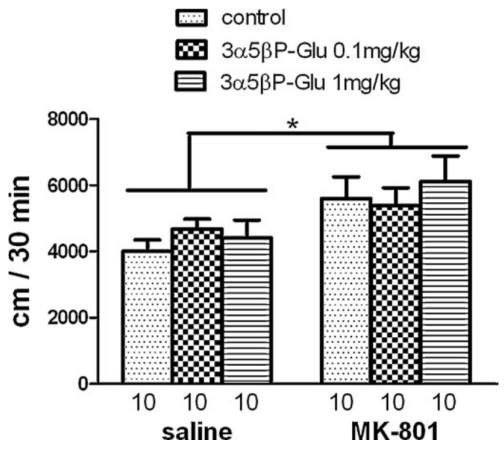


Figure 17: Effects of 3α5βP-Glu and MK-801 treatment on locomotion in the open-field test. Data are represented as mean ± SEM. *$p < 0.05$ main effect of MK-801 administration. Numbers below the bars denote the numbers of animals in particular groups.

4.3. Experiment 3: The effect of 3α5βP-Glu in NMDA lesion of hippocampi

4.3.1. Spatial learning in the Carousel maze

3α5βP-Glu ameliorated spatial learning deficit induced by hippocampal lesion as assessed by the number of entrances into the punished region (Fig. 18A). A Post-hoc test, measuring the significance of group effect ($F_{(10,53)} = 5.19$, $P < 0.0001$), revealed a considerable increase in "Number of Entrances" after an NMDA induced lesion ($P < 0.05$). However, 3α5βP-Glu ameliorated this deficit when given 30 min after surgery (at a dose of 0.1 or 1 mg/kg, $P < 0.05$ for both) or at a dose of 10 mg/kg given 3 h post-surgically ($P < 0.05$). When applied 24 h after surgery, no dose tested was found to be effective.

Cognitive deficit was also assessed using the "Maximum Time Avoided" parameter (Fig. 18B). A Two-way ANOVA test revealed a significant group effect ($F_{(10,53)} = 5.42$, $P < 0.0001$). As a subsequent post-hoc test specified, NMDA lesion induced deficit ($P < 0.05$, compared to the sham group) was blocked by 3α5βP-Glu only at a dose of 10 mg/kg administered 3 h post-surgery. To some extent, 3α5βP-Glu administration affected locomotor behavior. After calculating the significance of group effect ($F_{(10,53)} = 6.47$, $P < 0.0001$), a Turkey's post-hoc test found that untreated excitotoxic hippocampal lesions resulted in a mild, but insignificant hypolocomotion compared to sham operated rats. In addition, nearly all doses of 3α5βP-Glu treatments 24 h after surgery (with the exception of 0.1 mg/kg) decreased locomotor activity. To a lesser extent, this was also apparent after administrating 1 mg/kg, 3 h after NMDA infusion (Fig. 18C).

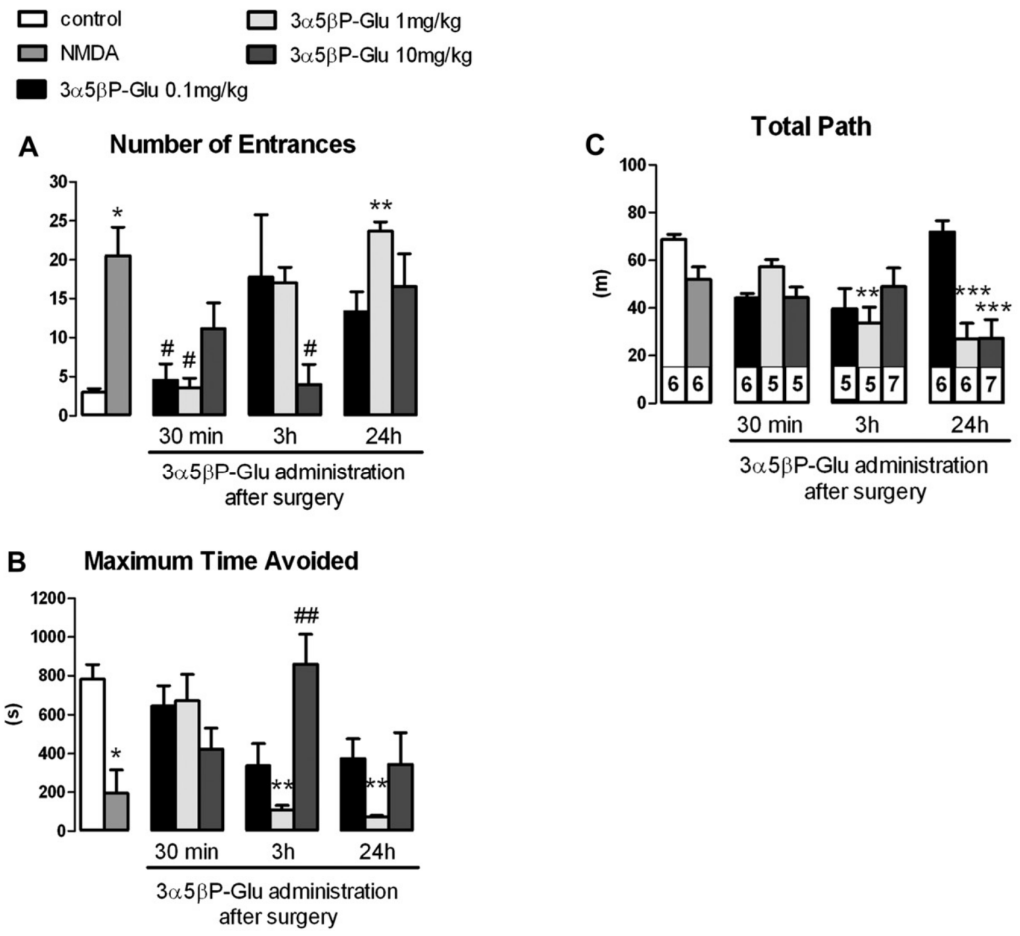


Figure 18: The effect of 3α5βP-Glu administration 30 min, 3 h, or 24 h after NMDA lesion in a behavioral spatial paradigm, the Carousel maze. Cognitive performance was assessed using "Number of Entrances" (A), and "Maximum Time Avoided" (B) parameters. Locomotor activity was assessed by measuring the total path length elapsed during a session (C). Data are presented from the last day of training as mean ± SEM. Number of animals in a group is given at the column base. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared to the control group; $\#P < 0.05$, $\#\#P < 0.01$ compared to the NMDA group.

4.3.2. Morphological damage and neuroinflammation

The NMDA lesion of hippocampi induced strong tissue damage and loss of tissue integrity. We observed damage of pyramidal and granular cells in dorsal hippocampus as well as significant amount of pyknotic cells. The lesion induced also strong FluroJade-B positivity indicating ongoing neurodegeneration. Additionally, the lesion induced strong neuroinflammation. Massive activation of microglia (detected with Iba-1 antibody) and reactive astrogliosis (detected with antibody against glial fibrillary acidic protein, GFAP). The pretreatment of animals with both memantine (5 mg/kg) and 3α5βP-Glu (1 mg/kg) ameliorated the acute neurotoxicity of NMDA as well as reduced neuroinflammatory response. Fig. 19 shows representative pictures of dorsal hippocampus at 7 days post injection (dpi).

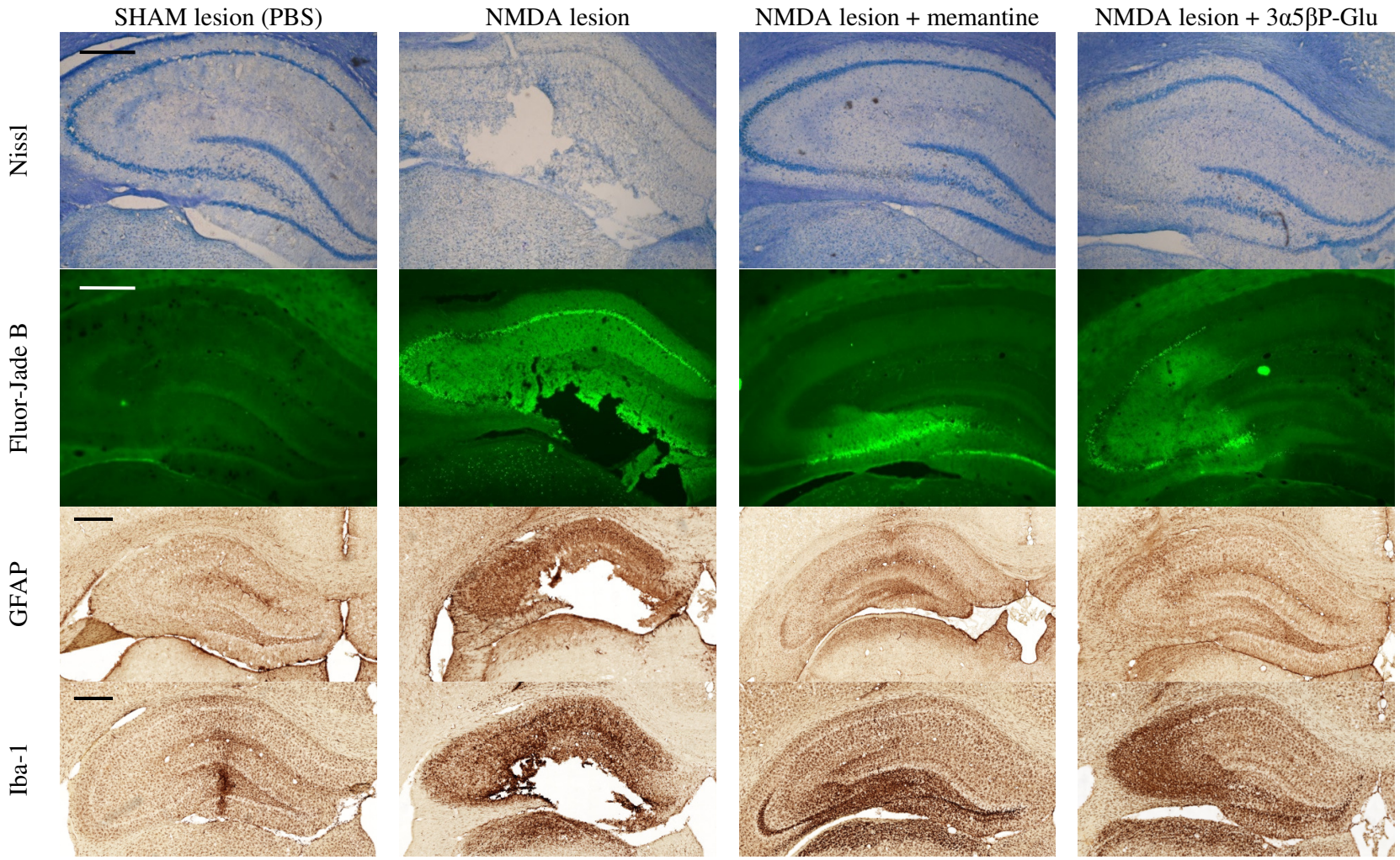


Figure 19: Representative pictures of ipsilateral dorsal hippocampus stained with Nissl, Fluoro-Jade B and antibodies against microglia (Iba-1) and astrocytes (GFAP) 7 days after unilateral NMDA lesion (1 µl of 90 mM NMDA). In comparison to NMDA lesioned animals, the pre-treatment of animals with memantine (5 mg/kg, i.p.) as well as 3α5βP-Glu (1 mg/kg, i.p.) reduced the damage at all levels. Scale bar: A = 500 µm.

4.4. Experiment 4: Morphological characterization of NMDA lesion

In this experiment, we characterized temporal progression of changes induced by unilateral infusion of NMDA (1 μl of 25 mM) into hippocampus. The lesion induced severe neurodegeneration of principal glutamatergic cells, pyramidal and granular neurons, in dorsal hippocampus. The damage of glutamatergic neurons remained focally and followed the diffusion of injected NMDA solution. 1 day post injection (dpi) the neurodegeneration was most prominent within cell body and later propagated to axon and dendrites. However, the damage did not spread into the contralateral hippocampus as well as did not propagate to other structures. Lesion also did not propagate into ventral part of ipsilateral hippocampal formation. Fig. 20 shows representative pictures of dorsal ipsilateral hippocampus 1, 3, 7 and 30 dpi. Sham lesion did not induced neurodegeneration (score 0). Only minor morphological signs of tissue damage in the proximity of cannula were observed. One-way ANOVA did not find significant effect ($P > 0.05$) in progression of damage score in any of studied subfields of hippocampal formation (CA1, CA3, dentate gyrus, hilus and subiculum; Fig. 21). Interestingly, the level of neurodegeneration measured as damage score of FJB positive cells did not change over 30 days. The neurons affected by NMDA were swollen; however, sustained their basic morphology.

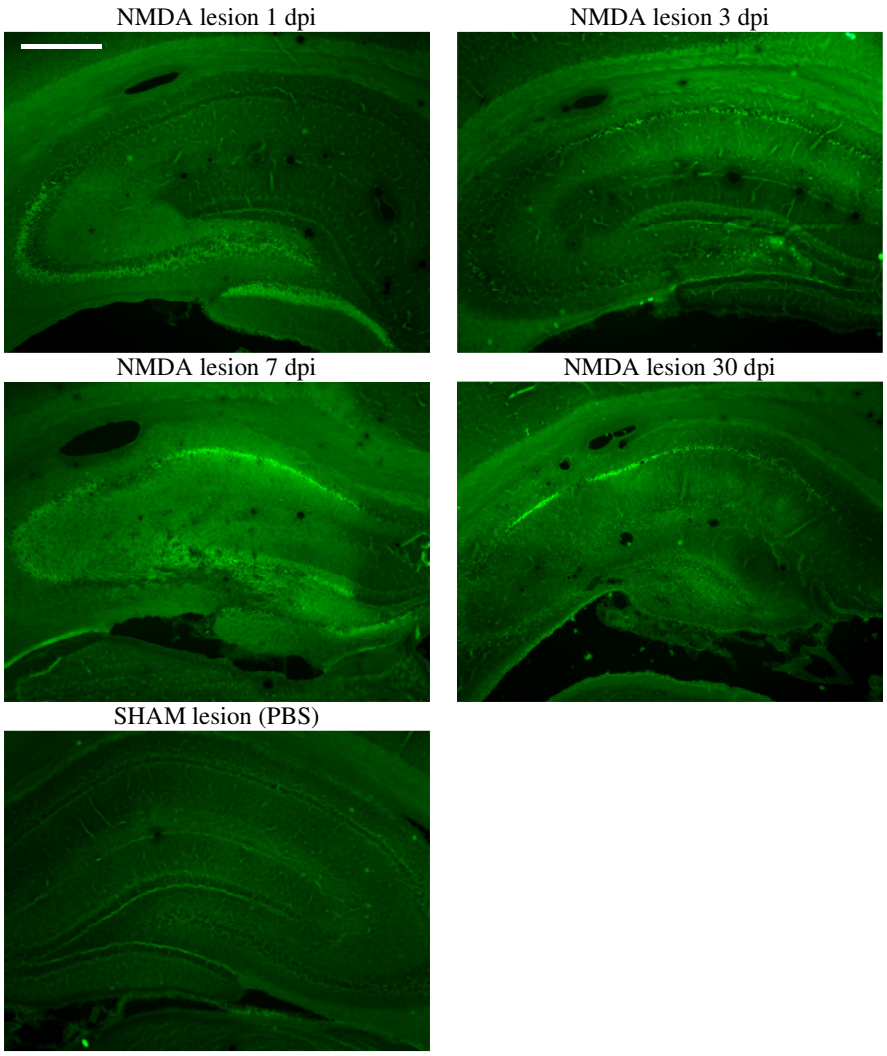


Figure 20: Representative images of Fluoro-Jade B staining of dorsal ipsilateral hippocampus at 1, 3, 7 or 30 dpi of NMDA or PBS (1 dpi). Scale bar: A = 500 µm.

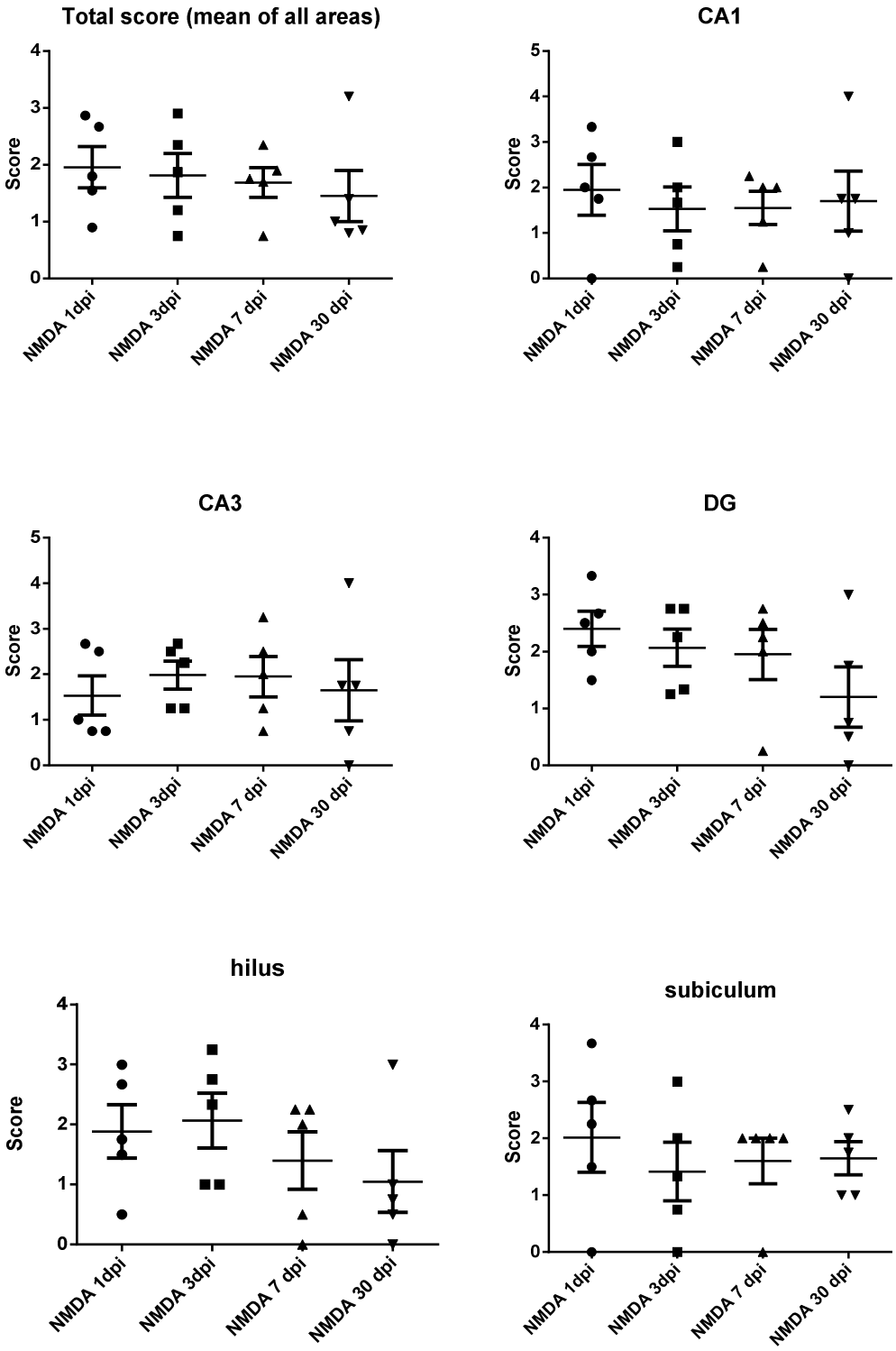


Figure 21: Neural damage score after NMDA lesion of hippocampus 1, 3, 7 and 30 dpi in CA1, CA3, dentate gyrus (DG), hilus and subiculum. No significant differences were observed among individual days post injection. Sham lesion induced no damage (score 0, data not shown) Score scale: 0 – 0-5%, 1 – 6-25%, 2 – 26-50%, 3 – 51-75%, 4 > 75%.

The utilization of specific antibodies against separate populations of GABAergic interneurons revealed that NMDA lesion did not induce their loss in hippocampus at any of studied time-points. Therefore, representative pictures only for 1 dpi are shown. The loss of calcium-binding proteins is a strong indicator of their neurodegeneration, but no ultimate evidence. Parvalbumin positive interneurons were not affected by NMDA lesion. We observed only mild and focal loss of parvalbumin positive interneurons 1 day after the lesion. Calretinin, calbindin and NPY positive interneurons were completely unaffected at all studied time points (Fig. 22).

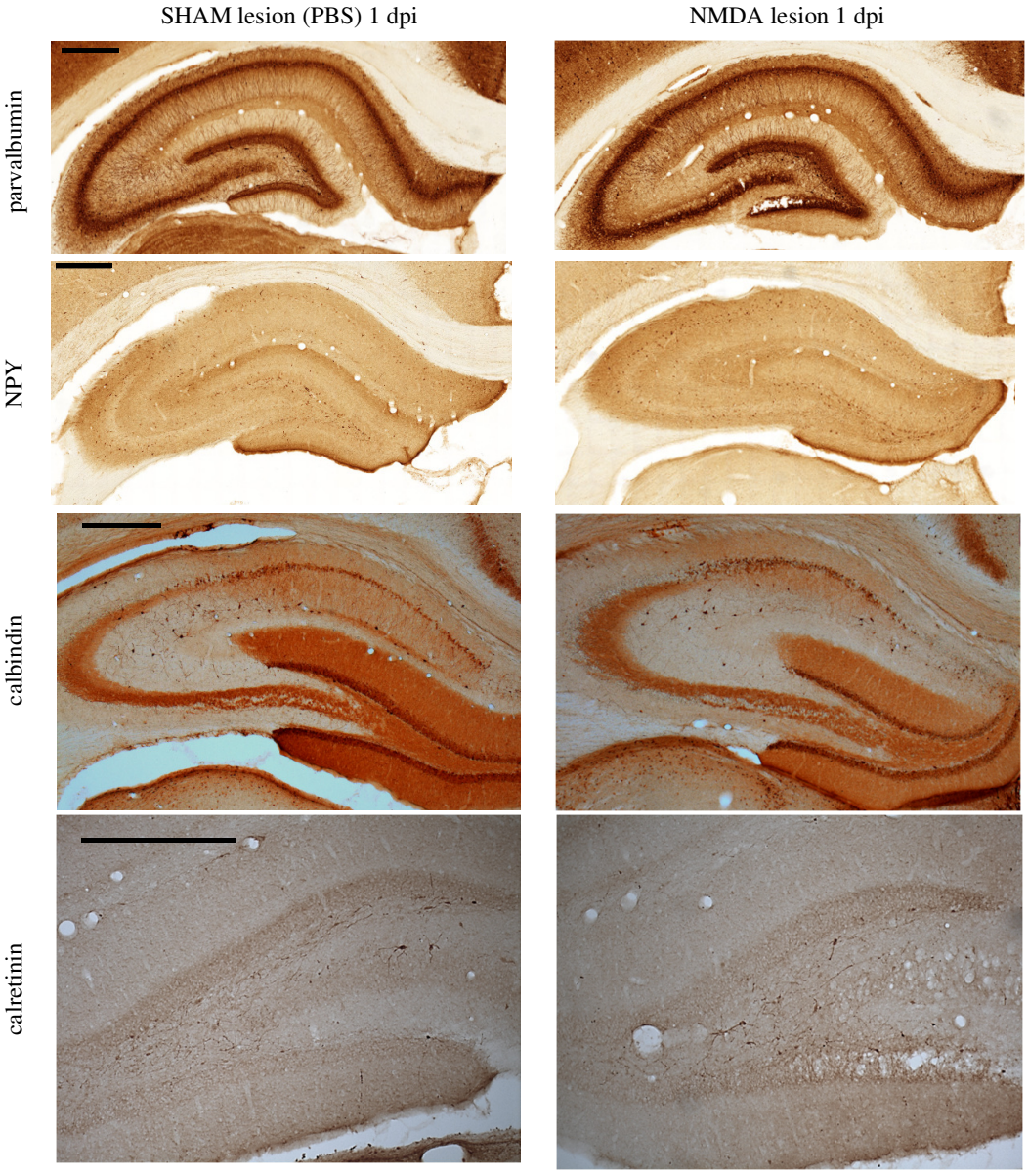


Figure 22: Distribution of parvalbumin, neuropeptide Y (NPY), calbindin and calretinin immunoreactivity 1 day after PBS or NMDA injection into hippocampus. Scale bar: A = 500 µm.

Next, we investigated the effect of NMDA lesion on neuroinflammation. The infusion of NMDA into hippocampus induced progressive hypertrophy and activation of microglia (Iba-1 immunoreactivity), which persisted also 30 dpi of NMDA (Fig. 23 and 24). Two-way ANOVA revealed effect of dpi ($F_{(3, 32)} = 4.156$, $P = 0.0135$), treatment ($F_{(1, 32)} = 51.02$, $P < 0.0001$) as well as their interaction ($F_{(3, 32)} = 6.277$, $P = 0.0018$). Bonferroni's multiple comparison test showed that NMDA lesion group differs from PBS group at 3, 7 and 30 dpi. Infusion of PBS did not induce activation of microglia.

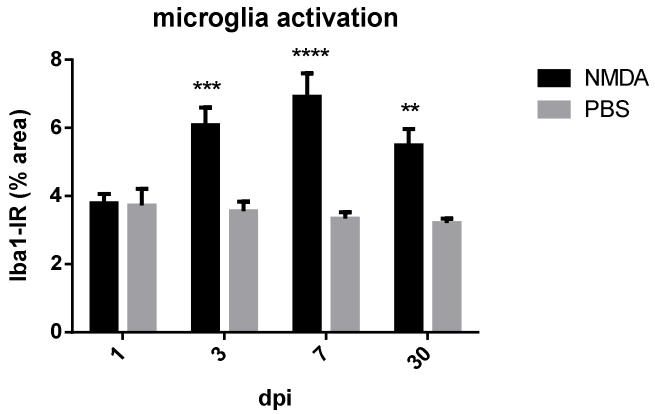


Figure 23: Quantitative analysis of hippocampal anti-Iba-1 immunoreactivity (IR) representing relative percentage of area covered by activated microglia. Values are given as mean ± SEM. ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

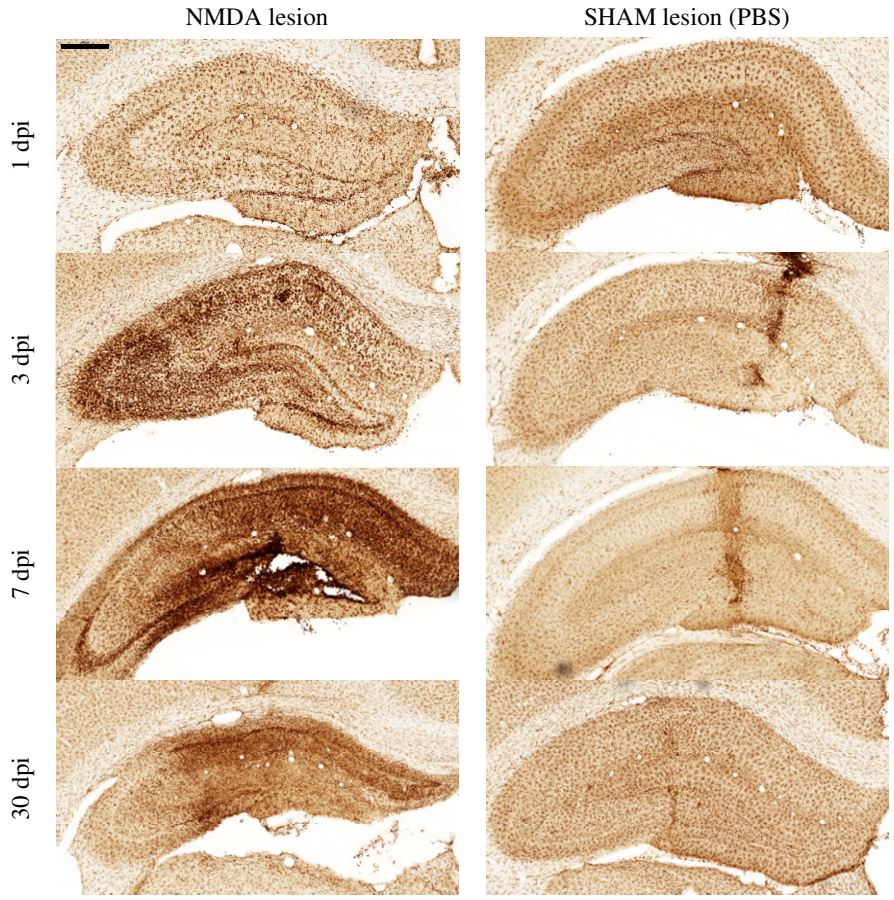


Figure 24: Representative images of immunoperoxidase staining using anti-Iba-1 antibody taken from the ipsilateral dorsal hippocampus at 1, 3, 7 or 30 days post injection (dpi) of NMDA or PBS. Scale bar: A = 500 µm.

The infusion of NMDA into hippocampus induced significant reactive astrogliosis at all studied time points (Fig. 25 and 26). Astroglial scar persisted even 30 dpi of NMDA. Two-way ANOVA revealed effect of dpi ($F_{(3, 32)} = 5.544$, $P = 0.0035$), treatment ($F_{(1, 32)} = 94.41$, $P < 0.0001$) as well as their interaction ($F_{(3, 32)} = 2.944$, $P = 0.0477$). Bonferroni's multiple comparison test showed that NMDA lesion group differs from PBS group at 1, 3, 7 and 30 dpi. Infusion of PBS induced only mild and local astrogliosis at the site of infusion cannula insertion.

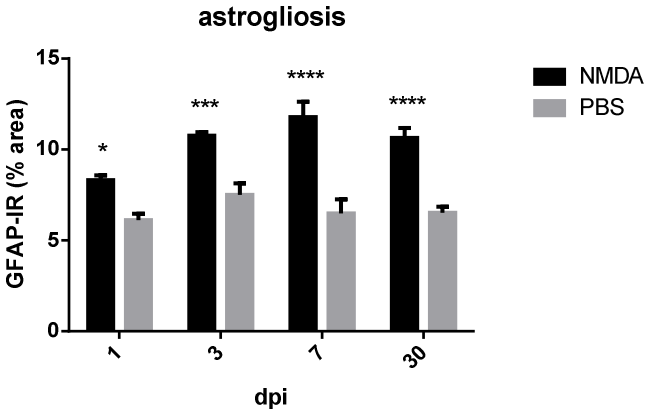


Figure 25: Quantitative analysis of hippocampal anti-GFAP immunoreactivity (IR) representing relative percentage of area covered by astrocytes. Values are given as mean ± SEM. * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$.

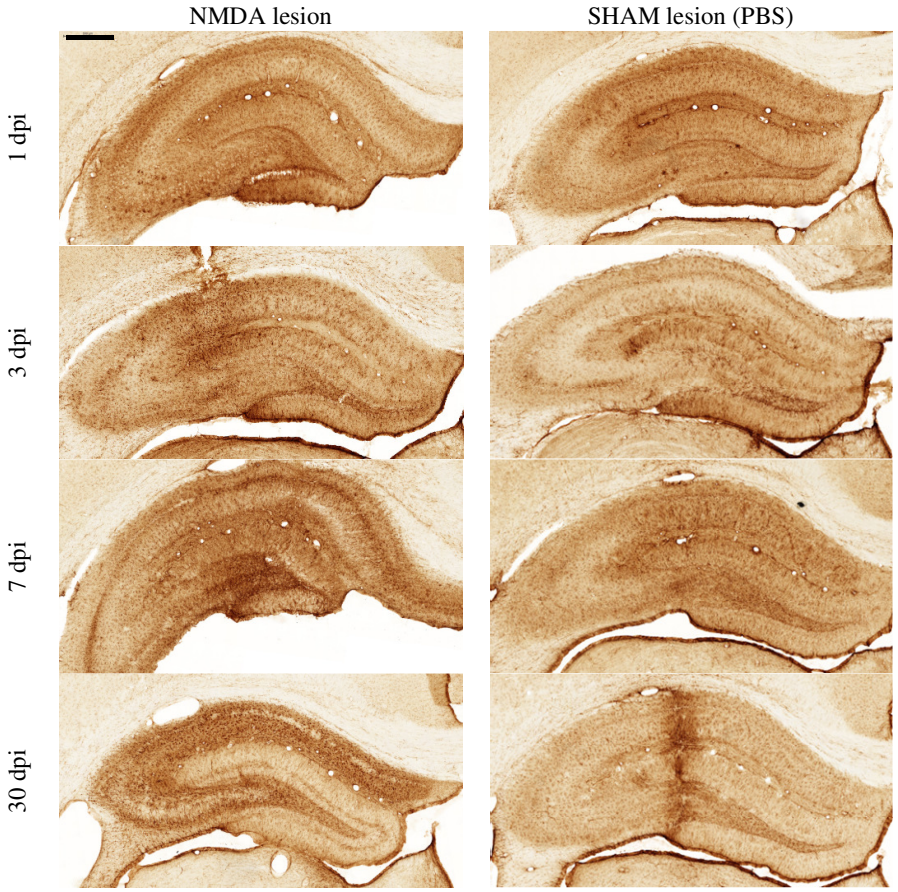


Figure 26: Representative images of immunoperoxidase staining using anti-GFAP antibody taken from the ipsilateral dorsal hippocampus at 1, 3, 7 or 30 days post injection (dpi) of NMDA or PBS. Scale bar: A = 500 µm.

In next experiment, we investigated the expression of NMDAR NR1 and NR2B subunits in hippocampus. Two-way ANOVA revealed effect of dpi for both subunits (NR1: $F_{(3, 31)}$= 5.235, P = 0.0049; NR2B: $F_{(3, 29)}$ = 4.385, P = 0.0116), treatment (NR1: $F_{(1, 31)}$= 20.81, P <

0.0001; NR2B: $F_{(1, 29)} = 22.87$, $P < 0.0001$) as well as their interaction (NR1: $F_{(1, 31)} = 20.81$, $P < 0.0001$; NR2B: $F_{(1, 31)} = 1.676$, $P = 0.2050$). Bonferroni's multiple comparison test showed that NMDA lesion group differs from sham lesion group at 1 and 3 dpi for both markers. Figures 27 and 29 show the quantification of relative optical density for NMDAR NR1 and NR2B subunits, respectively. The increased immunoreactivity correlated positively with FJB positive cells and increased neuroinflammation suggesting that the receptor overexpression is occurring in the lesion affected areas. The changes were reversible and already 7 dpi the NMDA lesioned animals did not differ from sham group ($P > 0.05$). However, representative pictures suggest that the overexpression of NR1 as well as NR2B subunit persisted in lesion affected areas (Fig. 28 and 30, respectively). The contralateral hippocampus was not affected in any group (Fig. 39).

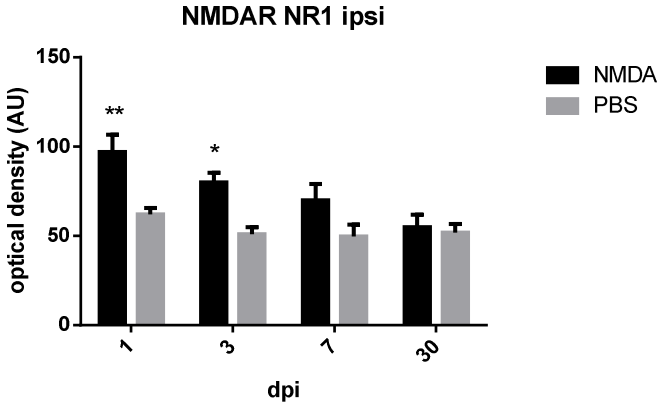


Figure 27: Quantitative analysis of immunoperoxidase staining relative optical density. Values for each hippocampus were normalized to neocortex. Values are given as mean ± SEM. * $P < 0.05$, ** $P < 0.005$.

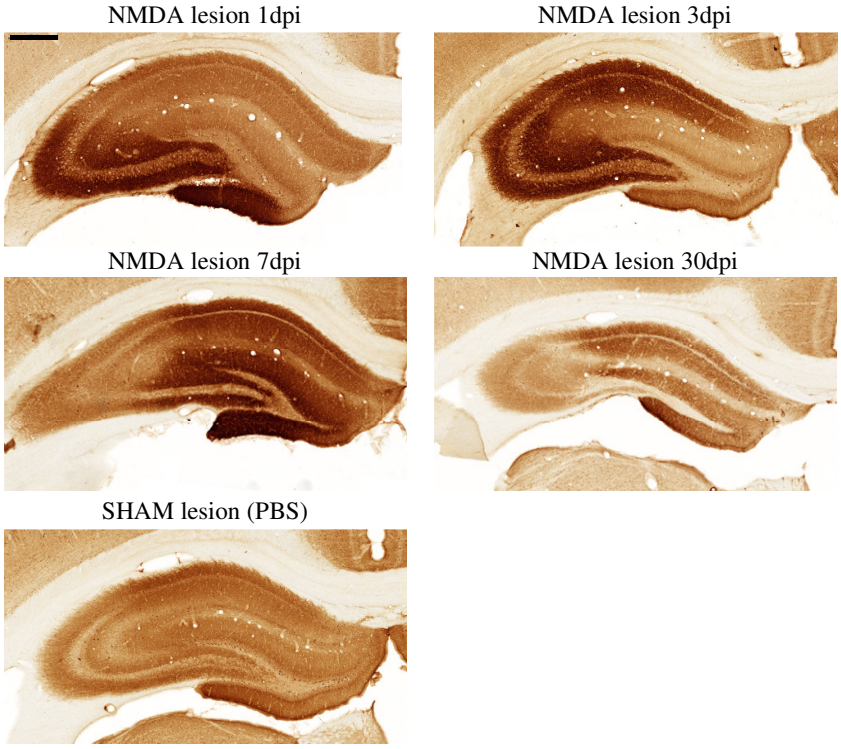


Figure 28: Representative images of immunoperoxidase staining using anti-NMDA receptor NR1 subunit antibody taken from the ipsilateral dorsal hippocampus at 1, 3, 7 or 30 days post injection (dpi) of NMDA. We did not observe significant changes in PBS injected hippocampus; therefore only one representative picture is shown. NMDA lesion induced overexpression of NMDA receptor NR1 subunit in ipsilateral hippocampus. The overexpression was significant 1 a 3 days post injection (dpi) of NMDA ($P < 0.05$). The overexpression correlated with activation of microglia. Scale bar: A = 500 µm.

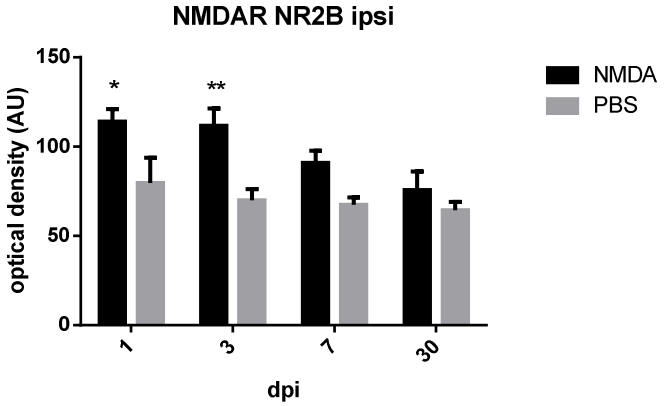


Figure 29: Quantitative analysis of immunoperoxidase staining relative optical density. Values for each hippocampus were normalized to neocortex. Values are given as mean ± SEM. * $P < 0.05$, ** $P < 0.005$.

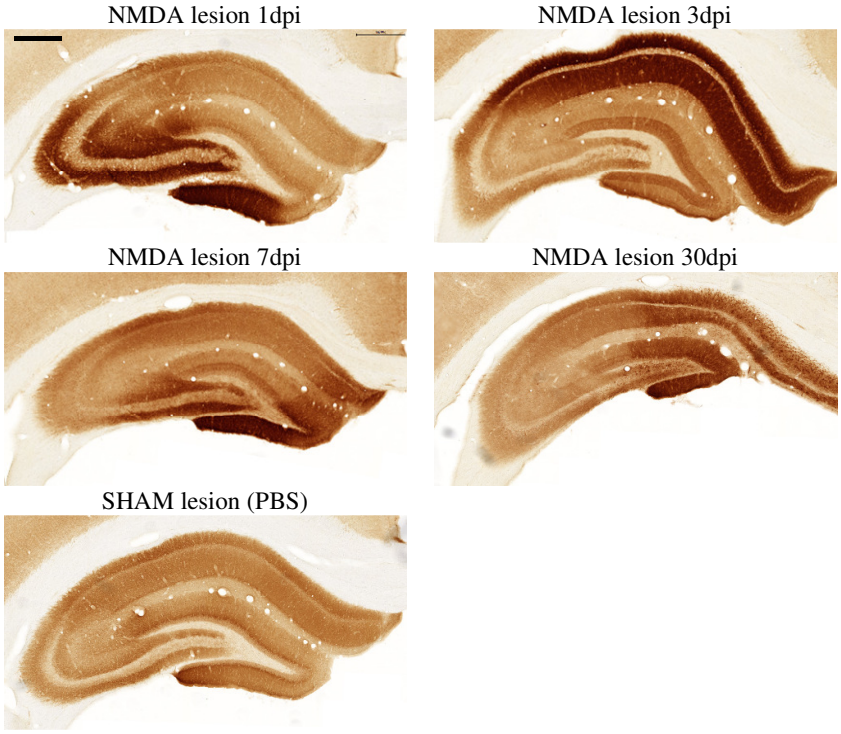


Figure 30: Representative images of immunoperoxidase staining using anti-NMDA receptor NR2B subunit antibody taken from the ipsilateral dorsal hippocampus at 1, 3, 7 or 30 days post injection (dpi) of NMDA. We did not observe significant changes in PBS injected hippocampus; therefore only one representative picture is shown. NMDA lesion induced overexpression of NMDA receptor NR2B subunit in ipsilateral hippocampus ($P < 0.05$). The overexpression was significant 1 a 3 days post injection (dpi) of NMDA. The overexpression correlated with activation of microglia. Scale bar: A = 500 µm.

Furthermore, we analyzed changes in the expression of main $GABA_A$ receptor α1 (Fig. 31 and 32), α2 (Fig. 33 and 34), α5 (Fig. 35 and 36) and γ2 subunits (Fig. 37 and 38). NMDA lesion significantly decreased the expression of $GABA_A$ receptor α5 subunit 30 dpi in ipsilateral hippocampus. Two-way ANOVA revealed effect of dpi ($F_{(3, 29)}= 3.243$, $P = 0.0363$), treatment ($F_{(1, 29)}= 5.293$, $P = 0.0288$) as well as their interaction ($F_{(3, 29)}= 2.973$, $P = 0.0480$). Bonferroni's multiple comparison test showed that NMDA lesion group differs from

PBS group at 30 dpi. Expression of other subunits was not significantly decreased; however, we observed mild loss of immunoreactivity in lesion affected regions. The contralateral hippocampus was not affected in any group (Fig. 39).

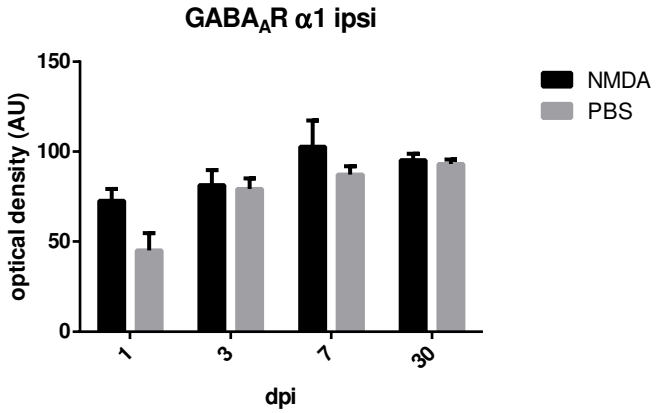


Figure 31: Quantitative analysis of immunoperoxidase staining relative optical density. Values for each hippocampus were normalized to neocortex. Values are given as mean ± SEM.

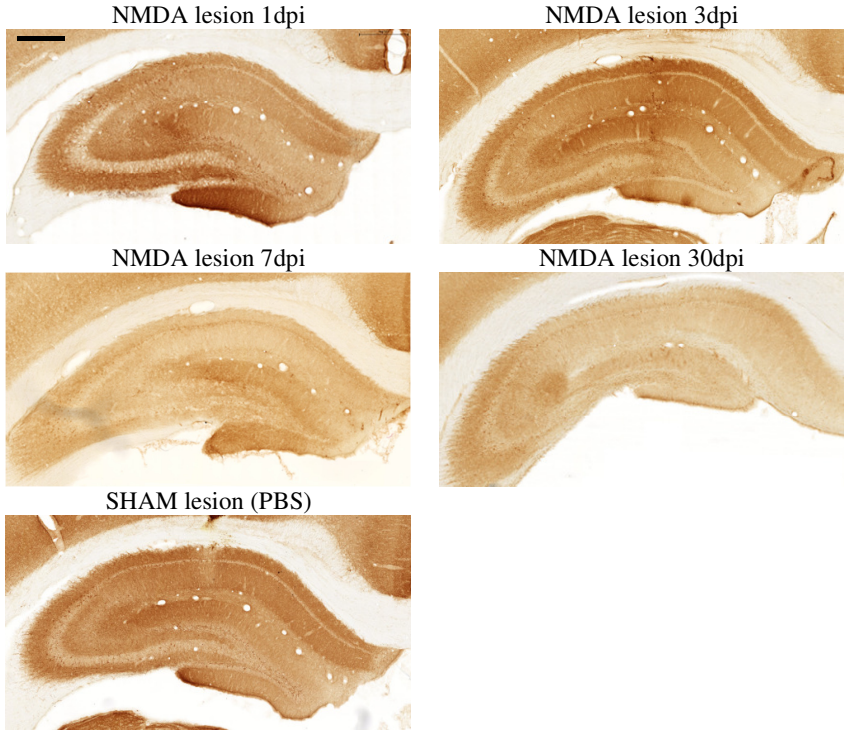


Figure 32: Representative images of immunoperoxidase staining using anti-GABA$_A$ receptor α_1 subunit antibody taken from the ipsilateral dorsal hippocampus at 1, 3, 7 or 30 days post injection (dpi) of NMDA. We did not observe significant changes in PBS injected hippocampus. NMDA did not induce significant changes in α_1 subunit expression ($P > 0.05$). We also did not observed loss of GABA$_A$ receptor α_1 interneurons at any time-point. Scale bar: A = 500 µm.

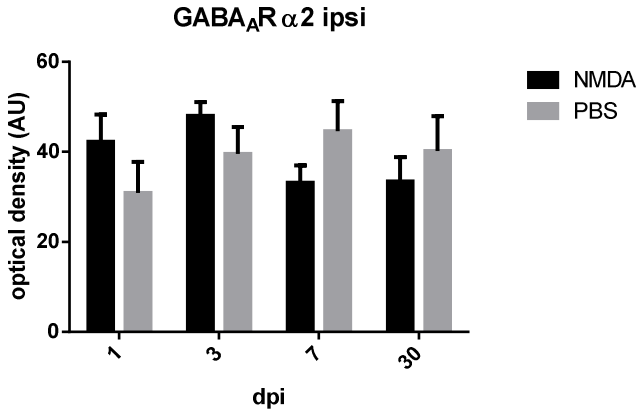


Figure 33: Quantitative analysis of immunoperoxidase staining relative optical density. Values for each hippocampus were normalized to neocortex. Values are given as mean ± SEM.

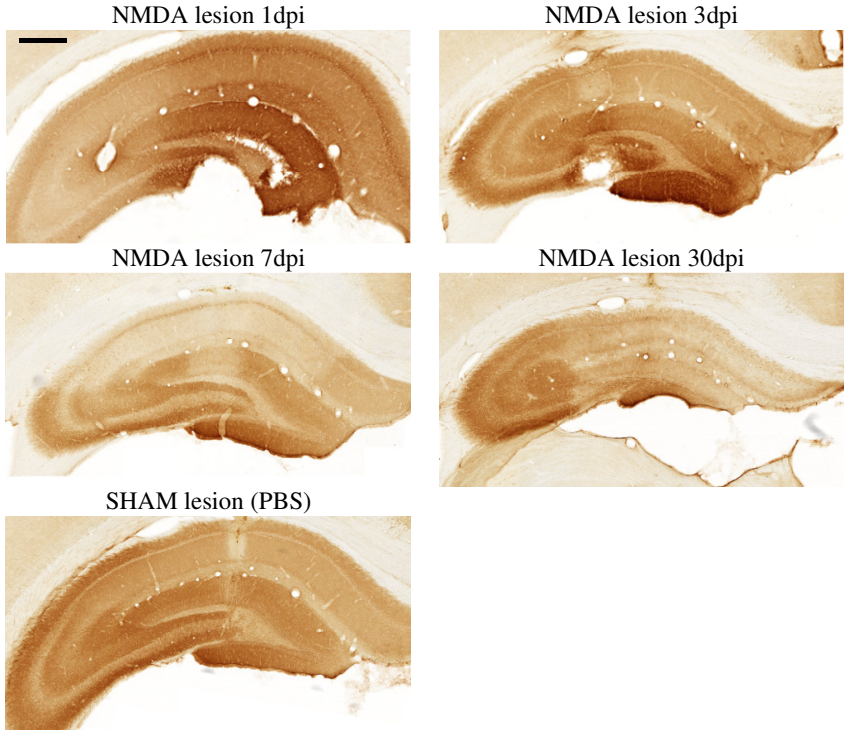


Figure 34: Representative images of immunoperoxidase staining using anti-GABA$_A$ receptor α2 subunit antibody taken from the ipsilateral dorsal hippocampus at 1, 3, 7 or 30 days post injection (dpi) of NMDA. We did not observe significant changes in PBS injected hippocampus. Quantification of relative optical density did not reveal significant changes between NMDA and PBS injected hippocampus ($P > 0.05$); however, we observed local loss of α2 subunit staining at days 7 and 30dpi that correlated with activation of microglia. Scale bar: A = 500 µm.

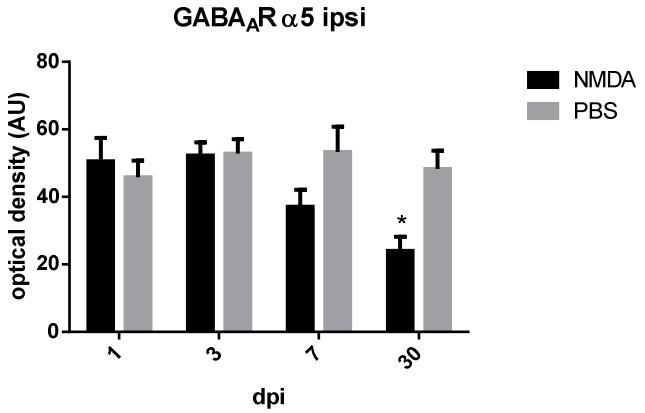


Figure 35: Quantitative analysis of immunoperoxidase staining relative optical density. Values for each hippocampus were normalized to neocortex. Values are given as mean ± SEM. * $P < 0.05$.

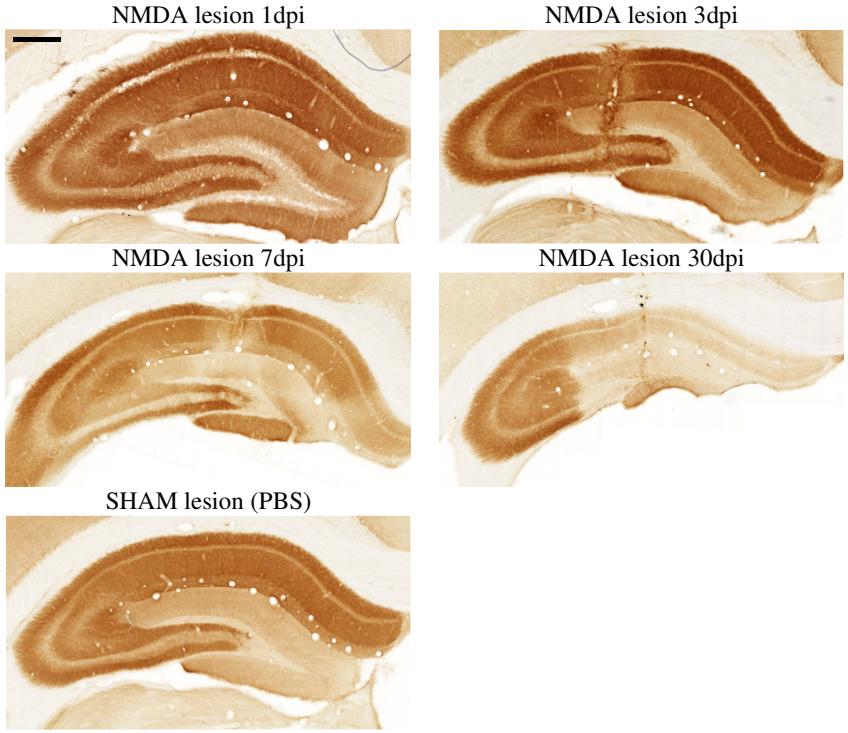


Figure 36: Representative images of immunoperoxidase staining using anti-GABA$_A$ receptor α5 subunit antibody taken from the ipsilateral dorsal hippocampus at 1, 3, 7 or 30 days post injection (dpi) of NMDA. We did not observe significant changes in PBS injected hippocampus. NMDA induced progressive loss of α5 subunit expression. The expression was significantly different compare to PBS sham lesion at 30 dpi ($P < 0.05$). Scale bar: A = 500 µm.

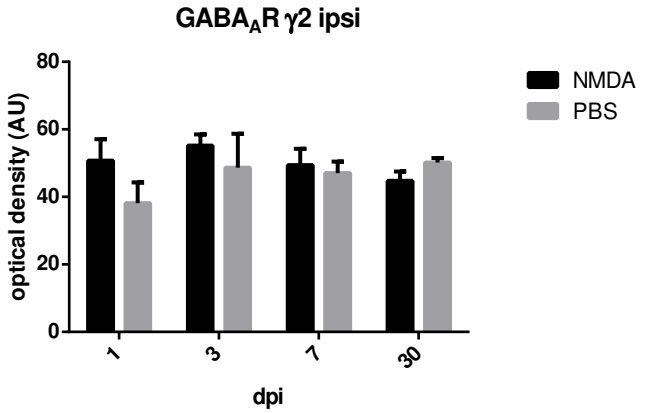


Figure 37: Quantitative analysis of immunoperoxidase staining relative optical density. Values for each hippocampus were normalized to neocortex. Values are given as mean ± SEM.

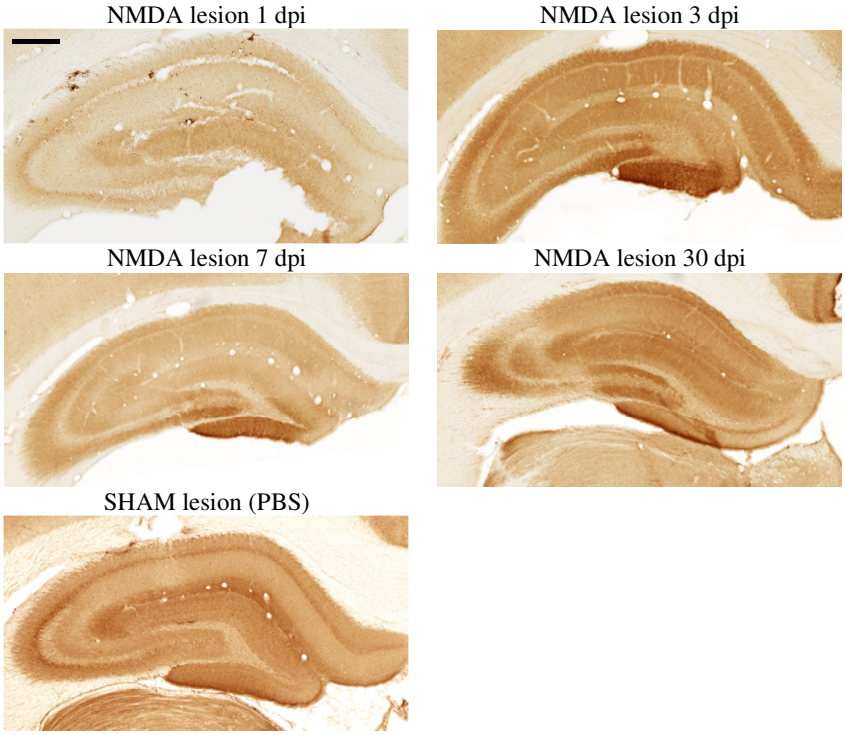


Figure 38: Representative images of immunoperoxidase staining using anti-GABA$_A$ receptor γ2 subunit antibody taken from the ipsilateral dorsal hippocampus at 1, 3, 7 or 30 days post injection (dpi) of NMDA. We did not observe significant changes in PBS injected hippocampus. Quantification of relative optical density did not reveal significant changes between NMDA and PBS injected hippocampus ($P > 0.05$). Scale bar: A = 500 µm.

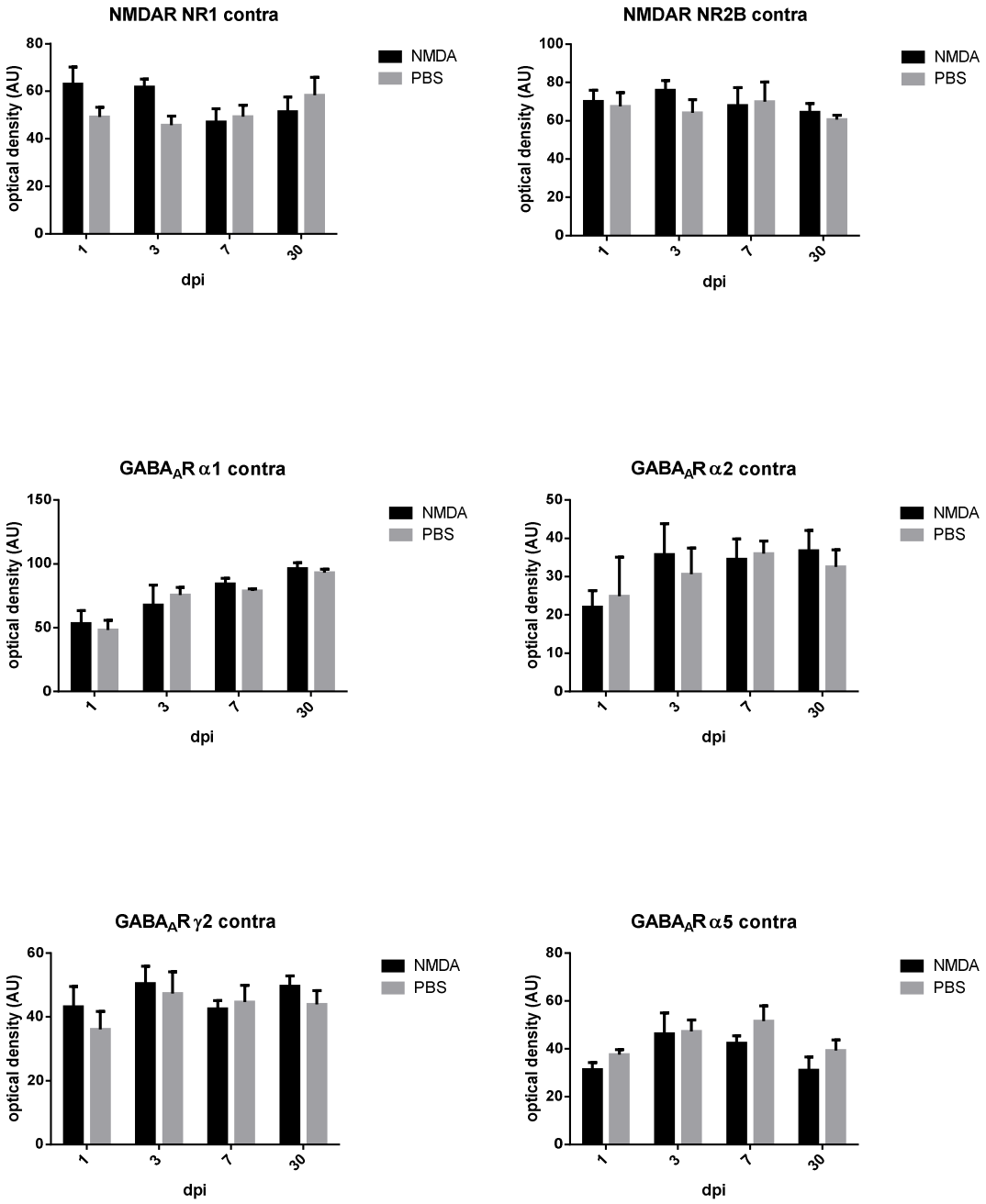


Figure 39: Quantitative analysis of immunoperoxidase staining relative optical density in contralateral (intact) hippocampus. Values for each hippocampus were normalized to neocortex. Statistical analysis did not reveal any difference between NMDA and PBS group ($p < 0.05$). Values are given as mean ± SEM.

5. DISCUSSION

5.1. Experiment 1

The inhibition of NMDA receptors has been linked to induction of psychotomimetic behavior (Bubenikova-Valesova V. et al., 2008). Therefore, we focused on the following behaviors: increased locomotor activity in a novel environment, information processing deficit (prepulse inhibition of the acoustic startle response) and memory impairment in passive and active avoidance tasks (Geyer M.A. and Swerdlow N.R., 2001; van den Buuse M., 2010). We found that 3α5βP-Glu did not induce psychotomimetic side effects. Additionally, it did not induce sedation. Similarly, 3α5βP-HS did not affect locomotion up to a dose of 20 mg/kg in rats (Sadri-Vakili G. et al., 2003). The reason for this convenient safety profile compared with other NMDA antagonists – for instance MK-801, phencyclidine, is unclear, but a distinct mode of action of neurosteroids such as 3α5βP-HS on NMDA receptors may be possible. These neurosteroids have a use-dependent but voltage- independent effect on NMDA receptors, possibly mediated by binding to the extracellular side of the NMDA receptor (Park-Chung M. et al., 1997; Sedlacek M. et al., 2008).

We propose that the absence of adverse side effects in this study may be related to the use-dependent action of 3α5βP-Glu that acts preferably on extrasynaptic and activated receptors leaving normal synaptic transmission unaffected. This hypothesis reflects the principle of memantine safety (Lipton S.A., 2005; Chen H. and Lipton S.A., 2006). Memantine is the most studied and clinically used use-dependent NMDA receptor antagonist (Minkeviciene R. et al., 2004; Zoladz P. et al., 2006), which is approved for the treatment of Alzheimer's disease. However, this work showed that memantine induces dose-dependent cognitive impairment in passive avoidance task after acute treatment as well as mild increase in locomotor activity after sub-chronic treatment in Carousel Maze. Disrupted memory formation by memantine in a radial maze was also reported (Wise L. and Lichtman A.H., 2007). A study of Creely et al. showed impaired navigation in a hole-board test and “PCP-like” stereotypes in adult rats at doses smaller than one-half the neuroprotective dose (Creely C. et al., 2006). In another study, memantine had no effect on memory and learning abilities in naive rats in the Morris water maze (Saab B. et al., 2011) and yet another study described enhanced memory retention in the Morris water maze with this drug (Minkeviciene R. et al., 2008).

Taken together, 3α5βP-Glu did not induce psychotomimetic side effects such as hyperlocomotion, sensorimotor gating deficit or cognitive deficit. Only the higher dose 10 mg/kg induced reduction in learning abilities; however, this effect was not significant. Contrary, memantine induced both mild increase in locomotor activity in Carousel maze and

significant cognitive impairment in passive avoidance task. These results suggest that 3α5βP-Glu may represent an NMDA antagonist devoid of psychotomimetic effects, even safer compound than memantine.

5.2. Experiment 2

We demonstrated precognitive effects of 3α5βP-Glu in an animal model of schizophrenia induced by the systemic administration of MK-801 at a dose of 0.1 mg/kg. This dose of MK-801 was selected because it elicits a cognitive deficit in the Carousel maze without locomotor activity sensitization (Stuchlik A. and Vales K., 2005). Higher doses of MK-801 (such as 0.2 mg/kg or 0.3 mg/kg) severely affect motor activity confounding the cognitive effects in the Carousel maze (Stuchlik A. and Vales K., 2005). The number of entrances into the to-be-avoided sector in the Carousel maze was significantly decreased after application of 0.001 mg/kg, 0.01 mg/kg, 1 mg/kg and 0.1 mg/kg of 3α5βP-Glu and MK-801 (compared to MK-801 alone; Fig. 16A). The lowest (0.0001 mg/kg) and the highest dose (10 mg/kg) of 3α5βP-Glu failed to produce procognitive effect (Fig. 16A) in this model. These results suggest a U-shaped dose-dependent effect of 3α5βP-Glu. This effect might be due to the binding of 3α5βP-Glu to $GABA_A$ receptor (Vyklicky L., unpublished results). It is conceivable that the lowest dose (0.0001 mg/kg) was not sufficient to inhibit NMDA receptors and normalize cognitive functions. In contrast, the highest dose (10 mg/kg) might have activated $GABA_A$ receptors, preventing the "stabilization" effect on the excitatory system, which was observed at the dose range of 0.001– 1 mg/kg. We have observed a similar U-shaped effect in experiment 3, where 3α5βP-Glu applied 30 min after the NMDA lesion showed neuroprotective effect at doses of 0.1 mg/kg and 1 mg/kg but not at 10 mg/kg. As noted above, higher doses of 3α5βP-Glu may interact with GABAergic neurotransmission and thus modulate general effects of 3α5βP-Glu.

Although administration of MK-801 either alone or in co-application with any dose of 3α5βP-Glu had no effect on locomotion in the Carousel maze (Fig. 16B), locomotor activity was slightly increased after MK-801 administration (0.1 mg/kg) in the open-field test and 3α5βP-Glu did not reduce this hyperlocomotion. This difference is probably due to the restraining effect of the task demands in the Carousel maze on spontaneous locomotion, which is absent in the open-field test. It is worth emphasizing that the hyperlocomotion was proposed as an experimental analogy of positive symptoms of schizophrenia (Bubenikova-Valesova V. et al., 2008). It appears that 3α5βP-Glu exerts procognitive effects in this animal model but no amelioration of positive symptoms.

In previous studies reported pro-cognitive effects of glycine binding site antagonists of NMDA receptor in the rodent models. Drug L-701, 324, antagonist of glycine-binding site,

suppressed amphetamine-induced behavioral changes in rodents (Bristow L.J. et al., 1996). The same results were obtained in phencyclidine induced model of schizophrenia (Karcz-Kubicha M. et al., 1999). Contrary to 3α5βP-Glu, these drugs additionally decreased locomotion activity (Karcz-Kubicha M. et al., 1999). Interestingly also PS, a positive allosteric NMDAR modulator, normalized animal behavioral abnormalities in the animal models of schizophrenia induced by either MK-801 or dopamine transporter knockout (Wong P. et al., 2015). PREG has only mild effect in this study. Another study has shown that the administration of pregnenolone led to some improvement of negative symptoms and cognitive deficit in schizophrenic patients (Marx C.E. et al., 2009). Considering that PREG has only minimal effect on NMDA and $GABA_A$ receptors, these data suggest that there might be another target that is responsible for PREG therapeutic effect.

Recent studies have suggested that impaired synthesis of neurosteroids might contribute to the pathophysiology of schizophrenia (Marx C.E. et al., 2006). Furthermore, atypical antipsychotics were found to alter neurosteroids concentrations (Marx C.E. et al., 2000 and 2006; Barbaccia M.L. et al., 2001). Decreased levels of these neurosteroids may be indicative of vulnerability to psychosis whereas increased levels may result in therapeutic benefit.

Taken together, 3α5βP-Glu ameliorated spatial cognitive deficit induced by MK-801 in rats. However, next experiments are needed to confirm its pro-cognitive effect. Utilization of another animal models such as withdrawal from sub-chronic phencyclidine administration or neurodevelopmental models could help us to better understand its therapeutic potential. Additionally, more complex battery of behavioral tests should be performed to investigate also other cognitive systems that are impaired in schizophrenia including working memory, attention and executive functions.

5.3. Experiment 3

Next we evaluated the neuroprotective and pro-cognitive effects of 3α5βP-Glu on spatial learning performance in rats with NMDA-induced lesions of the hippocampi. The intrahippocampal microinjection of NMDA significantly worsened performance of rats in the Carousel maze as measured by the "Number of Entrances" and "Maximum Time Avoided" parameters. Simultaneously, NMDA-lesioned rats had decreased locomotor activity during performance of the task. The Carousel maze is highly dependent on an intact hippocampus (Cimadevilla J.M. et al., 2001) and can be used to test cognitive coordination in certain configurations (Wesierska C. et al., 2005; Kubik S. et al., 2006). The administration of 3α5βP-Glu at particular time intervals after NMDA injection ameliorated such behavioral deficits in rats assayed in the Carousel maze compared to "lesioned-only" animals.

Furthermore, we found that 3α5βP-Glu and memantine pretreatment provided mild neuroprotection at morphological level as well. It decreased the level of neurodegeneration as well as neuroinflammation assessed as microglial activation and astrogliosis. Nevertheless, it did not fully protect from lesion development.

Such protective action on the behavioral consequences of excitatory damage is often observed after application of drugs inhibiting the NMDA receptor such as MK-801 (McDonald J. et al., 1990); however, many such drugs are excluded from the search for potential human neuroprotectants due to their significant side-effects ranging from sensory and motor disturbances to induction of schizophrenia-like symptoms. In fact, some of these drugs are used to induce animal models of this disorder (Bubenikova-Valesova V. et al., 2008).

The neuroprotective and pro-cognitive effects of 3α5βP-Glu is in accordance with several reports aimed at testing potential neuroprotective properties of other neuroactive steroid derivatives such as 3α5βP-HS. For example, systemic administration of 3α5βP-HS after focal cerebral ischemia induced by middle cerebral artery occlusion significantly reduced infarct size in mice (Weaver C.E. et al., 1997). Even when 3α5βP-HS was applied 30 min after the onset of ischemia the volume of cortical infarct was reduced by 39 %. This study also reported evidence supporting 3α5βP-HS neuroprotectivity *in vitro*. NMDA-induced cell damage was dose-dependently attenuated by co-application of 3α5βP-HS (Weaver C.E. et al., 1997). In another study, 3α5βP-HS significantly improved ischemia induced behavioral deficit in rabbits if administered up to 30 min following ischemia using an irreversible spinal cord ischemia model (Lapchak P.A., 2004). The same authors evaluated the neuroprotective properties of 3α5βP-HS following embolic stroke if given 5 min after embolization (Lapchak P.A., 2006). An additional synthetic analogue (3α-ol-5β-pregnan-20-one, l-Valine ester hydrochloride) showed good solubility in water and was capable of reducing edema in an animal model of traumatic brain injury (MacNevin C.J. et al., 2009). These results together with our present study suggest that neurosteroid substances and their derivatives may act protectively against excitotoxic damage to the brain and subsequent behavioral disturbances, via a mechanism related to the action of these substances on NMDA receptors.

5.4. Experiment 4

The NMDA lesion of hippocampus has been utilized by many researchers in the past to induce amnesia (Ferbinteanu J. and McDonald R.J., 2001; Ito R. et al., 2005) as well as to induce intense neuronal discharge (seizure activity) during the hours that follow its injection (Zaczek R. and Coyle J.T., 1982). In this model, single unilateral injection of NMDA into

hippocampus induced selective cell damage of CA1-CA3 pyramidal cells, dentate gyrus, hilar cells, and subiculum, but there was an absence of damage to areas and structures outside hippocampus. These data are in accordance with another study (Jarrard L.E. and Meldrum B.S., 2008) comparing effect of NMDA, ibotenic acid and kainic acid. However, compare to kainic acid lesion of hippocampus, another model of neurodegeneration and temporal lobe epilepsy, NMDA lesion does not produce that uniform and anatomically defined pattern of damage. Single injection of kainic acid into hippocampus is characterized by loss of hilar neurons, partial loss of CA1 and CA3 and progressive dispersion of dentate gyrus (Bouilleret V. et al., 2000) whereas NMDA lesion damage pattern was more variable and presumably followed the spread of injected solution. Kainic acid lesion is known to induce immediate loss of parvalbumin and calbindin positive interneurons in dentate gyrus and CA1 (Bouilleret V. et al., 2000). Surprisingly, NMDA lesion did not affect GABAergic interneurons at all studied time points.

Furthermore, NMDA lesion induced changes in expression of NMDA and $GABA_A$ receptor subunits. We found overexpression of NR1 and NR2B subunits of NMDAR 1 and 3 dpi. On the other hand, we found mild loss of GABA receptor subunits in affected areas. The α5 subunit was significantly downregulated at 30 dpi. The immunoreactivity of this subunit was found to be decreased 30 days after kainic acid lesion in CA1, CA3 and hilus, and increased in dentate gyrus (Bouilleret V. et al., 2000). $GABA_A$ receptors containing α5 subunit are localized preferentially extrasynaptically and generate a tonic conductance that regulates the excitability of pyramidal neurons in CA1 and CA3 regions of the hippocampus (Caraiscos V.B. et al., 2004). The NMDA lesion did not induce significant decrease in α1, 2 and γ2 subunits immunoreactivity. However, we observed mild loss in lesion affected areas. Kainic acid lesion is known to induce significant downregulation of α1, 2, 3 and γ2 subunits in CA1 and CA3 regions 30 dpi. Authors described also regional increase of α1, 2 and α5 in dentate gyrus (Bouilleret V. et al., 2000).

It seems that NMDA lesion breaks the balance between GABAergic and glutamatergic systems and makes hippocampus permanently more excitable. This hypothesis is strongly supported by a number of studies investigating NMDA as a pro-convulsive drug. The systemic administration of NMDA has been utilized to elicited epileptic motor seizures in developing rats aged from 7 to 25 days as well as in young adults (Mares P. and Velisek L., 1992). Another study showed that NMDA administration causes a unique seizure phenotype in the developing brain, with subsequent deficits in spatial learning and an increased susceptibility to PTZ seizures in adulthood (Stafstrom C.E. and Sasaki-Adams D.M., 2003). Moreover, upregulation of NMDA receptors in hippocampus has been described in the

pentylenetetrazol-induced "kindling" model of epilepsy (Ekonomu A. and Angelatou F., 1999).

Additionally, we found progressive activation of microglia and reactive astrogliosis. The neuroinflammation persisted even 30 days after the lesion. It is well known, that formation of glial scar is changing diffusion parameters and reducing volume of extracellular space (Sykova E. et al., 1999). This leads to increase of local concentration of neurotransmitters, metabolites as well as reactive oxygen species. For example, increased concentrations of glutamate can further shift the imbalance between glutamatergic and GABAergic system and facilitate the development of seizures.

Taken together, our data suggest that NMDA lesion of hippocampus may represent an interesting model of neurodegeneration and/or epilepsy characterized by severe neuroinflammation, overexpression of NMDAR at an early stage and downregulation of $GABA_A$ receptor subunits at 30 dpi.

6. CONCLUSIONS

The main aim of this thesis was to investigate the biological significance of 3α5βP-Glu. We investigated the effect of 3α5βP-Glu in naïve rats. Next, we evaluated effects of 3α5βP-Glu in animal models of schizophrenia and NMDA lesion of hippocampi. Furthermore, we morphologically characterized the NMDA lesion model.

As expected, 3α5βP-Glu did not induce significant psychotomimetic side effects such as hyperlocomotion, sensorimotor grating deficit or memory impairment. Additionally, it did not induce sedation that is typical for $GABA_A$ agonists. Even sub-chronic administration of 3α5βP-Glu had no effect on locomotor activity and learning in the Carousel maze. Other use-dependent NMDA antagonists (MK-801 and ketamine) induced dose-dependent hyperlocomotion and cognitive deficit.

Next, 3α5βP-Glu showed dose dependent pro-cognitive effects in animal model of schizophrenia; however, it had no effect on hyperlocomotion in this model. 3α5βP-Glu also ameliorated spatial learning deficit of rats induces by NMDA lesion of hippocampi in the Carousel maze and had mild effect on NMDA induced damage of hippocampus when applied before.

Additionally, the morphological analysis of hippocampal NMDA lesion revealed overexpression of NMDAR NR1 and NR2B subunits, 1 and 3 dpi, and downregulation of $GABA_AR$ α5 subunit, 30 dpi. The lesion was very conservative, did not spread to other structures and did not affect GABAergic interneurons. The lesion progression was accompanied with severe activation of microglia and astrogliosis.

Taken together, this thesis showed that neuroactive steroid 3α5βP-Glu, a use-dependent NMDAR antagonist and $GABA_AR$ modulator, does not induce psychotomimetic side effects typical for NMDA channel blockers. Furthermore, this thesis suggests that 3α5βP-Glu may represent a potential neuroprotectant to treat neurological diseases linked to excitotoxicity, as well as a drug to modify cognitive symptoms of psychiatric disorders linked to imbalance between glutamatergic and GABAergic neurotransmission. Indeed, further preclinical development is necessary to identify the most promising disease indications as well as other therapeutically interesting targets for neuroactive steroids.

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8. LIST OF PUBLICATIONS

a) Thesis relevant publications:

Impacted journals:

Rambousek L., Bubenikova-Valesova V., Kacer P., Syslova K., Kenney J., Holubova K., Najmanova V., Zach P., Svoboda J., Stuchlik A., Chodounska H., Kapras V., Adamusova E., Borovska J., Vyklicky L., Vales K. **Cellular and behavioural effects of a new steroidal inhibitor of the N-methyl-D-aspartate receptor 3α5β-pregnanolone glutamate.** Neuropharmacology. 2011;61(1- 2):61-8. **IF= 4. 72**

Vales, K.; **Rambousek, L.**; Holubova, K.; Svoboda, J.; Bubeníkova-Valesova, V.; Chodounska, H.; Vyklicky, L.; Stuchlik, A. **3α5β-Pregnanolone glutamate; a use-dependent NMDA antagonist; reversed spatial learning deficit in an animal model of schizophrenia.** Behavioural brain research. 2012;235:82-88. **IF= 3.63**

Patent applications:

Chodounska H., Kapras V., Vyklicky L., Borovska J., Vyklicky V., Vales K., Stuchlik A., **Rambousek L.** New pregnanolone compounds substituted in the 3-alpha-position with cationic group are N-methyl-D-aspartate receptor agonists useful for treating e.g. ischemic CNS injury, mood disorders, depression, schizophrenia and multiple sclerosis. WO2012110010-A1, 2012.

Borovska J., Cais O., Chodounska H., Kapras V., Kohout L., **Rambousek L.**, Stastna E., Stuchlik A., Vales K., Valesova V., Vyklickky L. New steroid anionic compounds used for production of pharmaceuticals treating such as ischemic damage of central nervous system, neurodegenerative changes and disorder, and for production of substances utilized in e.g. experimental research. WO2010136000-A2, 2010.

b) Publications not directly relevant to the thesis:

Impacted journals:

Rambousek L., Palenicek T., Vales K., Stuchlik A. The effect of psilocin on memory acquisition, retrieval and consolidation in rat. Frontiers in Behavioral Neuroscience. 2014; doi: 10.3389/fnbeh.2014.00180. **IF= 4.2**

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Palenicek, T.; Fujakova, M.; Brunovsky, M.; Horacek, J.; Gorman, I.; Balíkova, M.; **Rambousek, L**.; Syslova, K.; Kacer, P.; Zach, P.; Bubeníkova-Valesova, V.; Tyls, F.; Kubesova, A.; Puskarcíkova, J.; Höschl, C. Behavioral, neurochemical and pharmaco-EEG profiles of the psychedelic drug 4- bromo-2,5-dimethoxyphenethylamine (2C-B) in rats. Psychopharmacology. 2013;225:75-93. **IF= 3.99**

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Book chapters:

Syslova, K.; **Rambousek, L.**; Bubeníkova-Valesova, V.; Slamberova, R.; Novotny, P.; Kacer, P. Dopamine Analysis in Neuroscience Research. In Dopamine: Functions, Regulation and Health Effects; Kudo, E., Fujii, Y., Eds.; Nova Science Publishers: New York, 2012, ISBN: 978-1-61942-152-3.

Nekovarova, T.; Stuchlík, A.; Vales, K.; **Rambousek, L.**; Sumiyoshi, T. Cognitive Deficits in Pharmacological Rodent Models of Schizophrenia: Evaluation of Spatial Cognition. In Schizophrenia Research: Recent Advances; Sumiyosthi, T., Ed.; Nova Science Publishers: New York, 2012, ISBN: 978-1-61942-459-3.

Stuchlik, R.; Petrasek, T.; Hatalova, H.; **Rambousek, L.**; Nekovarova, T.; Vales, K. Behavioral Tests for Evaluation of Information Processing and Cognitive Deficits in Rodent Animal Models of Neuropsychiatric Disorders. In Neuropsychiatric Disorders; Burne, T. H. J., Ed.; InTech: Rijeka, 2012, ISBN: 979-953-307-169-4.