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Summary of the Dissertation

Effects of endogenous NMDA receptor modulators on neuronal morphology and synaptic plasticity

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1. Abstract

The NMDA receptor (NMDAR) plays a crucial role in cognitive processes like learning and memory, and its functional dysregulation is implicated in various mental disorders. Despite extensive research on the effects of ketamine and other NMDAR inhibitors, little is known about the impact of endogenous NMDAR modulators on neuronal structure and synaptic density, despite their dysregulation in mental disorders.

This study aimed to uncover the molecular and cellular changes induced by endogenous NMDAR modulators and their relevance to psychiatric disorders. We evaluated the effects of prevalent modulators - kynurenic acid, pregnenolone sulfate (PS), spermidine, and zinc - on primary cortical cultures over time. Various assays were used to measure cell viability, glutamate release, dendritic branching, synaptic protein expression, and GABA and BDNF levels. Additionally, we assessed the antidepressant-like effects of PS in a mouse chronic despair model using a number of behavioral tests.

Overall, our findings showed no significant impact of endogenous NMDAR modulators on glutamate release or neuronal viability in excitatory cells. However, PS increased their distal dendritic arborization, consistent with elevated BDNF expression and TrkB activation. While synaptic density remained stable, PS-treated neurons exhibited reduced PSD-95 puncta. Parvalbumin-positive inhibitory neurons treated with the modulators decreased dendritic branching without affecting GABA release or expression. Finally, PS demonstrated anxiolytic-like effects in mice subjected to the chronic despair model.

These results suggest that the observed increase in BDNF release, TrkB activation and dendritic field expansion may underlie the anxiolytic-like behavior induced by PS. Additionally, the heightened sensitivity of parvalbumin-positive neurons to structural changes induced by these modulators provides further insight into their potential implications for psychiatric disorders.

Keywords: NMDA receptor, dendritic branching, synaptic density, depression, mental disorders, neurosteroids, polyamines, kynurenic acid.

2. Abstrakt

NMDA receptor (NMDAR) je zásadní pro kognitivní procesy jako učení a paměť a poruchy jeho funkce se podílejí na patogenezi různých duševních poruch. Navzdory rozsáhlému výzkumu účinků inhibitorů NMDAR, jako např. ketamin, stále není objasněn vliv endogenních modulátorů NMDAR na strukturu neuronů a synaptickou hustotu, přestože právě jejich dysregulace je společných příznakem mnoha duševních poruch.

Cílem této studie bylo odhalit změny na molekulární a buněčné úrovni vyvolané endogenními modulátory NMDAR a jejich význam pro psychiatrické poruchy. Hodnotili jsme účinky hlavních modulátorů kyseliny kynurenové, pregnenolon sulfátu (PS), spermidinu a zinku na primární kortikální kultury v průběhu času. Byly zjištěny efekty na životaschopnost neuronů, uvolňování glutamátu, dendritické větvení, expresi synaptických proteinů a hladiny GABA a BDNF. Kromě toho jsme hodnotili potenciální antidepresivní účinky PS v myším chronic despair modelu za využití behaviorálních testů.

Studie neprokázala vliv endogenních modulátorů na uvolňování glutamátu nebo životaschopnost excitačních neuronů. PS však zvýšil jejich distální dendritickou arborizaci, což odpovídá zvýšené expresi BDNF a aktivaci receptoru TrkB. Zatímco synaptická hustota zůstala nezměněna, neurony ošetřené PS vykazovaly sníženou pozitivitu PSD-95 v imunofluorescenčním barvení. U inhibičních neuronů všechny modulátory snížily dendritickou arborizaci, aniž by ovlivnily uvolňování nebo expresi GABA. PS také vykazoval anxiolytické účinky v chronic despair modelu u myší.

Pozorované zvýšení hladin BDNF, aktivace receptoru TrkB a zvýšená komplexita dendritické arborizace může být podkladem anxiolytického efektu PS u myší. Výsledky této studie navíc poukazují na zvýšenou citlivost parvalbumin-pozitivních neuronů k endogenním NMDAR modulátorům, což poskytuje další vhled do jejich implikace u psychiatrických poruch.

Klíčová slova: NMDA receptor, dendritické větvení, synaptická hustota, deprese, duševní poruchy, neurosteroidy, polyaminy, kyselina kynurenová.

3. Introduction

Mental disorders, such as depression and anxiety, affect a significant proportion of the global population. The COVID-19 pandemic has exacerbated these conditions, emphasising the need for effective treatments (Organization, W.H., 2022). Although clinical diagnoses are based on observable symptoms, the molecular causes remain elusive. Dysregulation of neuronal circuits, particularly the balance between excitation and inhibition mediated by NMDA receptors (NMDARs), is implicated in psychiatric disorders. NMDAR dysfunction, which is often observed in these conditions (Myers et al., 2019), highlights their potential as therapeutic targets. However, the lack of safe and efficient medications poses significant challenges for drug development in brain disorders.

3.1 Function and structure of the NMDAR

Glutamate is the principal excitatory neurotransmitter in the nervous system. It exerts its effects through two major types of receptors: ionotropic glutamate receptors (iGluRs) and metabotropic glutamate receptors. There are three subtypes of iGluRs: AMPARs, KARs, and NMDARs (Madden, 2002). AMPARs primarily mediate the rapid component of excitatory postsynaptic currents (EPSCs), while KARs have a more limited role (Castillo et al., 1997; Twomey et al., 2019). NMDARs contribute to the slower, sustained component of EPSCs.

NMDARs are unique among iGluRs due to their slower gating kinetics, higher Ca^{+2} permeability, and voltage-dependent magnesium block. The NMDAR is a heterotetrameric complex composed of two obligatory GluN1 subunits and two GluN2 and/or GluN3 subunits, forming diverse receptor subtypes (Traynelis et al., 2010). These subunits exhibit distinct spatiotemporal expression profiles throughout development and across different brain regions. For example, GluN2B and GluN2D are prevalent in embryonic brains, while GluN2A expression increases postnatally and predominates in the adult central nervous system (CNS). The composition of NMDAR subunits influences receptor properties, with GluN2A and GluN2B subunits displaying differential roles in synaptic transmission and plasticity (Paoletti et al., 2013).

Structurally, NMDARs consist of four modular domains: the extracellular amino-terminal domain (ATD), ligand-binding domain (LBD),

transmembrane domain (TMD), and intracellular carboxyl (C)-terminal domain (CTD). NMDAR activation requires depolarization of the postsynaptic membrane, leading to the relief of Mg^{+2} block at the ion channel pore, and binding of glutamate and co-agonists (Vyklicky et al., 2014). Importantly, variations in NMDAR genes, particularly GluN1, GluN2A, and GluN2B, have been associated with psychiatric disorders, leading to changes in receptor properties and contributing to disease pathogenesis. These variants may result in hyperfunctional, hypofunctional, or unaltered receptor function, highlighting the intricate role of NMDAR dysfunction in psychiatric disorders (XiangWei et al., 2018).

3.2 Pharmacological modulation of NMDAR

3.2.1 Competitive antagonists

Competitive antagonists bind to the same binding site as the endogenous agonists without activating the receptor. Examples of competitive antagonists include D-AP5, AP7, and CGP-37849. These antagonists have shown anticonvulsant properties and broader effects such as neuroprotection and antidepressant activity (Fujikawa et al., 1994; Meldrum et al., 1988; Zivanović et al., 1999).

3.2.2 Open channel blockers

Open channel blockers, such as ketamine and MK-801, prevent ion flux by binding within the channel pore. Ketamine is commonly used as an anesthetic and is also known for its rapid-acting antidepressant effects (Krystal et al., 2024). Memantine, which is used in Alzheimer's treatment, preferentially inhibits extrasynaptic NMDARs, contributing to neuroprotection (Lipton, 2005; Xia et al., 2010).

3.2.3 Allosteric modulators

Allosteric modulators bind to specific sites on the NMDAR distinct from the agonist binding. Protons act as negative modulators, tonically inhibiting NMDARs (Jalali-Yazdi et al., 2018), while ethyl alcohol serves as a non-selective antagonist (Chandrasekar, 2013). Subunit-specific antagonists like ifenprodil and TCN-201 target GluN2B and GluN2A-containing receptors, respectively, offering insights into NMDAR subunit functions and implications in various conditions (Hansen et al., 2012; Williams, 2001).

3.3 Endogenous NMDAR modulators and psychiatric disorders

Despite extensive research into NMDAR structure and function, understanding how changes in NMDAR activity relate to symptoms of mental disorders remains a challenge. While exogenous antagonists such as ketamine have been studied, the role of endogenous modulators is less well understood. This section aims to summarise the key endogenous modulators of NMDARs and their potential involvement in the pathology of mental disorders.

3.3.1 Kynurenic acid

Kynurenic acid (KYNA) is an endogenous NMDAR antagonist derived from L-tryptophan. Kynurenine is synthesized from L-tryptophan by the enzymes IDO and TDO and subsequently converted to KYNA by kynurenine aminotransferases (Han et al., 2010). KYNA exhibits poor permeability across the blood-brain barrier (BBB) due to its inability to efficiently cross amino acid transporters (Fukui et al., 1991). KYNA acts as a non-competitive antagonist at the glycine co-agonist site of NMDARs and as a competitive antagonist at the glutamate recognition site. Interestingly, it has dual effects on AMPARs, acting as an antagonist at high concentrations and as a positive allosteric modulator at lower concentrations (Prescott et al., 2006). KYNA also antagonises α7 nicotinic acetylcholine receptors. Patients with schizophrenia and bipolar disorder have elevated levels of KYNA in the cerebrospinal fluid and brain tissue (Plitman et al., 2017). Animal studies support this, showing impaired cognitive function with increased brain KYNA levels (Chess et al., 2007). Knock-out mice with reduced KYNA levels show improved cognitive performance, supporting the idea that endogenous KYNA affects cognitive function (Potter et al., 2010).

3.3.2 Neurosteroids

Neurosteroids are brain-synthesised steroids with modulatory effects on ionotropic receptors, including NMDARs. Synthesised from cholesterol, pregnenolone (PREG), pregnanolone (PA) and allopregnanolone exert diverse effects on neuronal function (Rupprecht & Holsboer, 1999). Their modulation of NMDARs depends on sulphation of the C3 carbon group by

sulfotransferases. PREG sulfate (PS) enhances NMDAR-mediated Ca^{+2} influx, whereas PA sulfate (PAS) inhibits it. The direction of modulation is influenced by subunit composition, with PS potentiating GluN1/GluN2A and GluN1/GluN2B subunits and inhibiting GluN1/GluN2C and GluN1/GluN2D subunits (Malayev et al., 2002). Clinical studies have shown altered neurosteroid levels in psychiatric disorders. For example, patients with schizophrenia exhibit lower serum PREG levels, while those with Alzheimer's disease have increased PREG levels in certain brain regions (Marx et al., 2006; Ritsner et al., 2007). Furthermore, the addition of PREG therapy has been shown to benefit patients with bipolar disorder by improving depressive symptoms (Brown et al., 2014). Preclinical studies have demonstrated that PS is effective in ameliorating schizophrenia-like symptoms, improving spatial memory, exhibiting antidepressant-like effects, and attenuating conditioned fear stress (Plescia et al., 2013; Wong et al., 2015).

3.3.3 Polyamines

Polyamines, such as spermidine (SPD) and spermine, are aliphatic polycations that are found ubiquitously in the body. They are synthesized from ornithine, which is a product of arginine hydrolysis, and play crucial roles in various cellular functions, including cell proliferation and antioxidant effects. Although polyamines are present in both neurons and glial cells, astrocytes predominantly store spermine and spermidine (Coleman et al., 2004; Liu et al., 2008). Polyamines have a dual effect on NMDAR activity, with both stimulatory and inhibitory effects depending on factors such as binding sites and concentrations. They can increase NMDAR currents by enhancing channel opening frequency and glycine affinity, or decrease currents through voltage-dependent mechanisms and open channel block. For example, spermine enhances NMDA currents through glycine-independent stimulation and reduces receptor desensitization to glutamate (Williams, 1997). Schizophrenia is associated with changes in genes related to polyamine metabolism and increased levels of spermidine, suggesting their involvement in the disorder's manifestation. Studies on the behavioural effects of polyamines in animals have produced mixed results, with spermine administration impairing spatial learning but also improving memory in certain tasks (Conway, 1998; Frühauf et al.,

2015). Further research is needed to fully understand the impact of polyamines on learning and memory processes.

3.3.4 Zinc

Zinc is an essential trace element that plays a pivotal role in various neurobiological processes, from genetic mechanisms to ion channel activity. It is crucial for DNA replication, protein synthesis, immune responses, and oxidative stress regulation. In the brain, zinc exists as free ions in synaptic vesicles, particularly in zinc-enriched neurons (ZEN) throughout regions such as the cortex, amygdala, and hippocampus (Wenzel et al., 1997). Zinc has a functional role in modulating neurotransmitter release and synaptic function, both pre- and postsynaptically. It inhibits the NMDAR, particularly via GluN1/GluN2B receptors, and has biphasic effects on GluN1/GluN2A receptors (Chen et al., 1997). Zinc also regulates glutamatergic activity by decreasing presynaptic glutamate release and modulating enzymes involved in glutamate catabolism. Zinc homeostasis changes have been associated with several neurodegenerative diseases, such as Alzheimer's, Parkinson's, and Huntington's diseases, as well as depression and schizophrenia (Prakash et al., 2015). Patients with depression and schizophrenia present lower serum zinc concentrations, and zinc supplementation has demonstrated effectiveness as an adjuvant therapy (Mortazavi et al., 2015). Research using ZnT3 knockout mice has emphasised the significance of zinc in learning, memory, and synaptic function. Deficits in social and object recognition memory have been observed in adult ZnT3 knockout mice (Martel et al., 2011).

3.4 NMDAR dysregulation in psychiatric disorders

Dysregulation of NMDAR has been implicated in various psychiatric disorders. NMDAR hypofunction is a primary hypothesis for schizophrenia, while NMDAR hyperfunction is associated with excitotoxicity and neurodegeneration, suggesting an inverted-U-shaped dose-response curve (Lipton & Nakanishi, 1999). NMDAR modulators show promise in treating psychiatric disorders. Co-agonists such as D-serine, D-alanine, and glycine have improved negative symptoms of schizophrenia when used adjunctively (Kantrowitz et al., 2010). Memantine, which is an NMDAR antagonist, has demonstrated benefits in certain cases of Alzheimer's disease (Reisberg et

al., 2003). Preclinical experiments have shown that NMDAR antagonists such as ketamine, phencyclidine, and MK-801 induce behaviors that resemble schizophrenic-like symptoms, including cognitive and negative symptom-related behaviors (Lee & Zhou, 2019). It is interesting to note that ketamine has emerged as a fast-acting antidepressant at doses comparable to those that induce psychotic symptoms (Adell, 2020).

Finally, dendritic branching, spines, and synaptic density are dysregulated in many psychiatric disorders. Therefore, it is important to evaluate the structural and functional effects of endogenous NMDAR modulators on neurons, as the NMDAR is involved in these processes. This evaluation could have potential uses in treating psychiatric disorders.

4. Aims of the study

The aim of this project is to explore the structural and functional changes in neurons within primary cortical cultures following treatment with various NMDAR modulators and their potential implications for therapeutic use in psychiatric disorders.

Hypothesis:

Endogenous modulators of NMDAR are involved in the neurodevelopment of excitatory and inhibitory neurons by modulating the downstream signalling cascade of NMDARs. This modulation could elicit antidepressant-like effects in a chronic despair model in mice.

Objectives:

(i) Evaluate the morphological changes of excitatory/inhibitory neurons induced by endogenous NMDA receptor modulators.
(ii) Evaluate the synaptic changes of excitatory/inhibitory neurons induced by endogenous NMDA receptor modulators.
(iii) Measure the protein expression and activation of the BNDF/TrkB/ERK pathway.
(iv) Investigate the potential antidepressant-like effect of the most promising endogenous NMDAR modulator in a chronic despair model in mice.

5. Materials and methods

5.1 Primary cortical cultures

Approval was obtained from the NIMH Committee for Animal Research Ethics in accordance with the Czech Republic Animal Protection Code and EU Council Directive 2010/63/EU. The plates were coated with poly-L-lysine. Cortical cultures were prepared from E18 Wistar rat embryos by isolating the cortices and mechanically dissociating the cells. The dissociated cells were then filtered, counted, and plated at varying densities for different analyses. Half of the growth medium was changed every 4-5 days.

5.2 Treatments of cultures with endogenous NMDAR modulators

Kynurenic acid (150 nM), pregnenolone sulfate (50 μM), spermidine (50 μM), or zinc chloride (10 μM) were applied for varying exposure times. The compounds were diluted in bi-distilled water and added to cultures at a 1:100 ratio. Following treatment, the cultures underwent several assays, including immunocytochemistry, cell viability, glutamate release, ELISA, and western blot tests.

5.3 Cell viability assay

Cell viability was assessed using the MTS colorimetric assay. This method is based on the reduction of MTS tetrazolium compound to formazan in viable cells. The cultures were maintained in 100 μL of growth medium and treated with endogenous NMDAR modulators for 12 hours, 1 day, or 5 days at DIV19. After treatment, 10 μL of MTS was added to each well and incubated at 37°C and 5% CO_2 for 2 hours. Triton was used as a positive control. After shaking briefly, we measured the optical density (OD) at 490 nm using a plate reader.

5.4 Glutamate release measurements

Following treatment with endogenous NMDAR modulators for 1 and 5 days, cells were stimulated with pre-warmed Krebs-Ringer solution containing high potassium concentration and DL-TBOA (blocker of excitatory amino acid transporters). Glutamate levels were measured in the supernatant, while protein measurements were taken from cell lysates. To quantify glutamate concentration, samples were injected into the HPLC

system. The samples were resolved on a C18 column, and the fluorescence detection was used to monitor the derivatized glutamate. Glutamate levels were calculated using a calibration method normalized for the protein lysate content, using regression analysis.

5.5 Immunocytochemistry

Following treatment, the growth medium was replaced with a paraformaldehyde solution for fixation. The cells were then washed with PBS and blocked and permeabilized. Primary antibodies were applied overnight at 4°C. Coverslips were incubated with secondary antibodies and stained with Phalloidin for dendritic spine visualization. Finally, the samples were mounted and images were acquired using a laser scanning microscope. The analysis of the image was carried out using ImageJ with Synapse Counter and SNT plugin. The analysis was performed by an experimenter who was blinded to the treatment conditions.

5.6 ELISA and western blot

For protein analysis, the primary cortical cultures were washed with ice-cold PBS and then scraped in RIPA buffer with protease and phosphatase inhibitors. The protein concentration was determined using the BCA protein assay after sonication and centrifugation. The proteins were loaded onto a 4-20% TGX Stain-Free gel and run at 200 V. The gel images were captured using UV light to visualize total protein. The proteins were then transferred to a PVDF membrane and blocked with nonfat dry milk. The membranes were incubated with primary antibodies at 4°C overnight, washed, and then incubated with HRP-conjugated secondary antibodies. The protein bands were visualized using ECL substrate, and the band intensity was analyzed using Image Lab 6 software. GABA and BDNF levels were measured using ELISA kits, and their concentrations were calculated based on standard curves using a plate reader.

5.7 Behavioural tests

5.7.1 Animals

Data were collected from 15 wild-type C57Bl/6N mice housed in the animal facility at the University of Freiburg. Adult mice of both sexes (10-14 weeks old) were used under standardised conditions with a 12 h light/dark cycle

and free access to water and food. All procedures were approved by the German Animal Committees in accordance with national and international standards. Behavioural experiments were recorded and analysed off-line by a blinded observer.

5.7.2 Chronic despair model

Depressive-like symptoms were induced in mice using a previously described protocol involving 5 consecutive days of forced swim test. Mice were placed in water-filled beakers at 25°C for 10 min, dried, and returned to their cages. Immobility times during swimming were calculated as a measure of despair-like behaviour.

5.7.3 Drug administration

PS was injected intraperitoneally at 40 mg/kg, diluted in 0.1% Tween 80, 60 min prior to open field, three chamber test and forced swim tests on days 8-10.

5.7.4 Open field test

Mice were placed in a 40 × 60 cm rectangular arena with a 40 cm high wall of grey PVC to ensure low light intensity. They were allowed to move freely for 10 min. EthoVision XT software analysed variables including total distance travelled, time spent in the centre and centre entries.

5.7.5 Three-chamber test

The arena consisted of three adjacent chambers connected by open doors, each measuring 19 x 12 cm with walls 30 cm high. The test consisted of three 10 min sessions: habituation, sociability and social novelty. During habituation, the mice explored all three chambers. In the sociability session, an unfamiliar mouse was placed under one cup while an empty cup was placed opposite. In the social novelty session, a new, unfamiliar mouse was introduced under an empty cup. The EthoVision XT software analysed the interaction times with each cup and calculated the preference index accordingly.

5.7.6 Forced swim test

The procedure followed the protocol outlined in the Chronic Despair Model.

5.8 Data analysis and statistics

Data are presented as mean ± standard error of the mean (SEM). The Shapiro-Wilk test was used to assess data distribution. One-way ANOVA was used to compare treatment factors, followed by Dunnett's post hoc test. For treatments on different days, two-way ANOVA was used, followed by Tukey's post hoc test. An unpaired t-test was used for PS and vehicle experiments. Statistical significance was set at $p < 0.05$. All analyses were performed using R software (version 4.0.5) and RStudio (version 1.4.1717).

6. Results

6.1 Effect of endogenous NMDAR modulators on excitatory neurons

6.1.1 Endogenous NMDAR modulators do not alter cell viability and glutamate release

To assess potential toxicity on neuronal survival, we performed MTS viability assays on primary cortical cultures exposed to physiological concentrations of KYNA, PS, SPD and ZINC. Results showed no decrease in cell viability after 12 hours, 1 day (1DT) or 5 days (5DT) of treatment. We also investigated whether exposure to these modulators altered glutamate release. As basal glutamate levels were undetectable, we measured evoked release in the presence of DL-TBOA, a glutamate uptake inhibitor. Incubation with endogenous NMDAR modulators did not affect evoked glutamate release. Similarly, there were no differences in evoked glutamate release after 1DT and 5DT.

6.1.2 Pregnenolone sulfate promotes dendritic field expansion

To evaluate the structural changes caused by prolonged exposure to endogenous NMDAR modulators, cortical cultures were treated at DIV 14 for 5 days. Throughout all treatments, parameters such as total dendritic length, total number of dendritic branches, number of primary branches, number of branch points, and neuronal soma size remained unaltered. However, the Sholl analysis showed significant differences in branching intersections at 20 μm. Both PS and SPD decreased the number of intersections, but PS increased the number of branching intersections at 130, 150, 170 and 180 μm. Therefore, PS facilitated dendritic arborisation in remote compartments and promoted the extension of dendritic processes (see Figure 1).

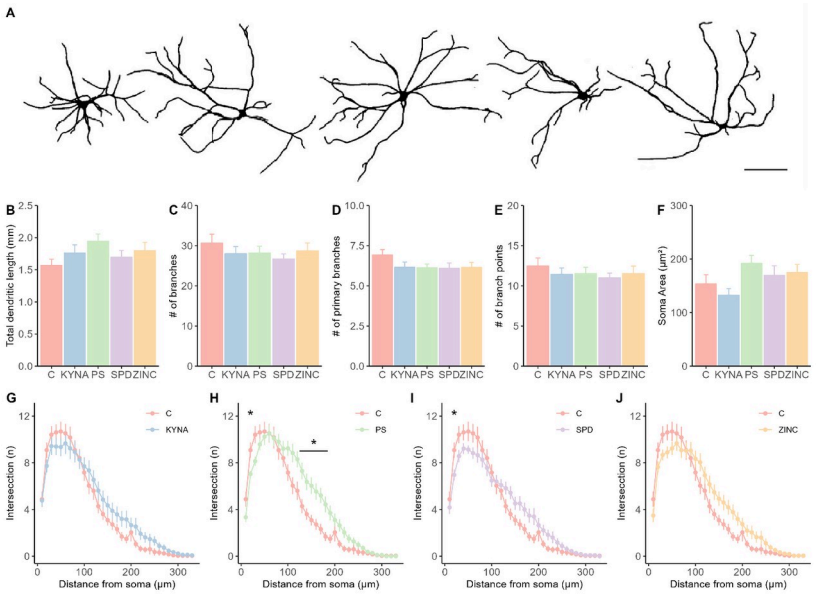

Figure 1. Dendritic branching and arbor complexity in cortical neurons. A Representative neurons. **B-F** NMDAR modulators did not affect total dendritic length, number of branches, number of primary branches, number of branch points, or soma area. **G-J** Sholl analysis displays intersections from soma distance. Mean ± SEM, n = 23-30 neurons per treatment, from three replicates. One-way ANOVA with Dunnett's post hoc. Scale bar = 100 µm.

6.1.3 Pregnenolone sulfate increases the expression of BDNF and TrkB activation

Binding of BDNF to its receptor TrkB leads to phosphorylation-dependent activation, initiating downstream signalling pathways crucial for dendritic branching and synaptic plasticity. We assessed BDNF after treatment for 1 and 5 days. Overall, there was a significant difference, with PS showing a tendency to increase BDNF levels (Figure 2). Given the differences observed only with PS treatment, we analysed the levels of phosphorylated TrkB (p-TrkB) and the downstream signalling mediator ERK1/2 (p-ERK1/2) after 1 and 6 hours of PS treatment using western blot analysis. After 1 hour, PS significantly increased TrkB phosphorylation and showed a trend towards increased total ERK1/2 expression. However, after 6 hours, TrkB activation was not observed, but there was a decrease in ERK1/2 phosphorylation (Figure 3).

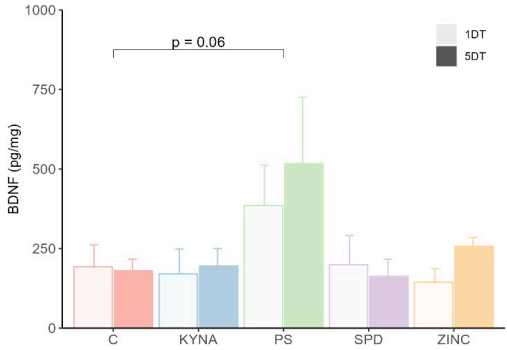

Figure 2. BDNF expression after exposure to endogenous NMDAR modulators. Protein levels of BDNF determined by ELISA (n = 4) after 1 and 5 DT. Means ± SEM. Two-way ANOVA followed by a Tukey's post hoc test.

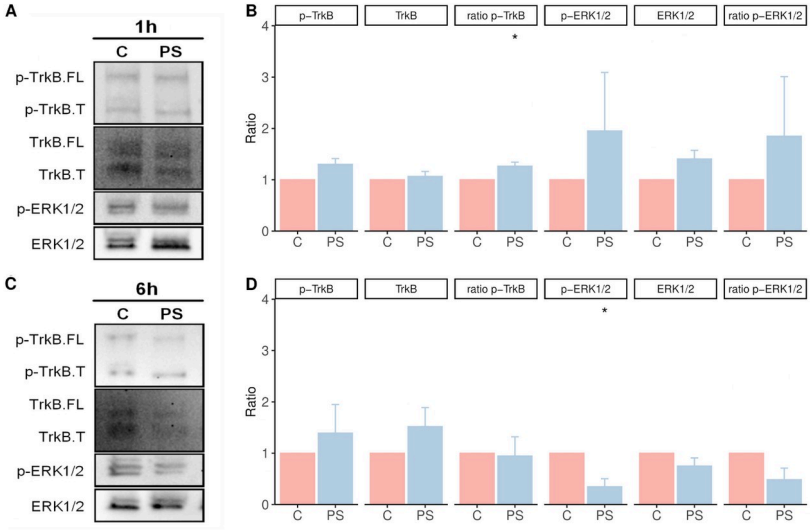

Figure 3. Western blot analysis of cultures treated with endogenous NMDAR modulators. **A,C** Representative immunoblots. **B,D** Corresponding protein quantification normalized to total protein load. Means ± SEM. Unpaired t-tests (n = 4).

6.1.4 Endogenous NMDAR modulators do not alter synaptic density

In proximal dendrites, a two-way ANOVA showed significant differences in synapse density, synapsin I/II density and PSD-95 density. However, Tukey's post hoc analysis indicated a decrease in PSD-95 density only by

PS compared to the control group. No differences were observed in distal dendrites. Western blot analysis of synaptic markers including GluN1, PSD-95 and synaptophysin after 1 and 5 days of treatment showed no significant differences.

6.2 Effect of endogenous NMDAR modulators on inhibitory neurons

6.2.1 Endogenous NMDAR modulators on inhibitory markers

After 5 days of treatment, endogenous NMDAR modulators did not affect intracellular or extracellular GABA concentrations.

6.2.2 Endogenous NMDAR modulators do not alter inhibitory synaptic proteins

Western blot analysis showed that the expression of gephyrin and VGAT, inhibitory postsynaptic and presynaptic proteins, respectively, was not altered by endogenous NMDAR modulators.

6.2.3 Endogenous NMDAR modulators decrease dendritic branching of parvalbumin-positive neurons

Treatment with endogenous NMDAR modulators for 5 days resulted in a decrease in total dendritic length, total number of dendritic branches and number of branch points in parvalbumin-positive inhibitory neurons (Figure 4B-E). Sholl analysis also revealed a decrease in the area under the curve and the maximum number of crossings (Figure 4F-H).

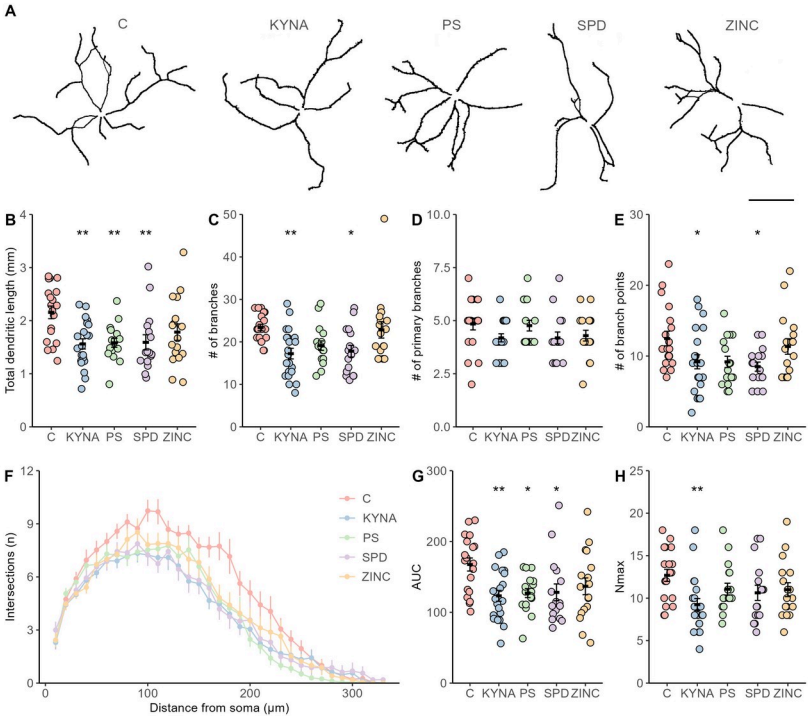

Figure 4. Dendritic branching and arbor complexity in parvalbumin-positive neurons. A Representative neurons. **B-E** Decreased dendritic branching and **F-H** arbor complexity. Means ± SEM. n = 16–21 neurons per treatment from 3 replicates. One-way ANOVA, Dunnett's post hoc. Scale bar = 100 µm.

6.3 Effect of pregnenolone sulfate on chronic despair model in mice

6.3.1 Anxiolytic-like effect of pregnenolone sulfate

During the OFT, the administration of PS resulted in an increase in the time spent in the center compared to the control group. However, no differences were observed in the total distance traveled or the number of entries to the center (Figure 5).

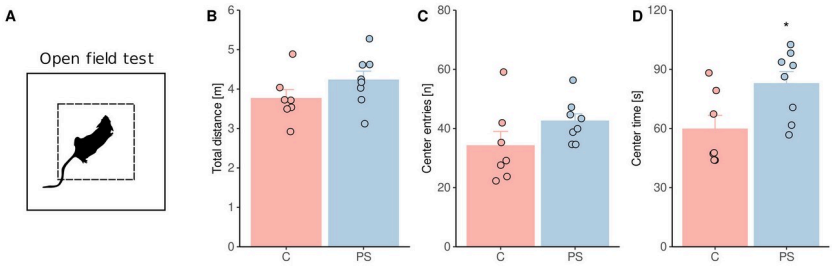

Figure 5. Decreased anxiety-like behaviors after PS administration. A Schematic diagram of the OFT. **B** Total distance traveled and **C** number of entries to the center are unchaged between groups. **D** PS increased the time spent in the center of the open field. Unpaired t-test. n = 7-8 per group. Means ± SEM.

6.3.2 Effect of pregnenolone sulfate on chronic distress model in mice

During the sociability and social novelty sessions of the 3-CT, During the sociability test, PS administration did no change the time spent with the mouse or empty glass and social index. No difference were found neither in preference for social novelty, since there was not difference in time spent with the familiar mouse or novel mouse and social novel index.

6.3.3 Effect of pregnenolone sulfate on chronic distress model in mice

In the FST, there were no differences in immobility time or latency of the first episode of immobility between the control and PS-treated groups.

7. Discussion

This study investigates the impact of endogenous NMDAR modulators on glutamate release, synaptic inputs, dendritic branching, and BDNF/TrkB/ERK signaling. The findings suggest that these modulators do not affect evoked glutamate release. However, PS enhanced distal dendritic arborization. Furthermore, PS treatment increased TrkB phosphorylation and showed a trend towards elevated BDNF expression after one hour. PS treatment led to a decrease in PSD-95 puncta density, but did not affect overall synaptic density. Additionally, the expression and release of GABA, gephyrin, and VGAT proteins remained unchanged. It is worth noting that all endogenous NMDAR modulators, except for ZINC, reduced dendritic branching and arbor complexity in parvalbumin-positive inhibitory neurons.

7.1 Glutamate release in not altered by endogenous NMDAR modulators

Glutamate release was unaffected by endogenous NMDAR modulators, as depolarization-induced glutamate release remained consistent over 1 and 5 days of treatment. Although presynaptic NMDARs usually enhance glutamate release through calcium-dependent mechanisms, our results showed no such alteration in glutamate release with the use of endogenous NMDAR modulators. Previous studies have reported that PS can enhance glutamate release through various mechanisms, including modulation of σ1 receptors and presynaptic NMDARs (Mameli et al., 2005; Meyer et al., 2002; Zamudio-Bulcock et al., 2011). However, our findings do not align with these reports. This difference may be due to variations in experimental techniques, which could be attributed to the higher temporal resolution of electrophysiological methods.

7.2 Pregnenolone sulfate increases dendritic field expansion

The evidence suggests that PS regulates dendritic arborization, but not branching, by increasing the dendritic field. SPD has a mild effect on reducing branching intersections close to the soma. Further analysis is necessary to elucidate the underlying mechanisms. There is ample evidence supporting the pivotal role of NMDAR in regulating dendritic complexity (Ewald et al., 2008). NMDAR activity can induce alterations in local structural proteins within postsynaptic compartments or activate long-range

signaling pathways involving MAPK and transcription factors, resulting in global responses (Cline & Haas, 2008). Research employing NMDAR agonists and antagonists has produced inconsistent findings concerning dendritic morphology, underscoring the intricacy of NMDAR-mediated effects. Expanding the reach of the dendritic tree without altering its total length can influence the distribution of synaptic input and intrinsic neuronal excitability. According to computational models, extending the distal dendrites enhances responsiveness to synaptic inputs that target the soma and proximal dendrites, potentially increasing firing activity and affecting synaptic integration and cortical network dynamics (Jorratt et al., 2023). These findings highlight the diverse impact of neurosteroids on neuronal morphology and connectivity.

7.3 Pregnenolone sulfate increases BNDF and TrkB activation

BDNF plays a crucial role in shaping neuronal architecture through TrkB receptor activation. It increases the branching of cortical and hippocampal neurons in dissociated cultures and organotypic slices (Horch & Katz, 2002). Therefore, the observed expansion of dendritic fields after PS treatment is consistent with the trend towards increased BDNF expression. TrkB activation depends on dimerization and subsequent phosphorylation of its intracellular tyrosine residues. Consequently, there was a significant increase in TrkB phosphorylation and a trend towards increased ERK1/2 expression after 1 hour of PS treatment. In contrast, phosphorylation of ERK1/2 was reduced after 6 hours of treatment, presumably due to a compensatory mechanism.

7.4 Pregnenolone sulfate decreased PSD-95 density, but not expression

PS reduces the density of PSD-95 puncta, particularly in proximal branches rather than distal ones. The lack of difference in total PSD-95 expression, as revealed by western blot analysis, may be due to the higher number of distal branches, which masks the results of the proximal branches. It is interesting to note that PSD-95 synaptic expression restricts dendritic branching by blocking NR2B-NMDAR synaptic clustering (Bustos et al., 2014). The decrease in PSD-95 expression with PS may involve a shift from PSD-95/GluN2A-NMDAR to SAP102/GluN2B-NMDAR complexes, potentially promoting increased arbor complexity. However, attempts to

verify this hypothesis by measuring the PS-induced alteration in the synaptic GluN2A/GluN2B subunit ratio using confocal microscopy were unsuccessful due to rapid fluorescence signal bleaching.

7.5 Endogenous NMDAR modulators decrease dendritic branching in parvalbumin-positive neurons

Fast-spiking parvalbumin-positive interneurons are the largest class of inhibitory neocortical cells. They play a critical role in regulating network synchrony and oscillations linked to learning and memory (Letzkus et al., 2011). The dendritic arbor architecture in GABAergic cultured neurons is altered by prolonged administration of the NMDA receptor antagonist MK801 and ketamine (Vutskits et al., 2006, 2007). Ketamine also impairs growth cone formation, synaptogenesis, dendritic development and maturation in human GABAergic projection neurons derived from human inducible pluripotent stem cells (X. Li et al., 2022). Therefore, our findings are consistent with previous studies using exogenous NMDAR antagonists.

7.6 Anxiolytic-like effect of pregnenolone sulfate in chronic despair model in mice

The “neurotrophin hypothesis” proposes a link between reduced hippocampal BDNF levels and depressive behaviours (Martinowich et al., 2007). Antidepressant treatment enhances BDNF expression. Since our results indicated that PS tends to increase BDNF expression, activates BDNF/TrkB/ERK pathway and dendritic field expansion, we tested its potential antidepressant and anxiolytic-like effect in chronic despair model. While PS exhibited an anxiolytic effect, no differences were observed in other behavioural tests. These findings are consistent with previous studies on PS's antidepressant and anxiolytic effects (Dhir & Kulkarni, 2008; Reddy & Kulkarni, 1997). However, these findings were obtained without a stress-inducing protocol. The protocol we used to model chronic despair involved forcing mice to swim for five consecutive days. This is based on the idea that depression in humans is induced by chronic rather than acute stress. Future studies could investigate changes in synaptic protein expression and dendritic spine density following PS treatment in mice exposed to the chronic despair model.

8. Conclusion

Abnormalities in glutamatergic neurotransmission, particularly those involving NMDAR dysfunctions, have been linked to various psychiatric disorders. However, understanding how these changes translate into mood disorders, addiction, and developmental brain diseases remains a challenge. Neurons and glial cells produce and release endogenous NMDAR modulators that affect neuronal processes and functions. Levels of these modulators have been observed to be altered in patients with mental disorders. The study examines the effect of endogenous NMDAR modulators on neuronal morphology and synaptic dynamics. PS appears to contribute to anxiolytic-like behaviour in mice by increasing BDNF release, activating TrkB, and expanding dendritic fields. Therefore, dysregulation of endogenous PS levels could impair the BNDF/TrkB signaling cascade, which may be related to decreased PS levels in patients with affective disorders, generalized social phobia, and anxiety disorder. Additionally, the research indicates that parvalbumin-positive neurons are particularly vulnerable to structural changes caused by endogenous NMDAR modulators, elucidating their role in neuronal circuitry and their potential significance in psychiatric pathophysiology.

9. References

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10. Overview of publications

10.1 Publications with IF related to the thesis

- **Jorratt P**, Ricny J, Leibold C, Ovsepian SV. Endogenous Modulators of NMDA Receptor Control Dendritic Field Expansion of Cortical Neurons. *Mol Neurobiol*. 2023;60(3):1440-1452. **(IF = 5.686).**
- **Jorratt P**, Hoschl C, Ovsepian SV. Endogenous antagonists of N-methyl-d-aspartate receptor in schizophrenia. *Alzheimers Dement*. 2021;17(5):888-905. **(IF = 16.655).**

10.2 Publications with IF non-related to the thesis

- Vicencio-Jimenez S, Delano PH, Madrid N, Terreros G, Maass JC, Delgado C, **Jorratt P**. Maintained Spatial Learning and Memory Functions in Middle-Aged α9 Nicotinic Receptor Subunit Knock-Out Mice. *Brain Sciences*. 2023; 13(5):794. **(IF = 3.333).**
- Syrová K, Šíchová K, Danda H, Lhotková E, **Jorratt P**, Pinterová-Leca N, Vejmola Č, Olejníková-Ladislavová L, Hájková K, Kuchař M, Horáček J, Páleníček T. Acute pharmacological profile of 2C-B-Fly-NBOMe in male Wistar rats-pharmacokinetics, effects on behaviour and thermoregulation. *Front Pharmacol*. 2023;14:1120419. **(IF = 5.988).**
- Šíchová K, Syrová K, Kofroňová E, Pinterova-Leca N, Vejmola Č, Nykodemová J, Palivec P, Olejníková L, Danda H, **Jorratt P**, Adam Š, Hiep BQ, Štefková-Mazochová K, Končická M, Kuchař M, Páleníček T. Pharmacokinetics, systemic toxicity, thermoregulation and acute behavioural effects of 25CN-NBOMe. *Addict Biol.* 2022;27(5):e13216. **(IF = 4.093).**
- Alvarez-Munoz H, Vicencio-Jimenez S, **Jorratt P**, Delano PH, Terreros G. Corticofugal and Brainstem Functions Associated With Medial Olivocochlear Cholinergic Transmission. *Front Neurosci*. 2022;16:866161. **(IF = 5.152).**
- Zaitsev AV, Smolensky IV, **Jorratt P**, Ovsepian SV. Neurobiology, Functions, and Relevance of Excitatory Amino Acid Transporters (EAATs) to Treatment of Refractory Epilepsy. *CNS Drugs*. 2020;34(11):1089-1103. **(IF = 6.497).**