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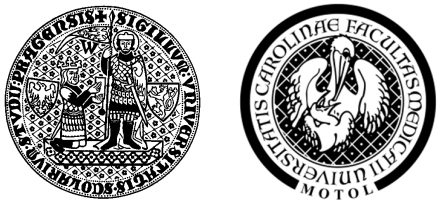

Biologický význam neuroaktivních steroidů v animálních modelech onemocnění mozku

Biological significance of neuroactive steroids in animal models of brain diseases

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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.

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.

1. INTRODUCTION

Neurosteroids are synthetized or metabolized de novo in the brain and modulate receptors for neurotransmitters. They are potent modulators of glutamatergic and γ-aminobutiric acid (GABA) receptors as well as other neurotransmitters receptors. Endogenous neurosteroids regulate many functions of the central and peripheral nervous system ranging from the development to complex behavior. Furthermore, their levels are altered during aging (Sierra A. et al., 2003) and under pathological conditions.

Neuroactive steroids represent molecules with high therapeutic potential. They are allosteric modulators of $GABA_A$ and N-methyl-D-aspartate (NMDA) receptors, the receptors responsible for the excitation-inhibition balance of the brain (Park-Chung M. et al., 1997 and 1999). Moreover, neurosteroids and neuroactive steroids readily cross the blood-brain barrier easily due to their lipophilic structure. Additionally, their structure is susceptible to a variety of synthetic modifications allowing fine tuning of their pharmacological properties. Results from preclinical and clinical studies suggests that neuroactive steroids may be a novel class of drugs for the treatment of epilepsy (Frye C.A. et al., 2000), depression (Urani A. et al., 2001), schizophrenia (Marx C.E. et al., 2009) and neurodegeneration (de Nicola A.F. et al., 2012).

Therefore, we have initiated the development of novel neuroactive steroids acting as inhibitors of NMDA receptors and modulators of $GABA_A$ receptor. Our lead molecule, 3α5β-pregnanolone glutamate (3α5βP-Glu), is rapidly absorbed after systemic administration and crosses the blood-brain barrier. 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).

First, we investigated if 3α5βP-Glu induces psychotomimetic side effects in naïve animals. Second, we tested its efficacy in the animal model of schizophrenia induced by systemic injection of NMDA channel blocker dizocilpine (MK-801). Administration of MK-801 induces hyperlocomotion and cognitive deficit, the behavioral correlates of schizophrenia symptoms. This model has been previously utilized to test antipsychotics and has been shown to have high pharmacological validity (Bubenikova-Valesova V. et al., 2008). Next, we investigated 3α5βP-Glu neuroprotective effect in animal model of excitotoxicity induced by intrahippocampal infusion of NMDA. Finally, we characterized the morphological changes induces by infusion of NMDA; in particular, the extent of neurodegeneration and changes in expression of NMDA and $GABA_A$ receptors subunits over 30 days.

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

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. 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.

We utilized the open-field test to evaluate the effect of 3α5βP-Glu on locomotor activity in naive rats and in an animal model of schizophrenia. Prepulse inhibition test (PPI) was utilized to evaluate the effect of 3α5βP-Glu on sensorimotor gating in naïve rats. Passive avoidance task and Carousel maze were used to evaluate effects of 3α5βP-Glu on learning and memory in naïve rats as well as in animal models of excitotoxicity and schizophrenia. The Carousel maze is a spatial cognitive task with high cognitive demands. In this task, animals have to avoid a "forbidden" sector on rotating arena. Their cognitive performance is assessed as number of entrances at 4^{th} day of training.

Excitotoxic lesion of hippocampus was induced by infusion of NMDA (95 and 25 mM in experiment 3 and 4, respectively) into hippocampus. Animals were fixed in stereotactic frame and anesthetized with isoflurane (1.5 – 2%) during this procedure. Sham operated animals received sterile PBS. Furthermore, we utilized histology and immunohistochemistry methods to evaluate potential neuroprotective effect of 3α5βP-Glu and to characterize the NMDA lesion of hippocampus. Animals were anaesthetized with mixture of ketamine (Vetoquinol) and xylasine (Alfasan) and transcardially perfused at a flow 50 mL/min with 250 ml, 4 °C, 4% paraformaldehyde. Free-floating sections (40 μm thick) were cut coronally on a sliding microtome (Leica). Sections were stained with Nissl, Fluoro-Jade B or immunohistochemistry with immunoperoxidase visualization was performed. Specific antibodies against activated microglia (Iba-1), astrocytes (GFAP), NMDA receptor subunits (NR1 and NR2B), $GABA_A$ receptor subunits ($GABA_AR$ α1, 2, 5 and γ2) and markers of GABAergic interneurons (parvalbumin, calretinin, calbindin, neuropeptid Y) were used. Densitometry measurements of $GABA_A$ and NMDA receptor subunits immunoreactivities were measured using ImageJ software (NIH, Maryland, USA). The optical density (OD) for each hippocampus was normalized to the optical density of neocortex ($OD = OD_{neocortex} - OD_{hippocampus}$). Four to six consecutive coronal sections from each animal were analyzed. 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.

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. 1.

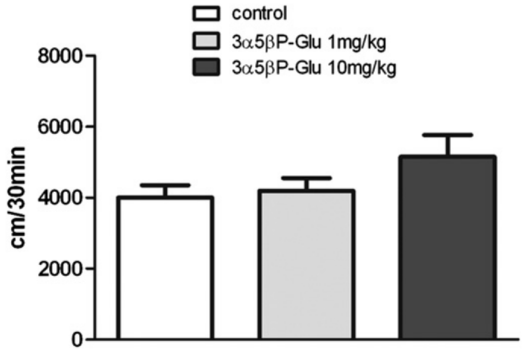

Figure 1: Acute effect of 3α5βP-Glu at doses 1 and 10 mg/kg i.p. administration on locomotion generated during 30 min in an open-field apparatus. There was no significant effect of 3α5βP-Glu on locomotor activity in a novel environment. Data are represented as mean ± SEM.

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

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. 2). One-way ANOVA test failed to confirm any group effect after prepulse 78 db ($F_{(2,27)}$ = 0.402, NS) and after 86 dB prepulse ($F_{(2,27)}$ = 0.6424, NS).

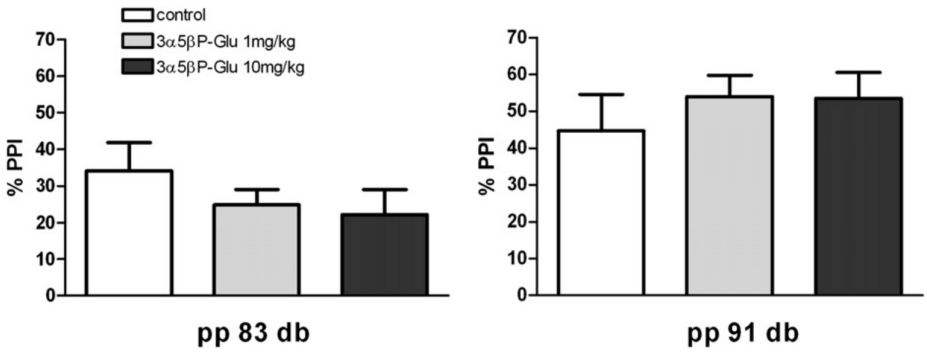

Figure 2: Effect of acute application of 3α5βP-Glu at dose 1 and 10 mg/kg i.p. on prepulse inhibition of the acoustic startle response [prepusle (pp) 83 and 93 dB]. There was no significant effect of 3α5βP-Glu on PPI. Data are represented as mean ± SEM.

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. 3). Control animals treated with PBS did not show any learning impairment in both parameters. One-way ANOVA revealed the effect of treatment ($F_{(9,26)}$ = 4.161, P=0.0003). 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).

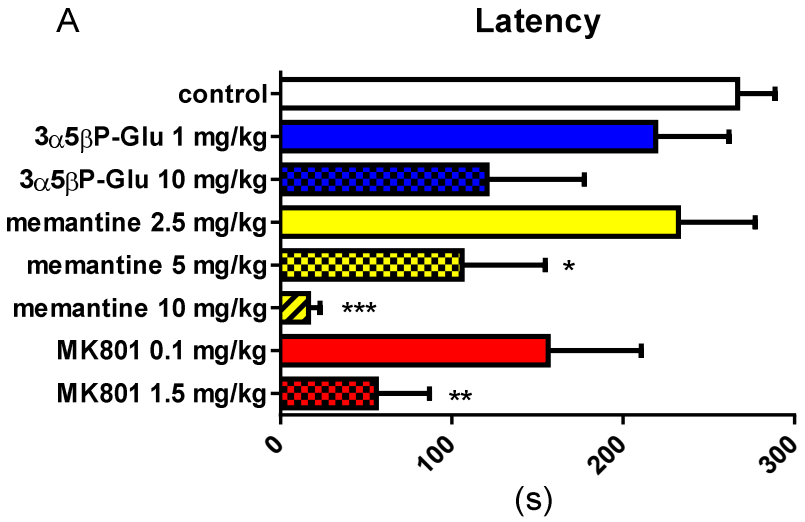

Figure 3: The latency when rats first entered the dark compartment (A) in the passive avoidance task after treatment with 3α5βP-Glu or other NMDA antagonists. Data are presented from the last day of training as mean ± SEM. *P < 0.05, ** P < 0.01, *** P < 0.001.

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

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 4th session of Carousel maze. Sub-chronic treatment with 3α5βP-Glu at doses 1 and 10 mg/kg significantly affects 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. 4.

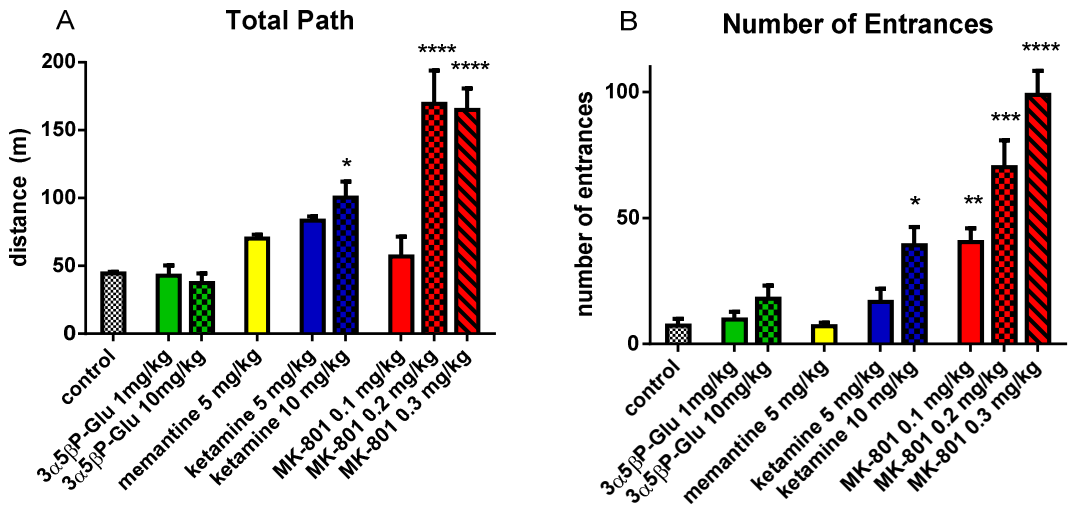

Figure 4: Locomotor activity (A, total path) and spatial learning performance (B, number of entrances) in the Carousel maze after sub-chronic treatment with 3α5βP-Glu or other NMDA antagonists. Data are shown from the last day of training as mean ± SEM. *$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

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

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 (Fig. 5). Two-way ANOVA revealed a significant interaction between the effects of 3α5βP-Glu 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 induce cognitive deficit, but it was ameliorated by 3α5βP-Glu at doses 0.001- 10 mg/kg.

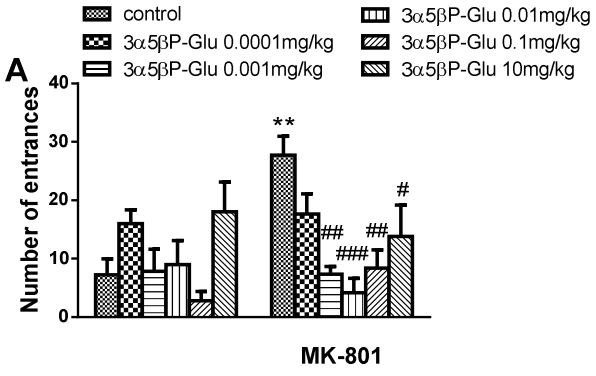

Figure 5: Administration of 3α5βP-Glu did not alter spatial learning at any dose in naive rats. MK-801 induced spatial learning deficit, which was reversed by 3α5βP-Glu administration at doses of 0.001- 10 mg/kg. Data are represented as mean ± SEM. **p < 0.01 compared to saline/saline group. #p < 0.05, ##p < 0.01, ###p < 0.001 compared to MK-801/saline group.

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

4.3.1. Spatial learning in the Carousel maze

3α5βP-Glu ameliorated hippocampal lesion induced cognitive spatial deficit as assessed by the number of entrances into the punished region (Fig. 6). 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.

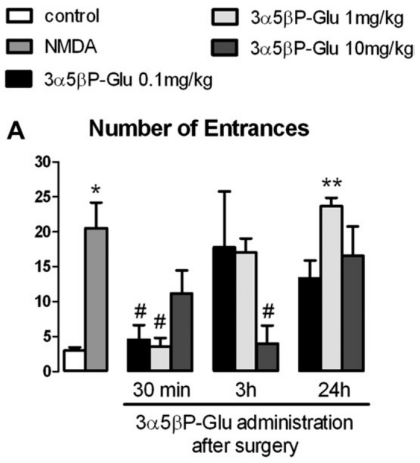

Figure 6: The effect of 3α5βP-Glu administration 30 min, 3 h, or 24 h after NMDA lesion in a behavioral spatial paradigm, the Carousel maze. Cognitive performance was assessed using 'Number of Entrances' (A). Data are presented from the last day of training as mean ± SEM. $*P < 0.05$, $**P < 0.01$, compared to the control group; $\#P < 0.05$ 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 Fluro-Jade 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. 7 shows representative pictures of dorsal hippocampus at 7 days post injection (dpi).

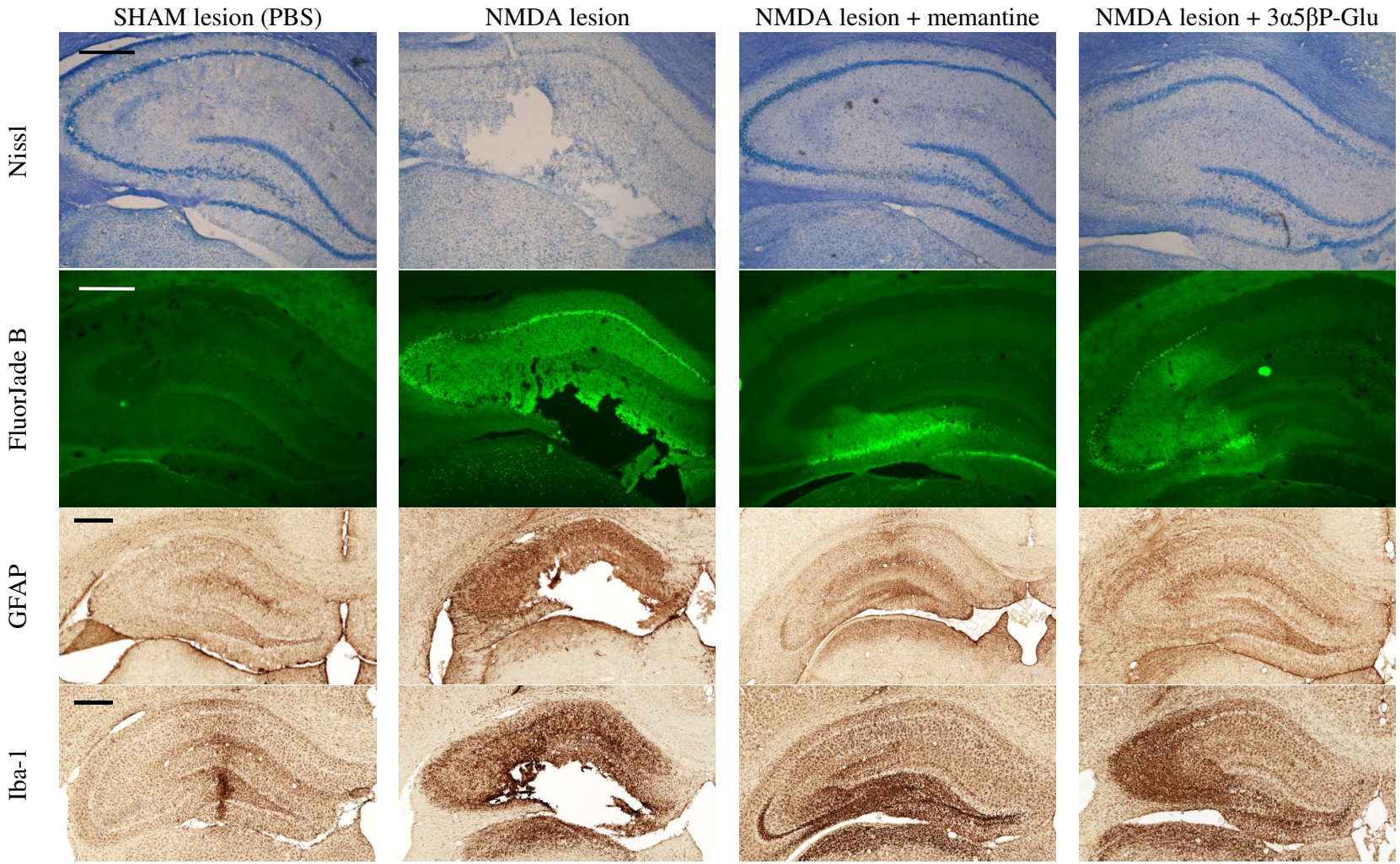

Figure 7: Representative pictures of ipsilateral dorsal hippocampus stained with Nissl, Fluoro-Jade B and antibodies against microglia (Iba-1) and astrocytes (GFAP) 7 days after unilateral NMDA lesion (1 µl of 90 mM NMDA). In comparison to NMDA lesioned animals, the pre-treatment of animals with memantine (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. 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. 8 shows mean score for all studies subfields of hippocampal formation. Interestingly, the level of neurodegeneration measured as damage score of Fluoro-Jade B positive cells did not change over 30 days. The neurons affected by NMDA were swollen; however, sustained their basic morphology.

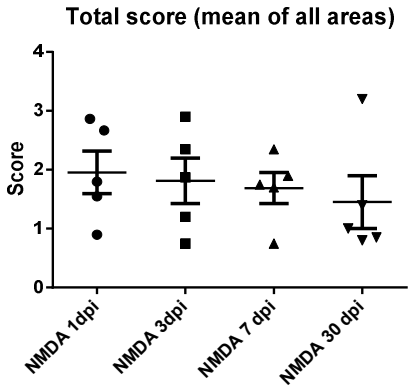

Figure 8: Neural damage score after NMDA lesion of hippocampus 1, 3, 7 and 30 days post injection (dpi). No significant differences were observed among individual days post injection. Sham lesion induced no damage (score 0, data not shown) Score scale: 0 – 0-5%, 1 – 6-25%, 2 – 26-50%, 3 – 51-75%, 4 > 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. 9).

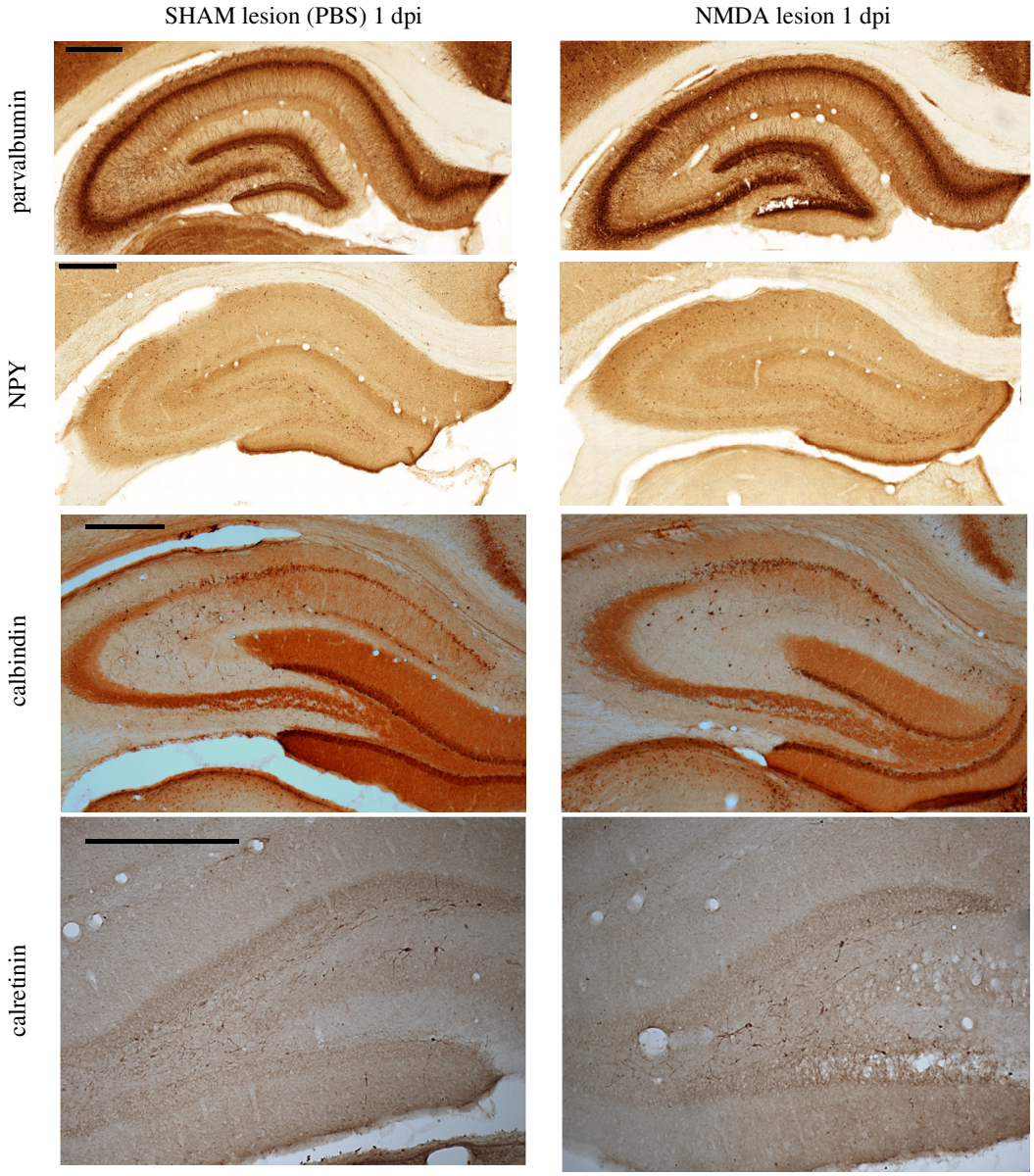

Figure 9: Distribution of parvalbumin, neuropeptide Y (NPY), calbindin and calretinin immunoreactivity 1 day after PBS or NMDA injection into hippocampus. Scale bar: A = 500 µm.

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.

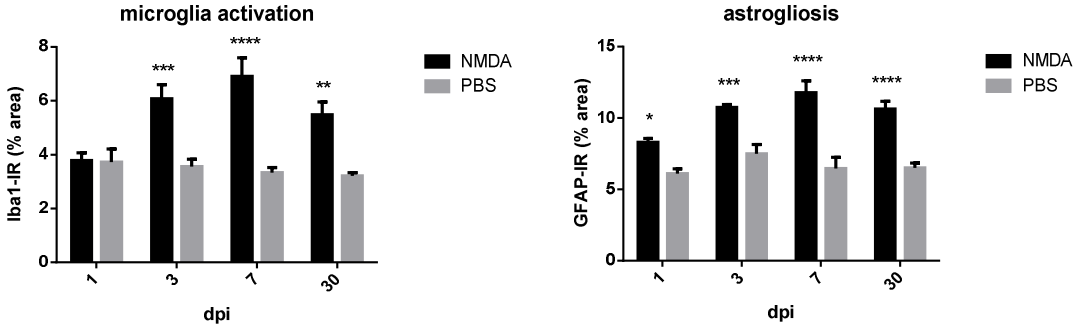

Figure 10: Quantitative analysis of hippocampal anti-Iba-1 and anti-GFAP immunoreactivity (IR) representing relative percentage of area covered by activated microglia. Values are given as mean ± SEM. ** P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.

The infusion of NMDA into hippocampus induced significant reactive astrogliosis at all studied time points (Fig 10). 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.

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. Figure 11 shows the quantification of relative optical density for NMDAR NR1 and NR2B subunits, respectively. The increased immunoreactivity correlated positively with Fluoro-Jade B 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).

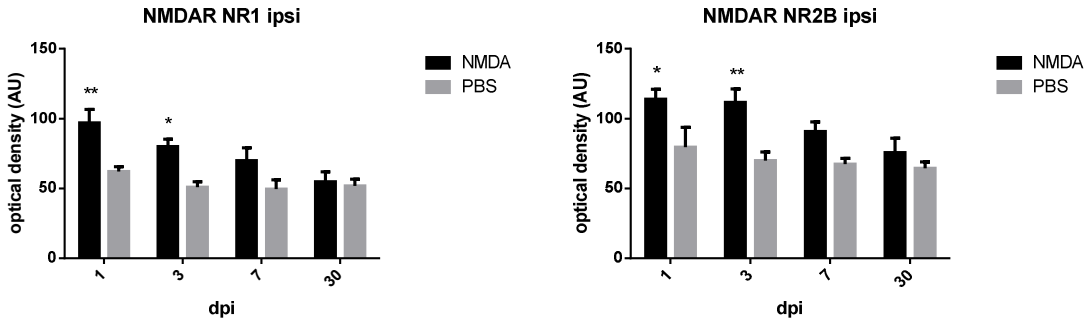

Figure 11: Quantitative analysis of NMDA receptor NR1 and NR2B subunits immunoperoxidase staining relative optical density. Values for each hippocampus were normalized to neocortex. Values are given as mean ± SEM. * $P < 0.05$, ** $P < 0.005$.

Furthermore, we analyzed changes in the expression of main $GABA_A$ receptor α1, α2, α5 and γ2 subunits. NMDA lesion significantly decreased the expression of $GABA_A$ receptor α5 subunit 30 dpi in ipsilateral hippocampus (Fig. 12). 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.

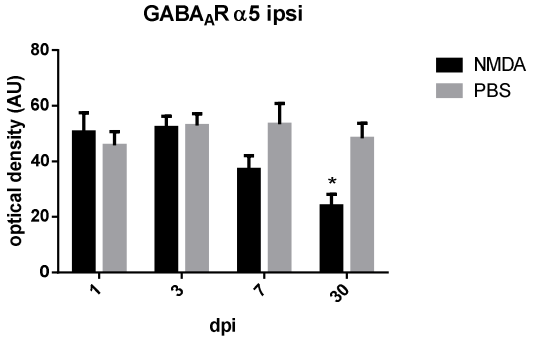

Figure 12: Quantitative analysis of $GABA_A$ receptor α5 subunit immunoperoxidase staining relative optical density. Values for each hippocampus were normalized to neocortex. Values are given as mean ± SEM. * $P < 0.05$, ** $P < 0.005$.

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. 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).

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). Memantine is the most studied and clinically used use-dependent NMDA receptor antagonist, 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 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 3α5βP-Glu in animal model of schizophrenia (compared to MK-801 alone). 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.

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., 2006; Barbaccia M.L. et al., 2001).

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" parameter. 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. 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) 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:

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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:

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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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