Charles University

Third Faculty of Medicine

Dissertation

Účinky endogenních modulátorů NMDA receptorů na morfologii neuronů a synaptickou plasticitu

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

Supervisor: MUDr. Tomáš Páleníček, PhD

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Abstract

Activation of the N-methyl-D-aspartate receptor (NMDAR) leads to downstream signaling that modulates structural and functional plasticity, which is crucial for cognitive processes such as learning and memory. Impairments in NMDAR activity are implicated in various mental disorders, including depression and schizophrenia. Despite the growing body of evidence on the effects and potential therapeutic benefits of ketamine and other NMDAR inhibitors, there is still a gap in undestanding the effects of endogenous NMDAR modulators. Specifically, their effects on the neuronal structure and synaptic density is unclear, despite the fact that their levels are dysregulated in patients with mental disorders.

The objective of this study is to elucidate the molecular and cellular changes resulting from endogenous NMDAR modulators and their potential implications in psychiatric disorders. To achieve this, we assessed the morphological and synaptic changes of excitatory and inhibitory neurons induced by the prevalent endogenous NMDAR modulators, including kynurenic acid, pregnenolone sulfate (PS), spermidine, and zinc at various time points.

We measured cell viability using the MTS assay and glutamate release using HPLC. Immunocytochemistry was used to measure dendritic branching and synaptic density. Western blot was used to measure the expression of synaptic proteins, while ELISA for measuring the expression of GABA and BDNF. The study also assessed the antidepressant-like effects of PS in a chronic despair model in mice using the open field test, three chamber test, and forced swim test.

We have not observed any significant changes in glutamate release, neuronal viability or dendritic branching. However, a comparison of the arbor complexity revealed that PS increased distal dendritic arborization, which is consistent with the tendency towards increased expression of BDNF and activation of the TrkB receptor. The density of glutamatergic synapses was consistent across all neuronal groups, except for those treated with PS, which exhibited a reduction in puncta of the scaffolding postsynaptic protein PSD-95. Parvalbumin-positive inhibitory neurons treated with the endogenous NMDAR modulators exhibited a decrease in dendritic branching and arbor complexity, without altering GABA release or expression. Finally, PS was found to reduce anxiety-like behaviour in a chronic despair model in mice.

The observed increase in BDNF release, activation of TrkB receptor and expansion of dendritic fields may contribute to the anxiolytic-like effect observed in mice. Furthermore, the results emphasise the increased sensitivity of parvalbumin-positive neurons to the endogenous NMDAR modulators, providing more insight into their implications in psychiatric disorders.

Abstrakt

Aktivace N-methyl-D-aspartátového receptoru (NMDAR) vede skrze intracelulární signální kaskády k modulaci funkční a strukturní plasticity, a sehrává tak zásadní roli v kognitivních procesech jako je učení a paměť. Poruchy funkce NMDAR se podílejí na patogenezi duševních onemocnění, jako je deprese či schizofrenie. Navzdory rostoucímu počtu studií popisujících účinky a potenciální terapeutické využití ketaminu a dalších inhibitorů NMDAR existuje stále významná mezera v pochopení účinků endogenních modulátorů NMDAR. Například není objasněný jejich vliv na neuronální růst, strukturu a synaptickou hustotu, přestože právě tyto procesy jsou u pacientů s duševními poruchami často dysregulovány.

Cílem této studie je objasnění molekulárních a buněčných změn způsobených endogenními modulátory NMDAR a jejich potenciálních účinků u psychiatrických poruch. Za tímto účelem jsme hodnotili morfologické a synaptické změny excitačních a inhibičních neuronů vyvolané významnými endogenními modulátory NMDAR kyselinou kynurenovou, pregnenolon sulfátem (PS), spermidinem a zinkem v různých časových bodech.

Životaschopnost buněk jsme měřili pomocí testu MTS a uvolňování glutamátu pomocí HPLC. Imunocytochemie byla použita k měření dendritického větvení a synaptické hustoty, western blot k měření exprese synaptických proteinů a ELISA k měření exprese GABA a BDNF. Studie dále hodnotila antidepresivní účinky PS v chronic dispair modelu u myší za využití open field, three chamber a forced swim testu.

Nebyly pozorovány žádné signifikantní změny v uvolňování glutamátu, životaschopnosti neuronů ani v dendritickém větvení. Srovnání složitosti dendritických větví však ukázalo, že PS zvýšil distální dendritickou arborizaci, což je v souladu se zvýšenou expresí BDNF a aktivací receptoru TrkB. Hustota glutamátergních synapsí byla srovnatelná ve všech skupinách kromě neuronů ošetřených PS, u nichž byl snížený počet synaptických postsynaptického scaffoldového proteinu PSD-95. Parvalbumin pozitivní inhibiční neurony vykazovaly následkem působení všech použitých NMDAR modulátorů snížení dendritického větvení a jeho složitosti, aniž by se změnilo uvolňování nebo exprese GABA. V chronic despair modelu u myší prokázal PS anxiolytický efekt.

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.

List of Abbreviations

3-CT	three chamber test
AHR	aryl hydrocarbon receptor
ALLO	allopregnanolone
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPAR	AMPA receptor
ANOVA	analysis of variance
Arc	activity-regulated cytoskeleton-associated protein
ATD	amino-terminal domain
BBB	brain-blood barrier
BCA	bicinchoninic acid assay
BDNF	brain-derived neurotrophic factor
CNS	central nervous system
CSF	cerebrospinal fluid
CTD	carboxyl-terminal domain
DIV	days in vitro
DMEM	dulbecco's modified eagle medium
DSM-IV-TR	diagnostic and statistical manual of mental disorders, text revision
DT	day-treatment
ELISA	enzyme-linked immunosorbent assay
EPSC	excitatory postsynaptic current
FBS	fetal bovine serum
FST	forced swim test
GABA	gamma-aminobutyric acid
GAD	glutamate decarboxylase
GFAP	glial fibrillary acidic protein
Gln	glutamine
GLT-1	glutamate transporter 1
Glu	glutamate
Glu-BS	glutamate binding site
Gly-BS	glycine binding site
GPR35	G protein–coupled orphan receptor 35
HBSS	hank’s balanced salt solution
HEPES	2-[4-(2-Hydroxyethyl)piperazin-1-yl]ethane-1-sulfonic acid
HPLC	high-performance liquid chromatography

IC_{50}	half maximal inhibitory concentration
IDO	indoleamine 2,3-dioxygenase
iGluR	ionotropic glutamate receptor
KAR	kainic acid receptors
KAT	kynurenine aminotransferases
KO	knock-out
KR	krebs–ringer
KYNA	kynurenic acid
LBD	ligand-binding domain
LPS	lipopolysaccharide
LTP	long-term potentiation
MAP2	microtubule-associated protein 2
MT	metallothionein
mTORC	mammalian target of rapamycin complex
nAChR	nicotinic acetylcholine receptor
NAD^+	nicotinamide adenine dinucleotide
NMDA	N-methyl-D-aspartate
NMDAR	NMDA receptor
OD	optical density
ODC	ornithine decarboxylase
OFT	open field test
PA	pregnanolone
PAS	pregnanolone sulfate
PBS	phosphate-buffered saline
PBST	PBS tween
PCP	phencyclidine
PFC	prefrontal cortex
PREG	pregnenolone
PS	pregnenolone sulfate
PSD-95	postsynaptic density protein 95
ROS	reactive oxygen species
RT	room temperature
SMOX	spermine oxidase
SMS	spermine synthase
SNAP25	synaptosomal-associated protein, 25kDa

SPD	spermidine
SRM	spermidine synthase
SSV	small synaptic vesicles
SULT	sulfotransferase
TDO	tryptophan 2,3-dioxygenase
TMD	transmembrane domain
VGAT	vesicular GABA transporter
VGCC	voltage-gated calcium channel
VGLUT1	vesicular glutamate transporter 1
VPAT	vesicular polyamine transporter
ZEN	zinc-enriched neuron
ZnT	zinc transporter

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1. Introduction

Mental disorders, such as depression, anxiety, schizophrenia and post-traumatic stress disorder, are characterised by disturbances in emotional regulation, cognition, behaviour, and sometimes perception. In 2019, one in eight people worldwide lived with a mental disorder, with anxiety and depressive disorders being the most prevalent. The following year, the number of people living with anxiety and depressive disorders increased by 26% and 28%, respectively, due to the COVID-19 pandemic (Organization, W.H., 2022).

The diagnosis of mental disorders is made clinically on the basis of observable symptoms and behaviours, usually using the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV-TR) and the World Health Organisation's International Statistical Classification of Diseases and Related Health Problems (ICD-10). The aetiology, including the molecular basis, remains incompletely understood (Lakhan et al., 2013).

The pathogenesis of certain psychiatric disorders may be linked to changes in the balance between excitation and inhibition in neuronal circuits (Sohal & Rubenstein, 2019). N-Methyl-D-aspartate receptors (NMDAR) are widely distributed throughout the brain and are essential for establishing and maintaining this balance (Paoletti et al., 2013). Dysfunction of NMDARs, which can cause an imbalance between excitation and inhibition, is frequently observed in psychiatric disorders (Myers et al., 2019; XiangWei et al., 2018). Therefore, NMDARs are important potential targets for drug discovery and development. Currently, there are few safe and effective medications for treating psychiatric disorders, and developing drugs for mental disorders poses many challenges (Becker & Greig, 2010; Berk, 2012; Pankevich et al., 2014).

1.1 Function and structure of the NMDAR

Glutamate is the major excitatory neurotransmitter in the nervous system and activates two different types of receptors: fast acting ionotropic (iGluRs) and slow acting metabotropic glutamatergic receptors (Kew & Kemp, 2005). iGluRs are organised as receptor-channel complexes that become permeable to non-selective cations upon binding to glutamate. This results in membrane depolarisation, which leads to the generation of action potentials when the threshold is reached. iGluRs are divided into three groups according to selective agonists: (1) kainate or kainic acid receptors (KARs), (2) α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) and (3) NMDARs (Madden, 2002).

AMPARs are recognised for their fast gating and desensitisation kinetics and are essential for the initial rapid component of the excitatory postsynaptic currents (EPSCs) (Twomey et al., 2019). On the other hand, KARs contribute modestly to the excitatory postsynaptic transmission

due to a limited subset of neurons expressing KARs postsynaptically and the small amplitude of KAR-mediated EPSCs (Castillo et al., 1997). The slow component of EPSCs is attributed to NMDARs, which have slow gating and desensitization kinetics and relatively weak desensitization. NMDARs are unique among iGluRs due to their high permeability to calcium ions. NMDAR-mediated calcium signalling plays a significant role in regulating synaptic development and plasticity. However, excessive NMDAR-mediated calcium influx may lead to neuronal damage or death and is implicated in the pathogenesis of numerous neurodegenerative disorders (Hansen et al., 2021; Traynelis et al., 2010) .

Native NMDARs are tetrameric assemblies formed by two obligatory GluN1 subunits and two GluN2 and/or GluN3 subunits, which occur in various molecular combinations (Ulbrich & Isacoff, 2008). Subunits exhibit different spatiotemporal expression profiles. Only GluN2B and GluN2D are expressed in the embryonic brain, while GluN2A expression begins shortly after birth and steadily increases to become widely and abundantly expressed in virtually every area of the adult central nervous system (CNS). As non-GluN1 subunits confer distinct physiological properties to the NMDAR, those expressed early in development play a crucial role in synaptogenesis and synaptic maturation. Meanwhile, those expressed in higher brain structures, such as the hippocampus and cortex, have central roles in synaptic function and plasticity in the adult CNS (Paoletti et al., 2013). In addition to the spatiotemporal expression profiles, different neuronal types express distinct NMDAR subunits, such as in adult hippocampus, where GluN2A and GluN2B mRNAs are prominent in CA1 and CA3 pyramidal cells, but GluN2C and GluN2D mRNAs occur in interneurons (Monyer et al., 1994). The composition of receptor subunits may play a role in the signaling of synaptic and extrasynaptic NMDARs, given the enrichment of the GluN2B subunit at peri- and extrasynaptic sites. However, it is an oversimplification to suggest that GluN2A subunits are exclusively located at synaptic sites while GluN2B subunits are segregated outside of synapses (Wyllie et al., 2013).

Figure 1A,B displays the four modules of NMDAR subunits, which consist of the extracellular amino-terminal domain (ATD) involved in subunit assembly and allosteric regulation, and the ligand-binding domain (LBD) formed by the two discontinuous segments S1 and S2. The LBD binds glycine in GluN1 and GluN3 subunits and glutamate in GluN2 subunits. The transmembrane domain (TMD) is composed of three membrane-spanning segments and a pore loop (M2). The intracellular carboxyl (C)-terminal domain (CTD) is a region involved in receptor trafficking and coupling receptors to signaling cascades (Vyklicky et al., 2014). The activation of NMDAR requires the relief of Mg^{+2} block at the ion channel pore, depolarization of the postsynaptic membrane by AMPAR, and two molecules of the agonist glutamate and two molecules of the co-agonist glycine for NMDAR composed of GluN1/GluN2 and only glycine for NMDAR

composed of GluN1/GluN3 (Pachernegg et al., 2012). Alternatively, additional co-agonists include D-alanine, L-alanine, D-serine, L-serine (P. E. Chen et al., 2008), as well as other agonists such as D-aspartate, D-glutamate, L-glutamate, NMDA, N-methyl-L-aspartate (Erreger et al., 2007), can be used.

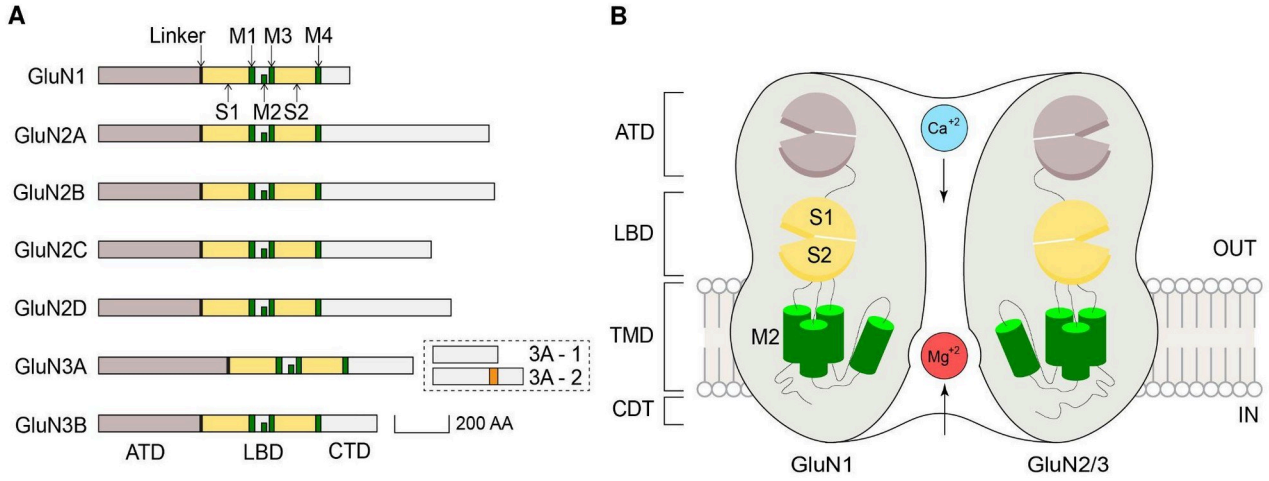

Figure 1. Molecular structure of NMDAR subunits. A GluN1 subunit encoded by one *GRIN1* gene (has eight splice variants) and four GluN2 (A-D) encoded by *GRIN2* (A-D) genes. Two GluN3 (A,B) encoded by *GRIN3* (A,B) are also shown. **B** Schematic representation of NMDAR and its subdomains, outlining the four domains of the subunits: (i) amino-terminal domain (ATD), (ii) ligand-binding domain (LBD) formed by S1 and S2 segments, (iii) transmembrane domain (TMD) containing the conducting pore (Ca^{+2}-permeable channel blocked by Mg^{+2} under hyperpolarised state), and (iv) intracellular carboxyl (C)-terminal domain (CTD). The figure is reproduced with permission from (Jorratt et al., 2021).

Several psychiatric disorders are linked to specific genomic variants that cause NMDAR dysfunction. These variants are widely considered to be one of the molecular bases of the prevalent comorbidities among many of these disorders (Hanada, 2020). Missense, nonsense, frameshift, splice site, and other disease-associated variants of NMDAR subunits are found particularly within genes encoding GluN1, GluN2A, and GluN2B subunits (Burnashev & Szepetowski, 2015; Strehlow et al., 2019). Functional analyses have revealed that genomic variations can cause a wide range of changes in NMDAR properties. These changes include alterations in agonist/co-agonist potency, single-channel conductance, receptor kinetics, calcium permeability, and pharmacological profile (Paoletti et al., 2013; XiangWei et al., 2018). Furthermore, variant NMDARs can be hyperfunctional (gain of function), hypofunctional (loss of function), or maintain unaltered receptor function in neuropsychiatric disorders (XiangWei et al., 2018). Genetic variants in other risk genes of psychiatric disorders or other factors can indirectly cause dysfunction of NMDARs (Banerjee et al., 2015; Freedman et al., 1995; Pitcher et al., 2011; Wei et al., 2014).

1.2 Pharmacological modulation of NMDAR

The NMDAR activity can be modulated by various ligands, such as competitive antagonists, open channel blockers and allosteric modulators.

1.2.1 Competitive antagonists

Competitive antagonists bind to the same binding site as the endogenous agonists without activating the receptor. Once bound, these antagonists block agonist binding, leading to reduced receptor activation. Competitive antagonists that selectively inhibit NMDAR currents include D-2-amino-5-phosphonovaleric acid (D-AP5) and 2-amino-7-phosphonoheptanoic acid (AP7), both of which are specific to the glutamate-binding site on the NMDAR. In animal models, D-AP5 and AP7 have demonstrated anticonvulsant properties (Meldrum et al., 1988; Zivanović et al., 1999). In addition, the potent glutamate-site antagonist CGP-37849 has a broad range of effects, including neuroprotection, anticonvulsant activity, as well as antidepressant and anxiolytic effects (Fujikawa et al., 1994; Gutnikov & Gaffan, 1996; Jessa et al., 1996; Papp & Moryl, 1994; Schmutz et al., 1990).

1.2.2 Open channel blockers

Open channel blockers prevent ion flux by binding to the NMDAR within the open channel pore. They are predominantly positively charged and bind to the receptor in a voltage-dependent manner. Channel blockers can be classified into three groups based on their mode of action: “trapping”, “partial trapping” and “foot in the door” blockers. Trapping blockers are small molecules that enter the channel pore and bind to it. They remain bound inside the pore even after agonist dissociation and channel closure. Therefore, the channel must reopen for the trapping blockers to unbind. Ketamine and MK-801 are examples of such trapping blockers. Ketamine has been used as an anesthetic since the 1960s. It exerts inhibitory effects through dual mechanisms: channel block at lower concentrations and negative allosteric modulation at higher concentrations (Orser et al., 1997). Although ketamine has potent analgesic properties at subanesthetic doses, its use as an analgesic is limited due to its dissociative effects at similar doses, which can cause psychotomimetic side effects (Ballard & Zarate, 2020). However, ketamine is still highly regarded for its rapid-acting and long-lasting antidepressant effects (Krystal et al., 2024). Partial trapping blockers hinder but do not completely prevent the channel closure and can escape the channel pore upon agonist unbinding. Memantine acts as a partial trapping blocker, with low-affinity to NMDAR, characterized by fast unbinding kinetics (Kotermanski et al., 2009) and widely used to treat Alzheimer’s disease (Lipton, 2005). Memantine preferentially inhibits extrasynaptic over synaptic NMDAR (Xia et al., 2010). Since the excessive activation of extrasynaptic NMDAR is one

of the major causes of excitotoxicity, preferential inhibition of extrasynaptic NMDAR by memantine is considered to underlie its neuroprotective effect (Bading, 2017). Finally, “foot in the door” blockers prevent channel closure and agonist dissociation while bound inside the channel. Consequently, agonists cannot unbind from the receptor while blocked by “foot in the door” blockers. 9-aminoacridine acts as a “foot in the door” blocker (Barygin et al., 2009).

1.2.3 Allosteric modulators

Allosteric modulators bind to specific sites on the NMDAR distinct from the agonist binding sites. Positive allosteric modulators enhance receptor function, while negative allosteric modulators inhibit it. Protons act as a negative allosteric modulator, tonically inhibiting NMDARs at physiological pH levels (Traynelis & Cull-Candy, 1990). Variations in extracellular proton concentrations, influenced by factors such as neurotransmitter release or ischemic conditions, can significantly impact NMDAR function (Giffard et al., 1990). Protons interact with multiple sites on the NMDAR, reducing its open probability and glycine affinity (Jalali-Yazdi et al., 2018). Ethyl alcohol acts as a non-selective NMDAR antagonist, with varying affinities for different subunits, particularly affecting those containing GluN2A and GluN2B (Chandrasekar, 2013). Subunit-specific NMDAR antagonists, such as ifenprodil and TCN-201, have been developed to distinguish responses among different NMDAR subtypes. Ifenprodil exhibits a strong inhibitory effect on GluN2B-containing receptors through a voltage-independent mechanism, reducing receptor open probability (Williams, 2001). In contrast, TCN-201 selectively inhibits GluN2A-containing NMDARs by reducing glycine affinity (Hansen et al., 2012). These modulators are useful for investigating the various roles of NMDAR subunits and their implications in physiological and pathological conditions.

1.3 Endogenous NMDAR modulators and psychiatric disorders

Although extensive research has been conducted on the structure and molecular characteristics of the NMDAR and its involvement in synaptic transmission, establishing a correlation between functional alterations and the symptoms of mental disorders remains a challenging task. Research has also been conducted to understand the effects of exogenous antagonists such as ketamine, phencyclidine, MK801, and others on neuronal excitability, synaptic functions, and plasticity. However, it is worth noting that there is currently limited knowledge regarding the role of endogenous modulators in these processes. Therefore, it is important to further elucidate the regulation of NMDAR functions by endogenous modulators, particularly for a better understanding of mental disorders related to NMDAR dysregulations. This section aims to provide

a summary of the main endogenous modulators of the NMDAR (Figure 2) and to examine their potential involvement in the pathology of mental disorders.

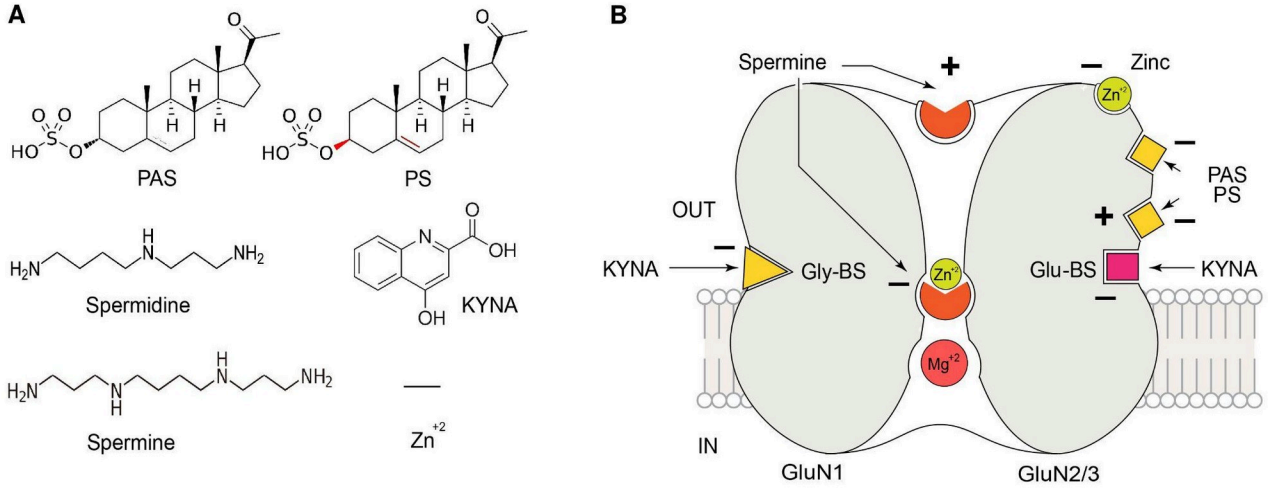

Figure 2. Endogenous modulators and their binding sites within the NMDAR. A Structure of endogenous modulators: pregnanolone sulfate (PAS), pregnenolone sulfate (PS), spermidine, spermine and kynurenic acid (KYNA). **B** NMDAR representation showing the binding sites of endogenous modulators. Symbols (+) and (-) indicate facilitatory and inhibitory effects, respectively. Glu-BS corresponds to the glutamate binding site, while Gly-BS represents the glycine binding site. The figure is reproduced with permission from (Jorratt et al., 2021).

1.3.1 Kynurenic acid

Kynurenic acid (KYNA) is an endogenous antagonist of NMDAR. It is derived from the essential amino acid L-tryptophan, which is involved in the production of serotonin and melatonin, protein biosynthesis, and the kynurenine pathway. This pathway is responsible for over 90% of tryptophan catabolism. Kynurenine is synthesized from L-tryptophan by indoleamine 2,3-dioxygenase (IDO) and tryptophan 2,3-dioxygenase (TDO) (Sainio et al., 1996) (Figure 3A). Subsequently, it undergoes irreversible transamination by four types of kynurenine aminotransferases (KAT I-IV) to produce KYNA (Han et al., 2010). Among them, KAT II is the major biosynthetic enzyme of KYNA in murine and human brain, being expressed in astrocytes and neurons, mainly GABAergic interneurons (Herédi et al., 2017). It is interesting to note that both IDO and TDO are expressed at low levels in the brain compared to peripheral organs (Dang et al., 2000). Unlike kynurenine, KYNA exhibits poor permeability across the blood-brain barrier (BBB) due to its inability to efficiently cross amino acid transporters (Fukui et al., 1991). As a result, it is primarily synthesized within the brain from precursors.

The antagonism of NMDAR by KYNA its non-competitive at the glycine co-agonist site (IC_{50} ~ 15 μM) (Kessler et al., 1989) and competitive at glutamate binding site at higher concentrations (200 - 500 μM) (Birch et al., 1988). Interestingly, KYNA has dual effects on AMPAR in a dose-dependent manner. At millimolar concentrations, it acts as an AMPAR antagonist, while at nanomolar to micromolar concentrations, it enhances AMPA currents through positive allosteric modulation (Prescott et al., 2006). In addition, KYNA is a potent non-competitive

antagonist of the α7 nicotinic acetylcholine receptor (nAChR) with an IC_{50} of approximately 7 μM (Hilmas et al., 2001). Table 1 summarises KYNA's targets.

Table 1. Targets of kynurenic acid.

Receptor	**Effect**	**Concentration**
NMDAR	IC_{50} (Glycine site)	15 μM
	IC_{50} (Glutamate site)	200 - 500 μM
KAR	IC_{50}	500 μM
AMPAR	Potentiate	0.03–30 μM
	Inhibit	3 mM
α7 nAChR	IC_{50}	7 μM
GPR35	EC_{50}	8 - 40 μM
AHR	EC_{50}	1-2 μM

nAChR: nicotinic acetylcholine receptors. AHR: aryl hydrocarbon receptor. GPR35: G protein–coupled orphan receptor 35.

In patients with schizophrenia and bipolar disorders, levels of KYNA in cerebrospinal fluid (CSF) and brain tissue are significantly higher compared to healthy controls (Plitman et al., 2017; A. K. Wang & Miller, 2018). Moreover, TDO level are significantly increased in postmortem frontal cortex and anterior cingulate cortex (Miller et al., 2004, 2006). The results of animal studies are generally consistent, with an increase in brain KYNA levels leading to impairments in pre-pulse inhibition (Erhardt et al., 2004), deficit of spatial working memory (Chess et al., 2007) and contextual fear memory and learning (Chess et al., 2009), as well as impairments auditory sensory gating (Shepard et al., 2003). Using knock-out mice with reduced endogenous KYNA levels due to the deletion of KAT II resulted in improved performance in object exploration and recognition, as well as spatial discrimination (Potter et al., 2010). These findings support the hypothesis that endogenous KYNA affects cognitive function.

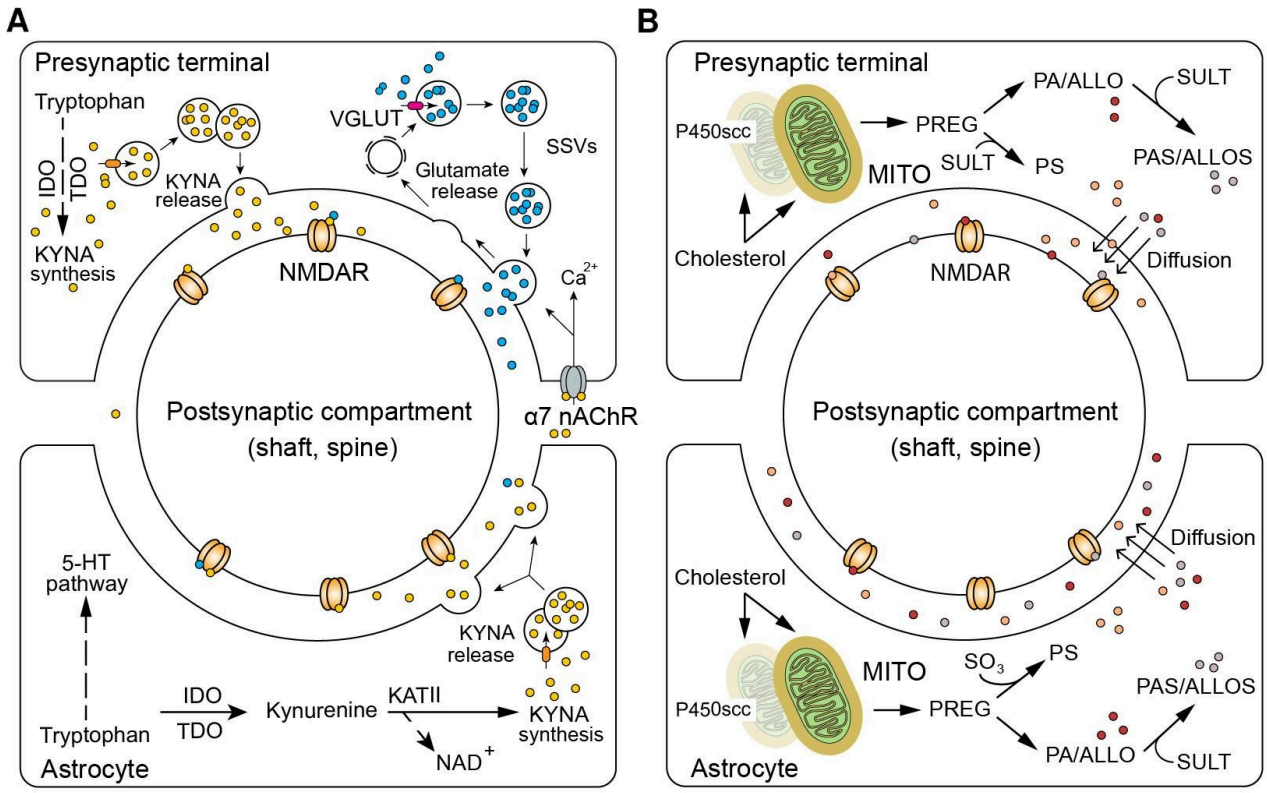

Figure 3. Schematic representation of the neurochemical origin and release of kynurenic acid (KYNA) and neurosteroids at glutamatergic synapses. A KYNA is a product of tryptophan metabolism synthesized from its precursor kynurenine. It is primarily released from astrocytes but can also be released from neurons. KYNA exhibits both excitatory and inhibitory effects on various receptors depending on its concentration. When released from glia or synaptic terminals, KYNA inhibits postsynaptic NMDARs. It binds to and blocks presynaptic α7 nAChRs. The mechanism of KYNA release remains unclear. **B** The synthesis of neurosteroids is initiated by the translocation of cholesterol into the mitochondria. Pregnenolone (PREG) is produced and converted into PREG sulfate (PS), pregnanolone (PA) sulfate (PAS), and allopregnanolone (ALLO) sulfate (ALLOS). Neurosteroids are released from neurons and astrocytes through diffusion, exerting modulatory effects on postsynaptic NMDARs. The figure has been reproduced with permission from (Jorratt et al., 2021). The abbreviations used in the figure are IDO for indoleamine 2,3-dioxygenase, TDO for tryptophan 2,3-dioxygenase, NAD^+ for nicotinamide adenine dinucleotide, KATII for kynurenine aminotransferase II, and SULT for sulfotransferase.

1.3.2 Neurosteroids

Neurosteroids are a class of steroids synthesized in the brain, independent of endocrine gland function (E.-E. Baulieu & Robel, 1990). The first step in this pathway involves translocating cholesterol across the inner mitochondrial membrane, where it is converted into pregnenolone (PREG) by the enzyme CYP11A1 (see Figure 3B). PREG, pregnanolone (PA) and allopregnanolone (ALLO) isoforms can be subsequently synthesized by the sequential enzymatic action of 3β-hydroxysteroid dehydrogenase, 5α/β reductase and 3α/β-hydroxysteroid dehydrogenase (E. E. Baulieu et al., 2001). These compounds have modulatory effects on ionotropic receptors, such as $GABA_A$, glycine, glutamate, and nicotinic acetylcholine receptors (Rupprecht & Holsboer, 1999), which are relevant in several neurophysiological and psychiatric processes.

The effects of PREG, PA and ALLO on NMDAR depend upon sulfation at the C3 carbon group site by SULTs (Schumacher et al., 2008). The ratio of sulfated to non-sulfated neurosteroids

plays a pivotal role in determining their modulatory activity. PREG sulfate (PS) enhances NMDA-mediated Ca^{+2} influx, while PA sulfate (PAS) inhibits it. PREG and PA have no effect on it (Weaver et al., 2000). The above statement suggests that the presence of a sulfate group on carbon C3 and the double bond between C5 and C6 are crucial in determining positive and negative modulation. It is important to note that the direction of modulation of the NMDA current by sulfated neurosteroids is dependent on the subunit composition. PS enhances currents mediated by GluN1/GluN2A and GluN1/GluN2B subunits, while inhibiting currents mediated by GluN1/GluN2C and GluN1/GluN2D subunits. Meanwhile, PAS was found to be more potent at inhibiting NMDA receptors that contain GluN1/GluN2C and GluN1/GluN2D subunits (Malayev et al., 2002). The effect of PS on NMDAR potentiation was increased when applied prior to the agonist. The rate constant of channel opening and closing suggests that the mechanism of PS is attributable to the increase in the peak channel open probability (Horak et al., 2004). In contrast, PAS reduced the frequency of NMDAR channel opening and mean time (Petrovic et al., 2005).

In clinical studies, it was found that the concentration of PREG in serum was lower in patients with schizophrenia compared to healthy controls (M. Ritsner et al., 2007). Furthermore, the addition of PREG to antipsychotic medications reduced the severity of negative symptoms of schizophrenia in patients in first episode cases, as compared to placebo (M. S. Ritsner et al., 2014). Additionally, subjects with Alzheimer's disease were found to have increased levels of PREG in the prefrontal and temporal cortex (Marx et al., 2006; Naylor et al., 2010). Patients with affective disorders (George et al., 1994), generalized social phobia (Heydari & Le Mellédo, 2002) and anxiety disorder (Semeniuk et al., 2001), have been found to have decreased levels of PREG and PS. In bipolar disorder, add-on therapy with PREG has been shown to improve depressive symptoms (Brown et al., 2014). Therefore, levels of neurosteroids may vary among different psychiatric disorders. Electrophysiological experiments conducted on hippocampal slices of rats have shown that PS enhances LTP in CA1 by potentiating the activity of NMDAR. This may mediate the improvement of memory and hippocampus-dependent functions (Sliwinski et al., 2004). In preclinical studies, PS has demonstrated efficacy in ameliorating schizophrenia-like symptoms in animal models of schizophrenia with knock-out of the dopamine transporter (Wong et al., 2015). Furthermore, PS improved the acquisition of spatial information and object discrimination in a spatial/visual task. It also potentiated the firing rate of neurons in the hippocampus and perirhinal cortex recorded *in vivo* (Plescia et al., 2014). Additionally, the level of endogenous PS in the hippocampus is correlated with the distance required to reach the hidden platform in the Morris water maze test in aged rats (Vallée et al., 1997). Moreover, PS attenuates conditioned fear stress and has an antidepressant-like effect (Noda, 2000; Plescia et al., 2013). Importantly, the observed results may be attributed to the indirect effects on NMDAR, as PS acts as

an agonist for σ1 receptors (Monnet & Maurice, 2006).

1.3.3 Polyamines

Polyamines are low molecular weight aliphatic polycations with two or more amino groups that readily interact with negatively charged molecules. Spermidine and spermine, the principal endogenous polyamines with 3 and 4 amino groups, respectively, are ubiquitously present throughout the body. Ornithine, a non-proteinogenic amino acid is the major precursor of their biosynthesis, which is formed from the hydrolytic cleavage of the amino acid arginine by arginase in the cytoplasm. Subsequently, ornithine undergoes decarboxylation by ornithine decarboxylase (ODC) to produce putrescine (Coleman et al., 2004). Aminopropyl groups are then successively transferred to putrescine to form spermidine and spermine (Pegg, 2009). It is important to note that this pathway is reversible, allowing for the conversion of spermine into spermidine and putrescine, and their concentrations may vary depending on the area and age (Liu et al., 2008). Polyamines are present in both glia cells and neurons, but spermine/spermidine-like immunoreactivity is predominantly enriched in astrocytes (Laube & Veh, 1997) which have low expression of ODC and spermidine synthase (Bernstein & Müller, 1999). Furthermore, glia cells have more efficient uptake machinery (Masuko et al., 2003), suggesting that polyamines are mainly produced in neurons and stored in glial cells (see Figure 4A).

Polyamines play a crucial role in various cellular functions, including cell proliferation, antioxidant effects, scavenging of ROS, and maintaining protein and nucleic acid stability and structure. Additionally, they modulate the activity of ion channels (Pegg, 2016). Polyamines have a dual effect on NMDAR, with both stimulatory and inhibitory effects depending on various factors and binding sites (Williams, 1997). Electrophysiological studies have demonstrated that polyamines can enhance NMDAR currents by increasing the frequency of channel opening and the receptor's affinity for glycine (Araneda et al., 1993; Williams, 2009). Conversely, polyamines can reduce NMDAR currents by inducing voltage-dependent reduction of single-channel amplitudes and/or by producing an open channel block (Araneda et al., 1999). These effects are partly due to direct molecular interactions between polyamines and the NMDAR channel complex. For example, spermine enhances NMDA currents through glycine-independent stimulation. It increases the open channel probability (Araneda et al., 1993) and reduces the desensitization of NMDAR to glutamate (Lerma, 1992) when glycine and glutamate are present in saturating concentrations. Additionally, spermine enhances the affinity of the GluN1/GluN2B NMDAR for glycine in glycine-dependent stimulation, which may involve a second spermine binding site (McGurk et al., 1990). On the other hand, spermine reduces the sensitivity of NMDAR to glutamate at GluN1/GluN2B receptors, which can mask the stimulatory effects of spermine at low concentrations of agonist (Williams, 1994).

Finally, spermine can also act as a voltage-dependent blocker of NMDAR from both the extracellular and intracellular sides of the channel pore, at a site related to the extracellular binding site for Mg^{+2}. However, sensitivity to internal spermine is probably too low to be physiologically relevant (Araneda et al., 1999).

The presence of a spermidine moiety in neuroleptics and hallucinogens has led to the proposal that endogenous polyamines may be associated with the psychotic manifestation of schizophrenia (Richardson-Andrews, 1983). Several genes involved in polyamine metabolism are altered in individuals with schizophrenia (Middleton et al., 2002). Evaluation of polyamine blood levels in patients with schizophrenia has shown a significant increase in spermidine levels and spermidine oxidase activity compared to healthy controls (Flayeh, 1988). In relation to mood disorders, there are no differences in polyamine levels in the hippocampus and frontal cortex of patients with depression (Gilad et al., 1995). However, there are increased levels of the precursor agmatine in plasma (Halaris et al., 1999). Animal models have been used to evaluate cognitive effects of polyamine administration. The direct intracerebroventricular administration of moderate to high doses of spermine produces a delayed neurotoxic effect that impairs spatial learning (Conway, 1998). However, other studies have indicated that spermine improves memory in a novel object recognition task and protects against LPS-induced memory deficits in mice through a mechanism involving GluN2B (Frühauf et al., 2015). Social recognition has also been also tested in rats, where the repeated injection of spermidine decreased the time spent exploring familiar rats (Mikolajczak et al., 2002). Therefore, further research is needed to better understand the effects of polyamines on learning and memory.

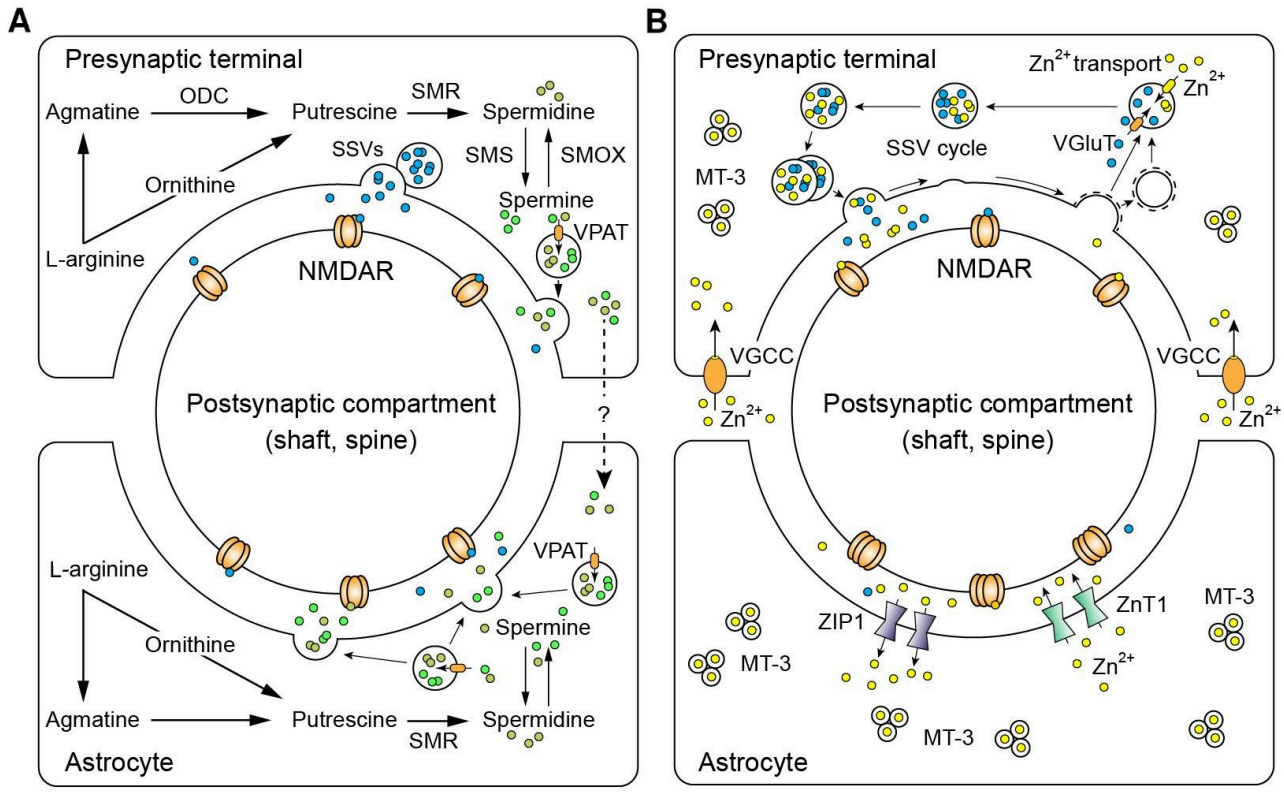

Figure 4 Schematic representation of the neurochemical origin and release of polyamines and zinc at glutamatergic synapses. A Polyamines are derived from L-arginine and are loaded and stored in secretory vesicles in neurons and astrocytes (Moriyama et al., 2020). **B** The entry of Zn^{+2} into neurons occurs mainly through activated voltage-gated calcium channels (VGCCs), as well as Ca^{+2} and Zn^{+2} permeable GluR2-lacking AMPA receptors (Sensi et al., 2009). Specific ZnT3 transporter loads Zn^{+2} into small synaptic vesicles. The release of Zn^{+2} into the synaptic cleft leads to the inhibition of the postsynaptic NMDARs. Astrocytes can export zinc to the extracellular space through the ZnT1 mechanism and uptake it through ZIP1. Zinc can be transported and stored in zinc-binding proteins (metallothioneins, MTs) in both neurons and astrocytes (Xu et al., 2019). The figure has been reproduced with permission from (Jorratt et al., 2021). The abbreviations used in the figure are ODC for ornithine decarboxylase, SRM for spermidine synthase, SMS for spermine synthase, SMOX for spermine oxidase and VPAT for vesicular polyamine transporter.

1.3.4 Zinc

Zinc is an essential trace element and the second most abundant transition metal in eukaryotic organisms. It is a potent regulator of fundamental neurobiological processes, ranging from genetic mechanisms to the activity of ion channels and receptors. Zinc's versatile functionality also makes it of major interest in an array of CNS diseases, including Alzheimer's disease, epilepsy, schizophrenia, and depression (Marger et al., 2014). Zinc is a crucial component of many proteins and plays a pivotal role in various cellular processes, including DNA replication and transcription, protein synthesis, maintenance of cell membrane integrity, cellular transport, immune responses, oxidative stress, apoptosis, and aging (among others) as one of the key cations and cofactors (Petrilli et al., 2017; Plum et al., 2010). It is widely distributed in all body tissues, particularly in muscle and bone. Around 5-15% of the total zinc in the brain exists as free or loosely-bound ions found in synaptic vesicles (Frederickson, 1989). These ions are taken up by zinc-enriched neurons

(ZEN) using the neuronal-specific zinc transporter ZnT3 from the cytosol (Wenzel et al., 1997). ZEN are present throughout the brain, but are particularly abundant in the cortex, amygdala, and hippocampus. They are likely a subgroup of glutamatergic neurons, as zinc-positive boutons also contain glutamate (Martinez-Guijarro et al., 1991). ZnT3 and vesicular glutamate transporter 1 (VGLUT1) were found in the same vesicle population (Salazar et al., 2005) (Figure 4B). Additionally, they were found in GABAergic and glycinergic neurons (Z. Wang et al., 2001).

Zinc regulates the neurotransmitter release at the presynapse, and is also co-released with transmitters, modulating synaptic functions at the postsynaptic membrane. The zinc released into the synaptic cleft acts as a neuromodulator, with potentiation and inhibition effects on many ion channels in the brain, depending on concentration and receptor subunit composition (Marger et al., 2014). In the context of NMDAR regulation, extracellular zinc inhibits the NMDA channel complex via the GluN1/GluN2B receptor with an IC_{50} of 9.5 μM. However, on GluN1/GluN2A, it has a biphasic effect; voltage-independent high affinity (IC_{50} = 5 nM) and voltage-dependent low affinity (IC_{50} = 79 μM) (N. Chen et al., 1997). This low affinity effect is likely due to the blockage of the channel pore. GluN1/GluN2C and GluNR1/GluN2D also bind to zinc, but with lower affinity (Rachline et al., 2005). Another mechanism of downregulating glutamatergic activity is through zinc's ability to decrease presynaptic glutamate release in the hippocampus by enhancing GABA release via potentiation of AMPA/kainate receptor (Takeda et al., 2004). Zinc also reduces the activity of glutamate dehydrogenase and glutamate decarboxylase, two enzymes involved in the glutamate catabolism (Ebadi et al., 1990).

As previously mentioned, changes in zinc homeostasis have been observed in various neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, Huntington's disease, depression, and schizophrenia (Prakash et al., 2015). A meta-analysis revealed that patients with schizophrenia and depression had significantly lower serum zinc concentrations (Joe et al., 2018; Swardfager et al., 2013). Zinc supplementation was found to be an effective adjuvant therapy for patients with schizophrenia and depression (Mortazavi et al., 2015; Nowak et al., 2003; Siwek et al., 2009). It has been suggested that synaptic zinc in the hippocampus enhances LTP in the CA1 region (Takeda et al., 2009) and modulates the mossy fiber LTP in the CA3 regions (Pan et al., 2011). To analyze the modulatory role of zinc in synaptic vesicles, ZnT3 KO mice have been used. While young KO mice showed normal learning, memory, and auditory pre-pulse inhibition compared with WT mice (Cole et al., 2001), adult KO mice had normal initial learning but required a longer latency to find a new platform in a standard water maze (Martel et al., 2011). The age-dependent decline in learning and memory in ZnT3 in KO mice was found to be associated with reduced protein levels of SNAP25, PSD-95, AMPAR, GluN2A and GluN2B (Adlard et al., 2010).

Furthermore, KO mice exhibited deficits in social and object recognition memory (Martel et al., 2011).

Table 2. Summary of the endogenous NMDAR modulators.

Modulator	*Effect on NMDAR*	*Binding site*	*NMDA Subunit*	*Behavioral effect*
Kynurenic acid	Inhibition	Non-competitive at glycine site	GluN1	Alterarion in pre-pulse inhibition, spatial working memory, contextual fear memory and learning, auditory sensory gating. Antidepressant-like effect
	Inhibition	Competitive at glutamate site	GluN2	
PAS	Inhibition	ATD in GluN2D, S1S2 in GluN2B	All GluN2, more potent in GluN2C and GluN2D	-
PS	Inhibition	ATD in GluN2D	GluN2C and GluN2D	Improve novel object recognition, social transmission, acquisition of spatial information and spatial/visual object discrimination. Attenuate conditioned fear stress and it has an antidepressant-like effect
	Potentiation	S1S2 in GluN2B	GluN2A and GluN2B	
Spermidine	-	-	-	Inhibited methamphetamine-induced behaviour
Spermine	Inhibition	Voltage-dependent pore blocker from extracellular and intracellular sides	All GluN2, more potent in GluN2A and GluN2B	Abolished the retention of the learnt platform position in Morris water maze, but improve memory in a novel object recognition task. Inhibited methamphetamine-induced behaviour
	Potentiation	Glycine-dependent potentiation: Increase affinity for glycine	GluN2A and GluN2B	
	Potentiation	Glycine-independent potentiation: reduction of tonic proton inhibition	ATD at the lower lobe of GluN1/GluN2B subunit interface	
Zinc	Inhibition	Voltage-dependent pore blocker (low affinity)	NR1/NR2A	Age-dependent deficit in learning and memory and deficient in social and object recognition memory in ZnT3-KO mice
	Inhibition	High affinity, voltage-independent	ATD domains of GluN2A (low sensitivity to GluN2B)	

PAS: Pregnanolone sulfate. PS: Pregnenolone Sulftate. The table is reproduced with permission from (Jorratt et al., 2021).

1.4 NMDAR dysregulation in psychiatric disorders.

Dysregulation of NMDAR has been linked to psychiatric disorders. Although NMDAR hypofunction is the primary hypothesis for schizophrenia, NMDAR hyperfunction has been associated with excitotoxicity and neurodegeneration, indicating an inverted-U-shaped dose-response curve (Lipton & Nakanishi, 1999). NMDAR modulators may have a potential therapeutic effects on psychiatric disorders. The use of NMDAR co-agonists, such as D-serine, D-alanine, and glycine, as adjunctive therapies has been shown to improve negative symptoms of schizophrenia (Heresco-Levy et al., 2004; Kantrowitz et al., 2010; Tsai et al., 2006). Additionally, memantine, an NMDAR antagonist, has been found to be beneficial in certain cases of Alzheimer's disease (Reisberg et al., 2003). In preclinical experiments, NMDAR antagonists, such as ketamine, phencyclidine and MK-801, induce a full range of behaviours that mimic schizophrenic-like symptoms, with robust changes in cognitive and negative symptom–related behaviours (G. Lee & Zhou, 2019).

However, ketamine has been found to act as a fast-acting antidepressant with a dosage (0.5 mg/kg) comparable to the one used to evoke psychotic symptoms in healthy volunteers (Krystal et al., 1994). It has been proposed that ketamine enhances modulatory function in mental status, alleviating depressive symptoms in patients but inducing psychotic symptoms in healthy controls (Adell, 2020). Figure 5 summarises the proposed mechanisms of the disinhibition hypothesis. Ketamine preferentially acts on GABAergic interneurons by blocking NMDARs, which leads to an increase in the firing of pyramidal neurons. This, in turn, induces an activation of the mammalian target of rapamycin complex (mTORC), which increases the expression of synaptic proteins such as Arc, PSD-95, GluR1, and synapsin I, as well as dendritic spine density (N. Li et al., 2010).

Neuronal subcompartments, such as dendritic branching, spines, and synaptic density, can change in response to activity. These elements are dysregulated in many psychiatric disorders (Forrest et al., 2018). Therefore, it is important to evaluate the structural and functional effects of endogenous NMDAR modulators on neurons, as NMDAR is involved in these procces (H. Wang & Peng, 2016). This evaluation could have potential uses in treating psychiatric disorders.

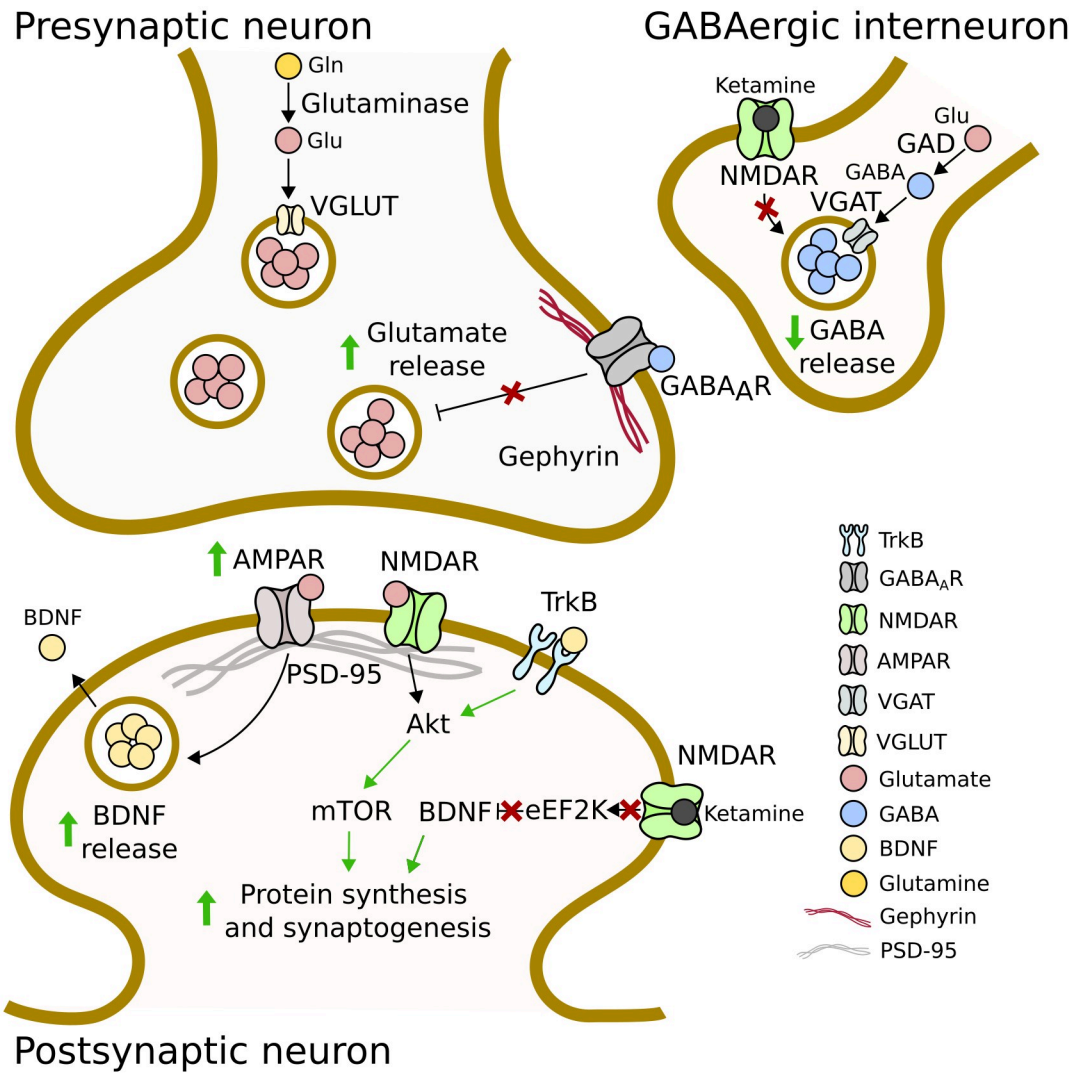

Figure 5. Proposed mechanisms of the disinhibition hypothesis. Ketamine selectively blocks NMDARs expressed on GABAergic inhibitory interneurons. This results in a decrease in inhibitory interneuron activity, leading to disinhibition of glutamatergic neurons and enhancing glutamatergic firing. The released glutamate binds to and activates post-synaptic AMPAR, which leads to the release of BDNF. The activation of TrkB/Akt/mTOR signalling enhances the production of proteins that promote the maturation and formation of synapses. The abbreviations used in the figure are GAD for glutamate decarboxylase, Gln for glutamine and Glu for glutamate.

2. 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.

3. Materials and methods

3.1 Primary cortical cultures

The experimental procedures have been approved by the NIMH Committee for Animal Research Ethics in accordance with the Animal Protection Code of the Czech Republic and the directive of the European Community Council (2010/63/EU). Prior to dissection, 24 well-plates with a 13 mm coverslip on each well, 6 well-plates, and 96 well-plates were coated with poly-L-lysine (25 μg/mL) overnight at 37° C in a 5% CO_2 humidified incubator. The samples were washed three times with distilled water for 5 minutes each and then left to dry. Primary cortical cultures were prepared from embryonic day 18 Wistar rats extracted from timed pregnant dams sacrificed by cervical dislocation. The abdomen was sprayed with 75% ethanol, and an incision was made into the womb. The foetuses were then removed and placed into a 100 mm Petri dish containing ice-cold phosphate-buffered saline (PBS). After removing them from their embryonic sac, the head was excised using scissors and placed into another 100 mm Petri dish containing ice-cold PBS. Once the skin, skull, and meninges were removed, the brains were isolated using elbow tweezers under a dissecting microscope. The cortices were then incubated in a 15 mL conical tube with ice-cold Hank's balanced salt solution (HBSS). These steps were performed in a laminar flow cell culture hood. After three washes in cold HBSS, the tissue was transferred to a 1.5 mL microcentrifuge tube and mechanically dissociated using a 0.9 mm needle followed by a 0.45 mm needle, three times each at a pace of one drop per second. The dissociated cells were filtered through a 100 μm pore cell strainer and then re-suspended in seeding medium, which consisted of DMEM supplemented with 10% FBS and 1% Penicillin-Streptomycin. The cells were counted using a hemocytometer, and trypan blue was added to distinguish dead cells. Dilutions were prepared as per the experimental requirements. Cells were plated at different densities depending on the intended analysis: (i) 20,000 cells/cm^2 on 13 mm coverslips in 24-well plates for immunostaining, (ii) 125,000 cells/cm^2 on coated 6-well plates for western blot and ELISA, and (iii) 125,000 cells/cm^2 on coated 96-well plates for MTS cell viability test. The primary cortical cultures were incubated at 37°C and 5% CO2. Over the course of the following day, the seeding medium was substituted with a growth medium consisting of Neurobasal medium, 2% B27 serum-free supplement, 2 mM L-Glutamine, and 1% Penicillin-Streptomycin. Every 4-5 days, half of the growth medium was replaced with fresh medium. Table 3 provides a summary of the products, including their catalog numbers. As no inhibitors of cell proliferation were added to the growth medium, the cortical cultures consisted of a mixture of neurons and glial cells. This was confirmed by immunofluorescence staining for neuronal and glial markers (Figure 6).

Table 3. Reagents and consumables used for the preparation and maintenance of neuronal cultures.

Reagent/Consumable	*Catalog number*
Hank's balanced salt solution (HBSS)	Thermo Fisher Scientific, 14175095
100-µm pore nylon filter	VWR, 732–2759
Coverslips	Marienfeld-Superior, 0117530
Neurobasal medium	Thermo Fisher Scientific, 21103049
Penicillin–Streptomycin	Thermo Fisher Scientific, 15070063
L-Glutamine	Thermo Fisher Scientific, 25030149
B27 serum-free supplement	Thermo Fisher Scientific, A3582801
Dulbecco's Modified Eagle Medium (DMEM)	Biowest, L0104-500
FBS	Biowest, S1810-500
0.9-mm needle	B. Braun, 4657519
0.45-mm needle	B. Braun, 4657683

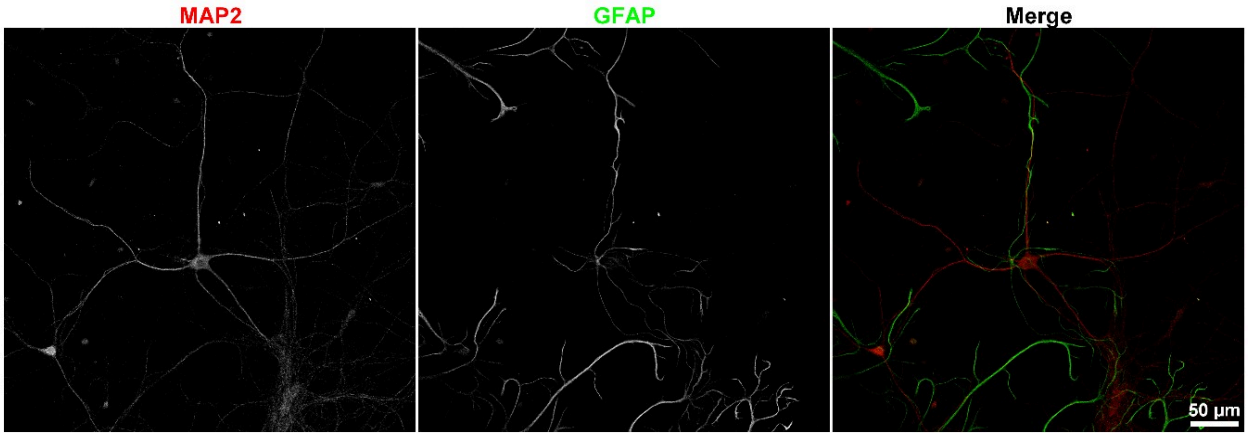

Figure 6. Representative confocal images of glia cells and neurons. Micrographs of GFAP and MAP2 double-stained cortical culture at DIV19.

3.2 Treatments of cultures with endogenous NMDAR modulators

Cultures were treated at days *in vitro* (DIV) 14 to evaluate mature neurons. The cultures were exposed to kynurenic acid, pregnenolone sulfate, spermidine or zinc chloride for 1 hour, 6 hours, 1 day and 5 days. The final concentrations of these compounds, which were used to mimic their physiological levels reported in the cerebrospinal fluid, are listed in Table 4. All compounds were diluted in bi-distilled water and added to the culture at a ratio of 1:100. Following the treatment period, the cultures underwent immunocytochemistry, cell viability assay, glutamate release, ELISA or western blot test.

Table 4. List of the endogenous NMDAR modulators used to treat neuronal cultures.

NMDAR modulator	*Concentration*	*Catalog number*
Kynurenic acid	150 nM	Sigma-Aldrich, K3375
Pregnenolone sulfate	50 µM	Sigma-Aldrich, P162
Spermidine	50 µM	Sigma-Aldrich, S0266
Zinc Chloride	10 µM	Sigma-Aldrich, 208086

3.3 Cell viability assay

Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) colorimetric assay. This assay is based on the reduction of the MTS tetrazolium compound to generate formazan product in living cells. The cultures were kept with 100 µL of growth medium and treated with endogenous NMDAR modulators for 12 hours, 1 day, or 5 days. Subsequently, 10 µL of MTS was added to each well and incubated at 37°C and 5% CO_2 for 2 hours (at DIV19). Triton was used as a positive control. Following a 20-second shake, the optical density (OD) was measured at 490 nm using a plate reader (Tecan Infinite M200 Pro). The data were expressed as a percentage of cell viability using the formula:

$$(OD_{sample}/OD_{control}) \times 100.$$

3.4 Glutamate release measurements

A modified version of the method described in (Kristofikova et al., 2008), involving amino acid derivatization by dansyl chloride and fluorescence detection, was employed. Following treatment with endogenous NMDAR modulators for 1 and 5 days, the growth medium was aspirated and cells were stimulated for 10 minutes with pre-warmed (38ºC) Krebs-Ringer (KR) solution containing high potassium concentration (75 mM NaCl, 50 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mM NaH_2PO_4, 10 mM glucose, 20 mM HEPES pH 7.4) and 0.3 mM DL-TBOA (blocker of excitatory amino acid transporters) to prevent glutamate clearance. Glutamate levels were measured in the supernatant, while protein measurements were taken from the cells dissolved in 0.5 mL of 0.2 N NaOH (in triplicate) using BSA as a standard, as described elsewhere (Peterson, 1977), but modified for microplates. To quantify the glutamate concentration, 0.1 mL of supernatant was mixed with 0.1 mL of 200 mM $NaHCO_3$/10 mM EDTA and 0.4 mL of dansyl chloride (1.25 mg/mL dissolved in acetone) and incubated at 60°C for 1 hour in the dark. The samples were lyophilized using the Hanil module 4080C centrifuge vacuum concentrator and then reconstituted in 0.2 mL of H_2O. After centrifugation at 15,000 g for 10 minutes at 4°C, 20 µL of the samples were

injected into the HPLC system (Thermo Scientific Dionex Ultimate 3,000 with a quaternary pump, fluorescence detector, and autosampler). The samples were resolved on a C18 column (Separon SGX C18, 3 × 150 mm), and the fluorescence detection (330 nm excitation and 550 nm emission) was used to monitor the derivatized glutamate. The separation conditions were optimized for measuring glutamate. Isocratic chromatography was performed for 15 minutes at a flow rate of 0.4 mL/min using the "separation" mobile phase (50 mM KPO_4 pH 7.2/8% acetonitrile/14% methanol), followed by the "chase" mobile phase (50 mM KPO_4 pH 7.2/70% methanol) for 5 minutes. The column was then re-equilibrated with the "separation" mobile phase for 10 minutes. Glutamate levels were calculated using a calibration method (1.25, 2.5, 5, and 10 μM) and presented after normalization for the protein lysate content, using regression analysis. One of the standards was injected after every sixth sample.

3.5 Immunocytochemistry

Following treatment, the growth medium was removed and replaced with paraformaldehyde (4% in PBS and 2% sucrose) for 15 minutes at room temperature. The cultures were then washed three times with PBS and incubated in a blocking solution (10% FBS in 0.1% Triton X-100 in PBS) for 1 hour at room temperature. The use of 0.1% Triton-X facilitates cell permeabilization, allowing the antibody to penetrate the cell membrane for the detection of intracellular proteins, while FBS blocks nonspecific binding of antibodies. Cells on coverslips were incubated overnight with rabbit anti-glial fibrillary acidic protein (GFAP), chicken anti-microtubule-associated protein 2 (MAP2), mouse anti-postsynaptic density protein 95 (PSD-95), guinea pig anti-synapsin I/II or mouse anti-parvalbumin (concentrations are summarised in Table 5) in 0.1% Triton-X 100 in PBS at 4°C. Dendritic spines were stained using Alexa Fluor 405 Phalloidin, which labels F-actin enriched in spines (Thermo Fisher, catalogue #A30104). The coverslips were washed three times with PBS the following day and then incubated with the corresponding secondary antibody for 1 hour. The antibody was diluted in 0.1% Triton-X 100 in PBS at room temperature. After washing the coverslips with PBS, the cells were mounted onto microscope slides using Fluoroshield mounting media (Sigma-Aldrich, F6182). Image acquisition was performed using a Leica DMi8 (Leica Microsystems) laser scanning microscope controlled by LAS X software (Leica Microsystems). For dendritic tree morphology studies, we used a 40x oil objective with a numerical aperture (NA) of 1.3. For synapse density counting, we used a 63x oil objective with an NA of 1.4, Z-stacks at 0.3 μm step size, and 2.6x digital zooms. We analyzed the images using ImageJ with Synapse Counter (Dzyubenko et al., 2016) and SNT v3.2.11 plugin (Arshadi et al., 2021). An experimenter blinded to treatment conditions performed the image analysis.

3.6 ELISA and western blot

For protein analysis, the primary cortical cultures were washed with ice-cold PBS and then scraped in 300 µL RIPA buffer (Sigma-Aldrich, 20-188) with Protease and Phosphatase Inhibitor Cocktail (Sigma-Aldrich, PPC1010-1ML). The samples were then transferred to 1.5 mL microcentrifuge tubes and incubated on ice for 15 minutes. After that, the samples were sonicated three times for 3 seconds each, centrifuged at 13,000 g for 5 minutes at 4°C to pellet cell debris, and the supernatant was transferred to a new 1.5 mL microcentrifuge tube. The protein concentration was determined using the BCA protein assay. The samples were stored at -80°C. To load the samples, 20-40 µg of proteins were mixed with 4x Laemmli sample buffer and heated at 90º C for 10 minutes on a heating block. A 4-20% TGX Stain-Free gel (Bio-Rad, 4568094) was run at 200 V and then activated by exposure to UV light for 2.5 min to visualize the proteins using the ChemiDoc™ MP system. The proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Bio-Rad, 1704272) using the Trans-Blot® Turbo™ transfer system for 10 minutes. A stain-free blot image was captured to measure the total protein in each sample lane. Subsequently, the blot was blocked with 5% nonfat dry milk (in PBS) for 1 hour and incubated overnight at 4°C with primary antibodies in 1% nonfat dry milk (concentrations are summarized in Table 5). The membranes were subsequently washed five times with PBST (0.1% Tween-20 in PBS) and then incubated with the corresponding horseradish peroxidase-conjugated antibody. After one hour, the membranes were washed again and incubated with Clarity ECL substrate (Bio-Rad, 1705061) for five minutes. The optical density and intensity of the protein bands on the TGX Stain-Free gels were analyzed using Image Lab 6 software (Bio-Rad). For stripping, the membrane was washed in PBST for 5 minutes, incubated with Restore™ PLUS Stripping Buffer (Thermo Fisher Scientific, 46430) for 10 minutes, and then washed three times with PBST for 5 minutes each. The levels of GABA and BDNF were measured using an ELISA kit (LDN Labor Diagnostika Nord #BAE-2500R and Biosensis #BEK-2211, respectively) following the manufacturer's guidelines. Concentration was calculated based on the standard curve after measuring the absorbance using a plate reader (Tecan Infinite M200 Pro).

Table 5. List of antibodies used for immunocytochemistry and western blot.

Antibody	*Concentration*	*Experiment*	*Catalog number*
Anti-ERK1/2	1:500	WB	Thermo Fisher Scientific, 13-6200
Anti-Gephyrin	1:1000	WB	Synaptic systems, 147111
Anti-GFAP	1:500	IHC	Dako, Z033429
Anti-MAP2	1:10000	IHC	Abcam, ab5392
Anti-GluN1	1:1000	WB	Thermo Fisher Scientific, 32-0500
Anti-phospho-ERK1/2	1:500	WB	Thermo Fisher Scientific, 44-680G
Anti-phospho-TrkB	1:500	WB	Thermo Fisher Scientific, PA5-38077
Anti-PSD-95	1:500	IHC - WB	Abcam, ab192757
Anti-synapsin I/II	1:1000	IHC	Synaptic Systems, 106004
Anti-synaptophysin	1:1000	WB	Abcam, ab32594
Anti-TrkB	1:500	WB	Thermo Fisher Scientific, MA5-14903
Anti-VGAT	1:1000	WB	Synaptic systems, 131004

IHC = Immunocytochemistry, WB = Western blot.

3.7 Behavioral tests

3.7.1 Animals

The experimental data was collected from 15 wild-type C57Bl6N mice acquired from Janvier (Le Genest-Saint-Isle, France) and housed at the animal facility of the University of Freiburg. Adult mice of both sexes, aged 10-14 weeks, were used in behavioral experiments. The mice were kept under standardized conditions with a 12 hour light/dark cycle and provided with free access to water and food. The animal review boards in Germany (Regierungspräsidium Freiburg) previously approved all procedures, and the animal care use protocols adhered to national and international standards. A trained observer, who was blinded to the treatment, recorded all behavioral experiments (Figure 7) on video and analyzed them offline.

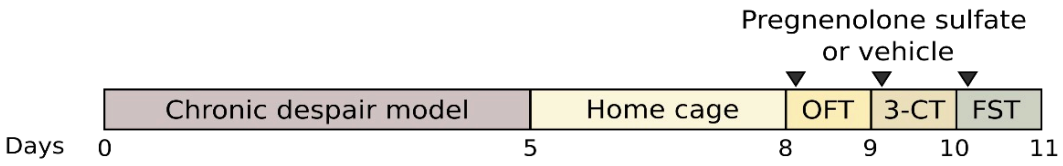

Figure 7. Timeline of the behavioral experiments. OFT = Open field test; 3-CT = Three chamber test; FST = Forced swim test. Arrowheads indicate the drug administrations.

3.7.2 Chronic despair model (CDM)

A previously well-described protocol (Vestring et al., 2021) was used to induce depressive-like symptoms in mice. Female and male mice were exposed to 5 consecutive days of forced swim test during the induction phase. This involved placing the mice in beakers filled with water at 25°C for 10 minutes. After swimming, the animals were gently dried and returned to their home cages. The water was changed before each trial. Immobility times were defined as the cumulative time during which the animal was passively floating.

3.7.3 Drug administration

PS (Sigma-Aldrich, P162) was injected intraperitoneally at a dose of 40 mg/kg, diluted in 0.1% Tween 80, 60 minutes before the open field test (OFT), three chamber test (3-CT), and forced swim test (FST) tests on days 8-10 (Figure 7). The volume of injection was adjusted according to the weight of the animal, and the control was administered using the same vehicle.

3.7.4 Open field test

The experiment was conducted in a rectangular arena measuring 40 × 60 cm, enclosed by a 40 cm high wall made of grey PVC with a luminous intensity of less than 10 lux. The mice were placed in the center of the arena and allowed to move freely for 10 minutes. The variables analyzed using EthoVision XT software (Noldus, Netherlands) were total distance travelled, time spent in the center, and center entries.

3.7.5 Three chamber test

The arena comprised three adjacent chambers, each measuring 19 x 12 cm, with 30 cm high walls connecting them via open doorways. The test consisted of three 10-minute sessions: habituation, sociability, and social novelty. During the habituation session, the mice were allowed to explore the arena and the three chambers. In the sociability session, a previously unknown mouse (stranger 1) was introduced under a cup in one of the side chambers, while an identical empty cup was placed in the opposite chamber. During the social novelty session, a previously unencountered mouse (referred to as 'stranger 2') was introduced and placed under an empty cup. The time spent interacting with each cup was analysed using EthoVision XT software (Noldus, Netherlands), and the preference index was defined as follows:

$$\text{Sociability index} = \frac{\text{Stranger 1} - \text{Empty}}{\text{Stranger 1} + \text{Empty}}$$

$$\text{Social novelty index} = \frac{\text{Stranger 2} - \text{Stranger 1}}{\text{Stranger 2} + \text{Stranger 1}}$$

3.7.6 Forced swim test

The procedure was performed as described in the CDM protocol.

3.8 Data analysis and statistics

The data are presented as mean ± standard error of the mean (S.E.M.). The distribution of data was assessed using the Shapiro-Wilk test. To compare the treatment factor, one-way ANOVA was used, followed by Dunnett's post hoc test. When comparing treatments on different days of treatment (treatment x days), we used two-way ANOVA, followed by Tukey's post hoc test. For the experiment performed only with PS and vehicle, we used an unpaired t-test. Statistical tests with a *p-value* < 0.05 were considered significant. We conducted all statistical tests using R software (version 4.0.5) and RStudio (version 1.4.1717).

4. Results

To investigate the effect of endogenous NMDAR modulators on morphology and synaptic density, primary cortical cultures were exposed to kynurenic acid (KYNA), pregnenolone sulfate (PS), spermidine (SPD), and zinc (ZINC). The results were then compared to untreated cortical cultures. Prior to treatment, the cortical cultures were observed on an inverted bright-field microscope with phase contrast. Figure 8 displays representative pictures at different developmental stages.

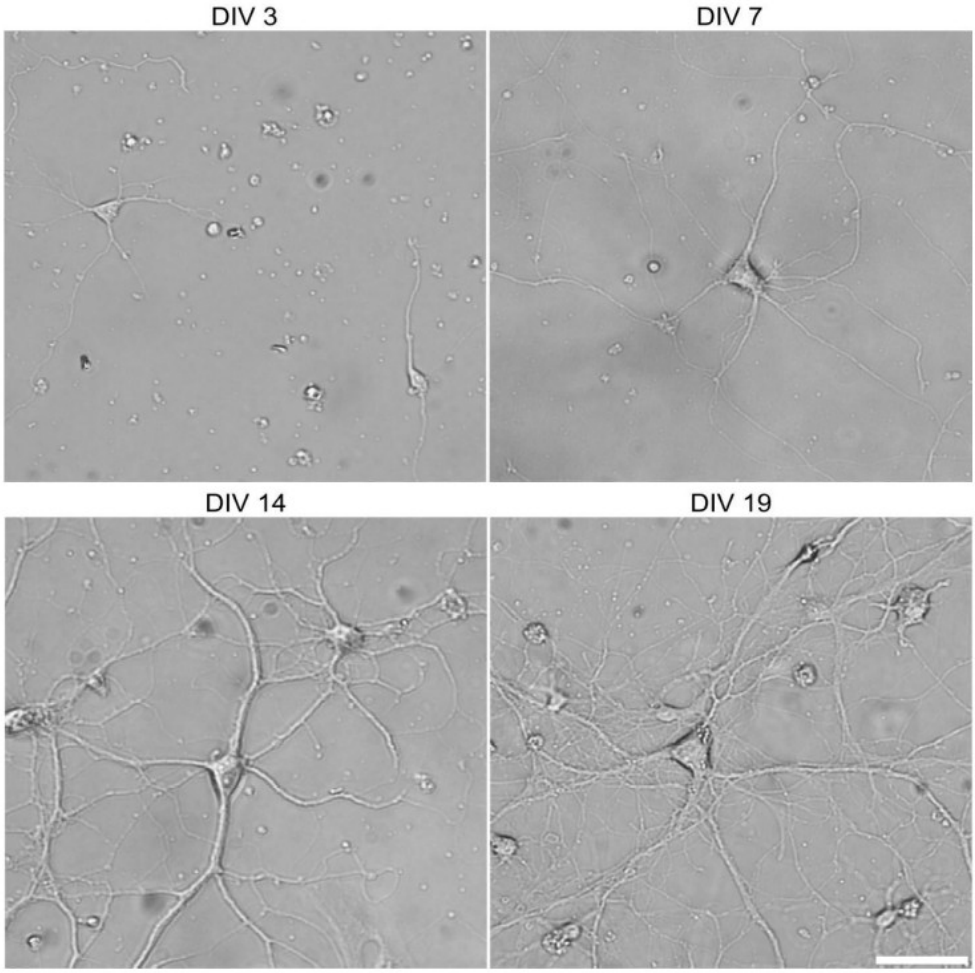

Figure 8. Primary cortical culture. Representative images obtained from cells plated at a density of 20,000 cells/cm^2 during DIV 3, 7, 14, and 19, using an inverted bright-field microscope with phase contrast. The scale bar is 50 μm.

4.1 Effect of endogenous NMDAR modulators on excitatory neurons

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

As NMDAR regulates neuronal viability, we conducted an MTS viability test to determine whether exposure to physiological concentrations of KYNA, PS, SPD, and ZINC causes any toxicity or alters neuronal survival in primary cortical culture. The results from two biological replicates indicate that treatment for 12 hours, 1 day (1DT), and 5 days (5DT) did not decrease cell

viability (Figure 9) (one-way ANOVA; 12 h $F_{(1,4)} = 0.809$, $p = 0.525$; 1 day $F_{(1,4)} = 1.493$, $p = 0.216$; 5 days $F_{(1,4)} = 1.894$, $p = 0.123$).

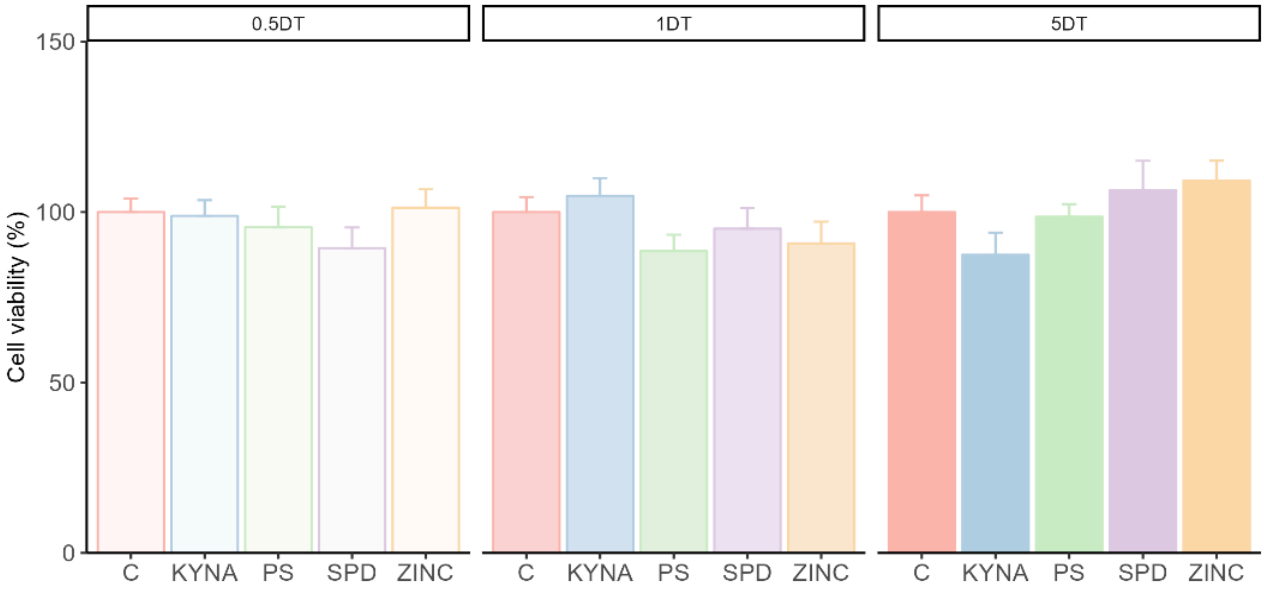

Figure 9. Cell viability of cortical culture after exposure to endogenous NMDAR modulators. The results of the MTS assay after 12 h (0.5 DT) and 1 and 5 DT are presented. The control level is considered as 100%, and the data is expressed as means ± S.E.M. The statistical analysis was performed using one-way ANOVA. The experiment was conducted with 6-7 technical replicates from two biological replicas.

Subsequently, we investigated whether exposure to endogenous NMDAR modulators alters glutamate release. Basal glutamate levels were undetectable, so we measured the evoked release of glutamate in the presence of DL-TBOA, an inhibitor of glutamate uptake. To assess evoked glutamate release, we incubated untreated cortical neurons with high K^+ Krebs-Ringer (KR) solution and the endogenous NMDAR modulators for 10 minutes. The percentage of evoked glutamate release per protein did not differ in this treatment (one-way ANOVA; $F_{(1,4)} = 0.778$, $p = 0.549$) (see Figure 10, "Acute"). We then evaluated evoked glutamate release using high K^+ KR solution after 1DT and 5DT. The percentage of evoked glutamate release per protein did not differ for either treatment (one-way ANOVA; 1DT $F_{(1,4)} = 0.458$, $p = 0.766$; 5DT $F_{(1,4)} = 1.293$, $p = 0.292$).

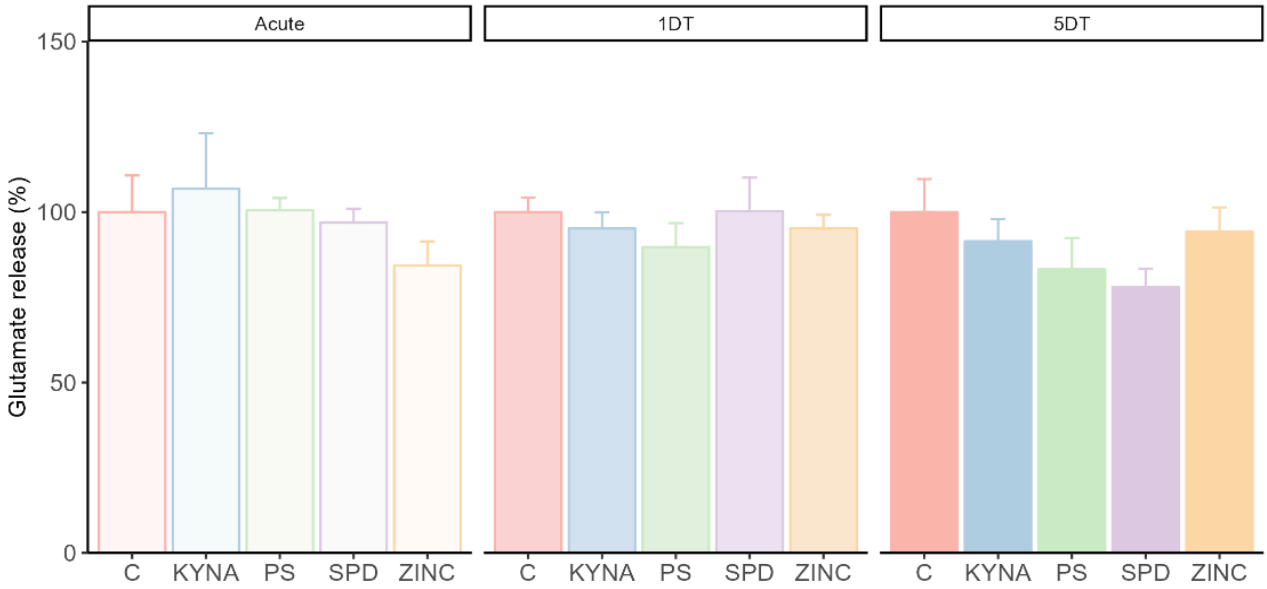

Figure 10. Evoked glutamate release of cortical neurons treated with endogenous NMDAR modulators. Glutamate release was evoked using 10 minutes of incubation with 50 mM K^+ in Krebs-Ringer solution, both with endogenous NMDAR modulators (acute treatment) and after 1 and 5 DT. The control level is taken as 100%, and the data is presented as means ± S.E.M. The statistical analysis was performed using one-way ANOVA. The experiment was conducted with 3-4 technical replicates from two biological replicas.

4.1.2 Pregnenolone sulfate promotes dendritic field expansion

We investigated whether prolonged exposure to endogenous NMDAR modulators can induce measurable structural changes, as NMDAR is central to shaping synaptic function and plasticity. Cortical cultures were treated at DIV 14 for 5 days and stained to measure morphological changes (Figure 11).

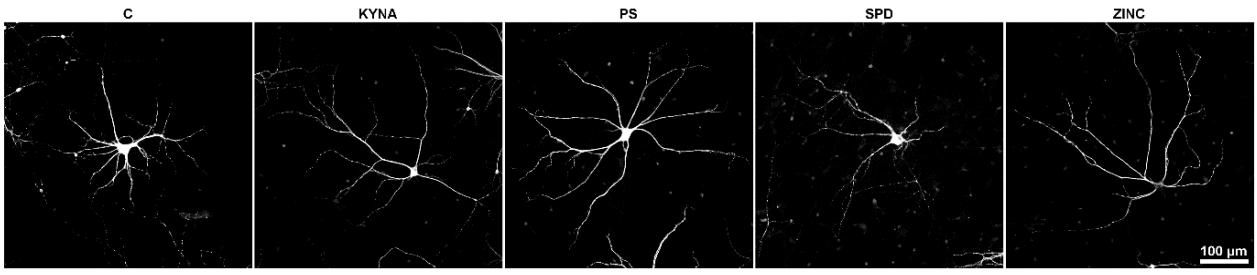

Figure 11. Representative confocal images of cortical neurons after exposure to endogenous NMDAR modulators. Cortical cultures were treated at DIV 14 for 5 days for branching reconstruction.

Under all treatments, the total dendritic length (Figure 12B), the total number of dendritic branches (Figure 12C), the number of primary branches (Figure 12D), the number of branch points (Figure 12E), and neuronal soma size (Figure 12F) remained unchanged (one-way ANOVA; total dendritic length $F_{(1,4)} = 1.13$, $p = 0.348$; number of branches $F_{(1,4)} = 0.57$, $p = 0.682$; the number of primary branches $F_{(1,4)} = 1.28$, $p = 0.281$; the number of branch points $F_{(1,4)} = 0.39$, $p = 0.816$; soma

area $F_{(1,4)} = 3.06$, $p = 0.019$) (no difference between groups and control in Dunnett's post hoc test). We then performed a Sholl analysis to measure arbor complexity based on the number of branching intersections of concentric circles along the distance from the soma. The differences in the means of these tests reached statistical significance in cortical cultures exposed to PS and SPD. While both PS and SPD significantly decreased the number of intersections at 20 µm (one-way ANOVA; $F_{(1,4)} = 2.51$, $p = 0.045$), only PS significantly increased the number of intersections at 130, 150, 170 and 180 µm (one-way ANOVA; 130 µm $F_{(1,4)} = 2.55$, $p = 0.042$; 150 µm $F_{(1,4)} = 2.74$, $p = 0.031$; 170 µm $F_{(1,4)} = 2.80$, $p = 0.029$, 180 µm $F_{(1,4)} = 2.60$, $p = 0.040$). These experiments indicate that, at physiological concentrations, PS facilitates dendritic arborisation in remote compartments and promote the expansion of dendritic projections (Figure 12H).

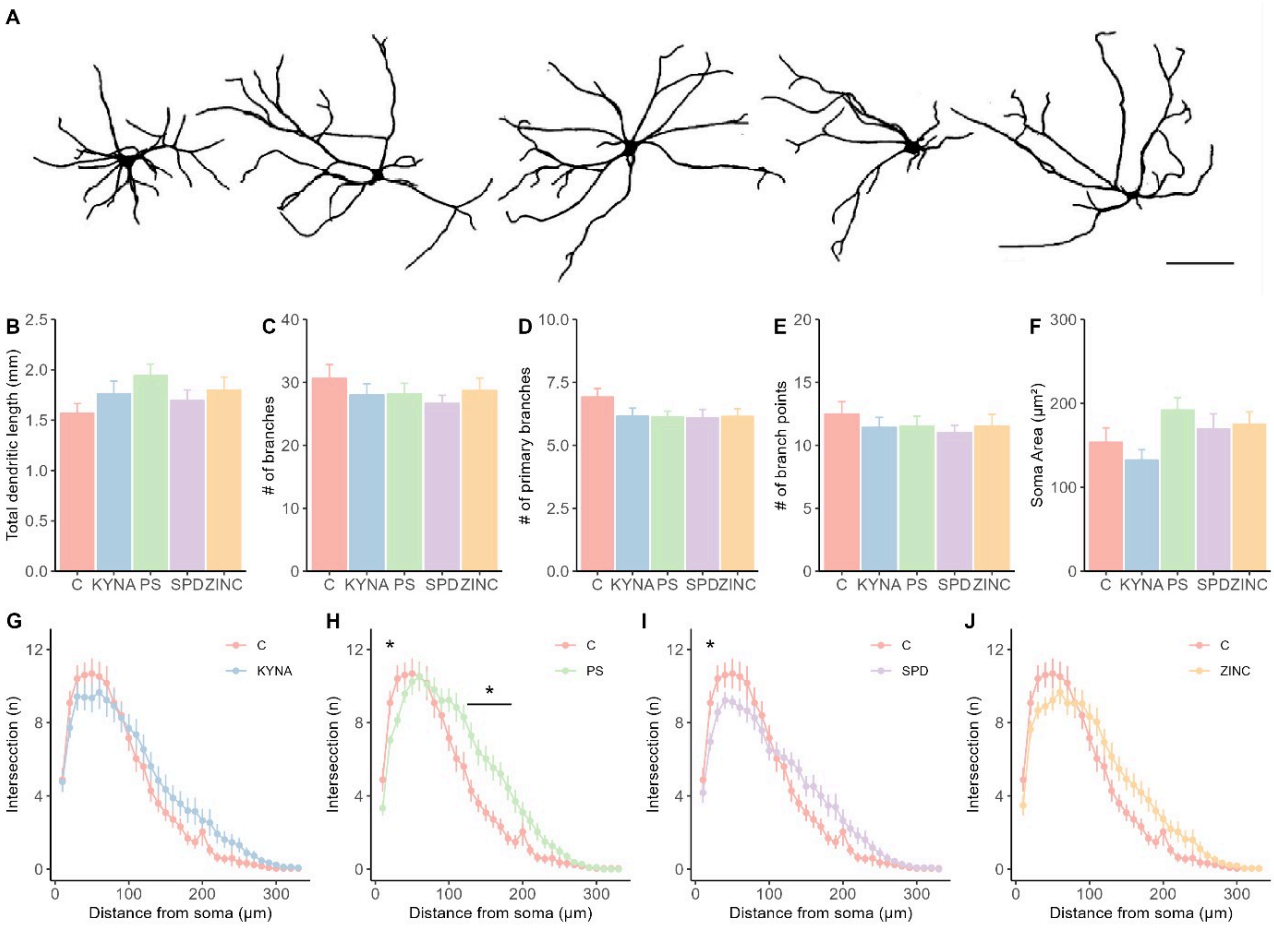

Figure 12. Analysis of dendritic branching and arbor complexity in cortical neurons treated with different NMDAR modulators for 5 days. **A** Representative examples of reconstructed neurons. **B–F** Endogenous NMDAR modulators did not produce a difference in the **B** total dendritic length, **C** number of branches, **D** number of primary branches, **E** number of branch points, and **F** soma area. (**G–J**) Sholl analysis shows the number of intersections along with the distance from the soma for different treatments. Data are presented as mean ± S.E.M. n = 23-30 randomly selected neurons per treatment from three biological replicates. One-way ANOVA followed by Dunnett's post hoc. Scale bar = 100 µm.

4.1.3 Pregnenolone sulfate increases the expression of BDNF and TrkB activation

Binding of BDNF to TrkB leads to phosphorylation-dependent activation of TrkB. Downstream signalling occurs through the extracellular regulated kinase (ERK) and Akt pathways, which are key regulators of dendritic branching and synaptic plasticity. Thus, we measured BNDF expression from lysate after treatment with the endogenous NMDAR modulator for 1 and 5 DT using ELISA. Taking both time-point treatments together, there was a significant difference (two-way ANOVA; $F_{(1,4)} = 3.136$, $p = 0.029$), where PS had a tendency of increased BNDF levels (Tukey's post hoc, $p = 0.061$, Figure 13).

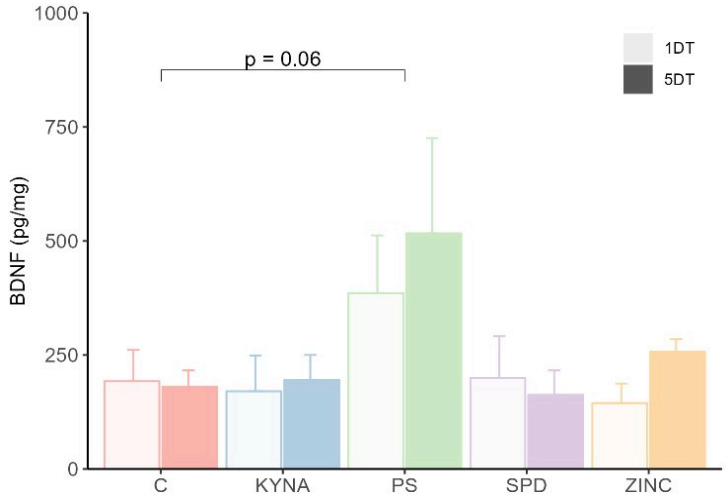

Figure 13. BDNF expression after exposure to endogenous NMDAR modulators. Protein levels of BDNF determined by ELISA (n = 4 biological replicas) in cortical cultures after 1 and 5 DT. The data is presented as means ± S.E.M. Two-way ANOVA was followed by a Tukey's post hoc test.

As there were differences in the expression of BDNF only after treatment with PS, we analysed levels of phosphorylated (active) TrkB (p-TrkB), as well as the signal mediator ERK1/2 (p-ERK1/2) after 1 and 6 hours of treatment with PS. The western blot analysis (Figure 14) showed that after 1 hour of treatment with PS, the ratio of TrkB phosphorylation (p-TrkB/TrkB) significantly increased (1.264 ± 0.156, unpaired t-test, $p = 0.04$) and there was a tendency towards an increase in the total expression of ERK1/2 (1.40 ± 0.34, unpaired t-test, $p = 0.09$) (Figure 14B). However, TrkB activation was not observed after 6 hours, but there was a decrease in ERK1/2 phosphorylation (0.35 ± 0.31, unpaired t-test, $p = 0.03$) (Figure 14D).

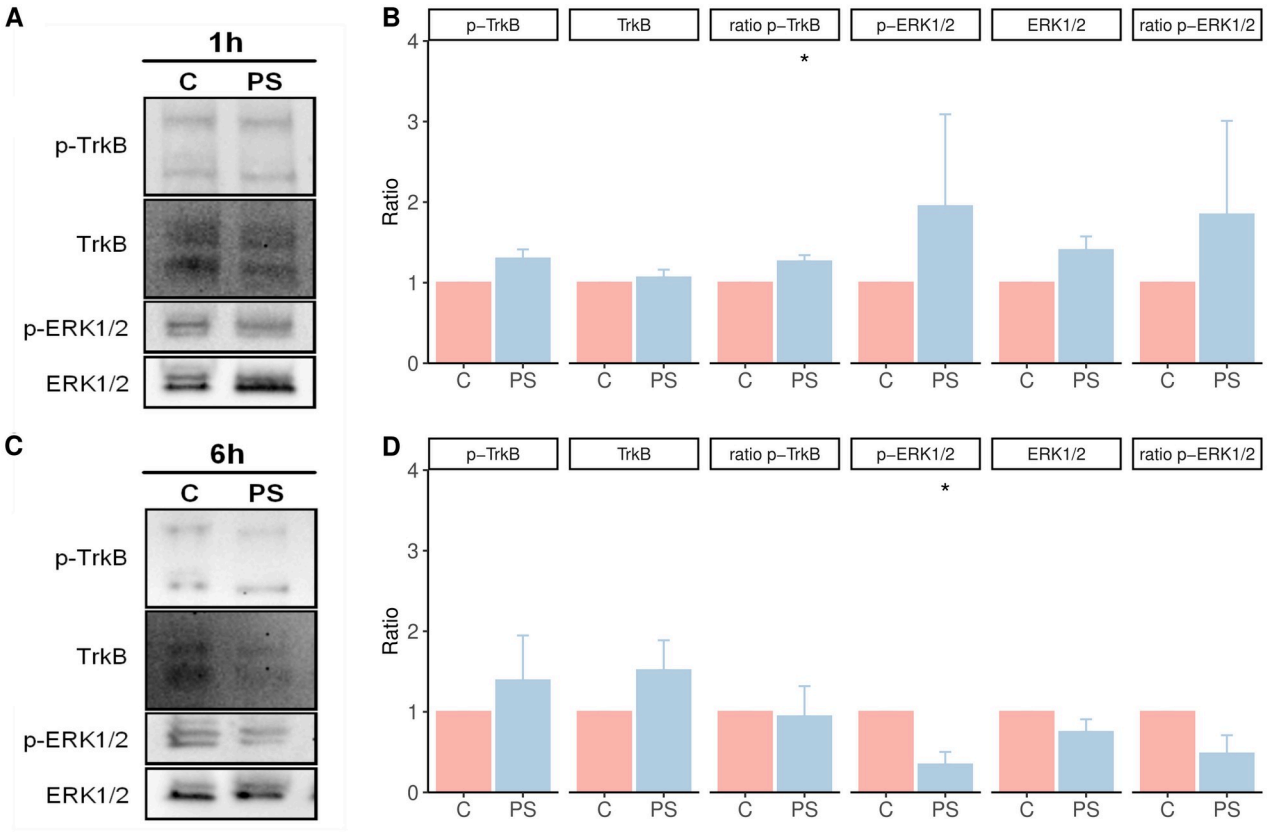

Figure 14. Analysis of western blot from cortical cultures treated with endogenous NMDAR modulators for 1 and 6 hours. A,C Representative immunoblots of the p-TrkB, total TrkB, ratio of p-TrkB, p-ERK1/2, total ERK 1/2 and ratio of p-ERK1/2 for the different 1- and 6-hours treatments. **B,D** Quantification of the corresponding proteins normalized to the total load protein of the stain-free gel (TGX Stain-Free BioRad). The data is presented as means ± S.E.M. Unpaired t-test. n = 4 biological replicas.

4.1.4 Endogenous NMDAR modulators do not alter synaptic density of cortical neuron

To assess changes in dendritic spine morphology and density of excitatory synaptic inputs, cortical cultures were treated at DIV 14 for 1 and 5 days. Attempts to count and identify dendritic spines using phalloidin staining were unsuccessful due to ambiguities over morphological differences of spines captured with our settings and rapid bleaching of the fluorescence signal (Figure 15).

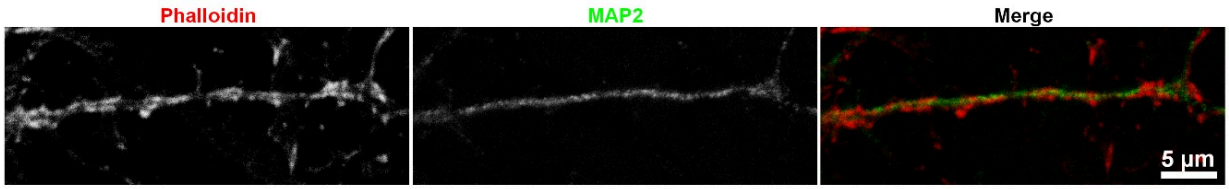

Figure 15. Fluorescent staining of dendritic spines. A dendritic branch (MAP2), bearing multiple phalloidin-positive dendritic spines.

Figure 16A displays images of samples stained with MAP2, synapsin I/II (presynaptic marker), and PSD-95 (postsynaptic marker) antibodies. We examined the effects of the endogenous NMDAR modulators on synaptic inputs in proximal (< 100 mm) versus distal (> 100 mm) dendritic segments to investigate the potential differential sensitivity. When analysing proximal dendrites (Figure 15B-D), a two-way ANOVA (treatment x days) revealed a significant difference in synapse density, which is defined as the colocalization of pre- and postsynaptic markers (two-way ANOVA; $F_{(1,4)} = 2.498$, $p = 0.043$), synapsin I/II density (two-way ANOVA; $F_{(1,4)} = 2.774$, $p = 0.027$), and PSD-95 density (two-way ANOVA; $F_{(1,4)} = 2.81$, $p = 0.026$). However, Tukey's post hoc analysis showed that, compared to the control group, the only significant difference was a decrease in PSD-95 density by PS ($p = 0.04$). There was no difference in the same immunofluorescence features of distal dendrites (see Figure 17).

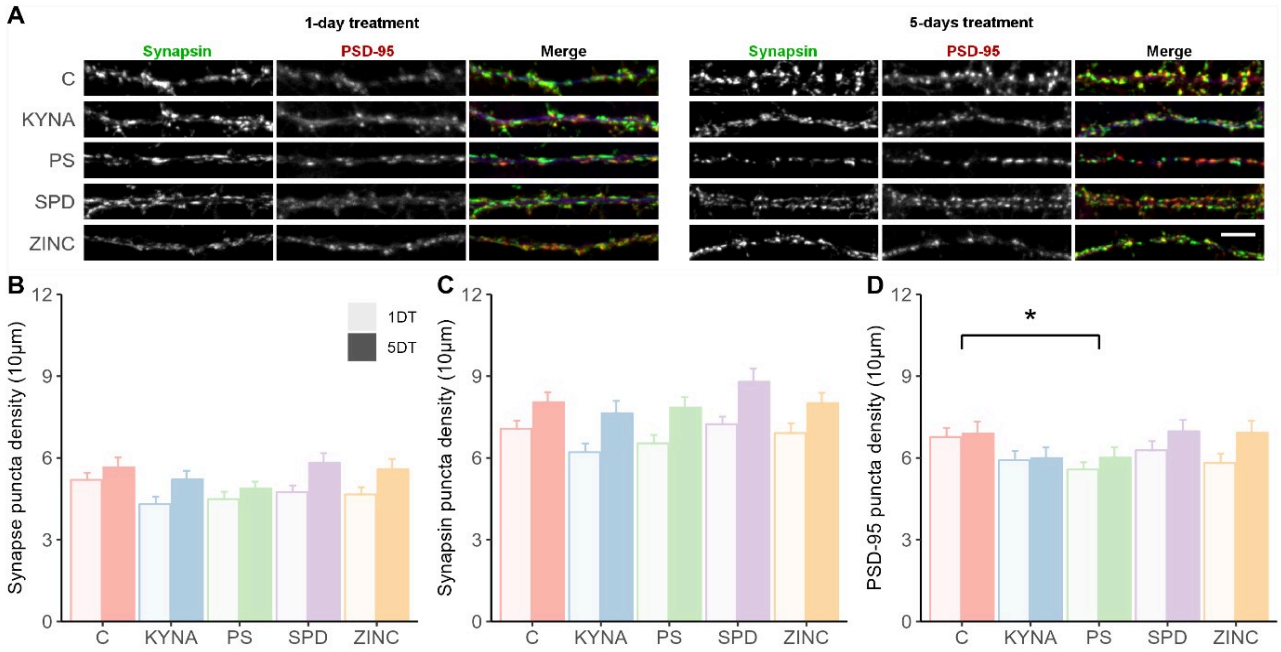

Figure 16. Analysis of synaptic density in cortical cultures treated for 1 or 5 days with endogenous NMDAR modulators. A Representative pictures of neuronal branches taken < 100 µm from the soma of 1 and 5 day treatment. **C–E** Quantification of synapse density (synapsin and PSD-95 colocalized puncta), presynaptic density (synapsin puncta) and postsynaptic density (PSD-95 puncta) from branches proximal (< 100 µm) from the soma. Data are represented as means ± S.E.M. n = 30–39 from three biological replicas. One-way ANOVA followed by Tukey's post hoc. Scale bar = 5 µm.

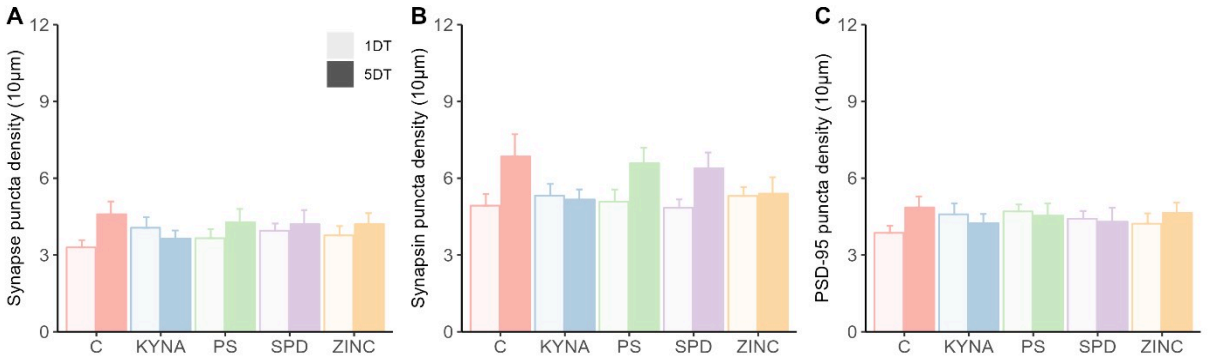

Figure 17. Analysis of synaptic density in cortical cultures treated for 1 or 5 days with endogenous NMDAR modulators. A–C Quantification of synapse density (synapsin and PSD-95 colocalized puncta), presynaptic density (synapsin puncta) and postsynaptic density (PSD-95 puncta) from branches distal (> 100 µm) from the soma. Data are represented as means ± S.E.M. n = 8–20 from three biological replicas. One-way ANOVA.

To complement the assessment of synaptic density, the protein expression of synaptic markers was measured through western blot. The total protein expression of the mandatory NMDAR subunit GluN1, PSD-95, and the presynaptic marker synaptophysin were measured after 1DT and 5DT (Figure 18A). The western blot results indicate that PSD-95 levels are consistent with previous findings; however, no significant difference was found. Additionally, no differences were found in the expression of synaptophysin and GluN1 (Figure 18B).

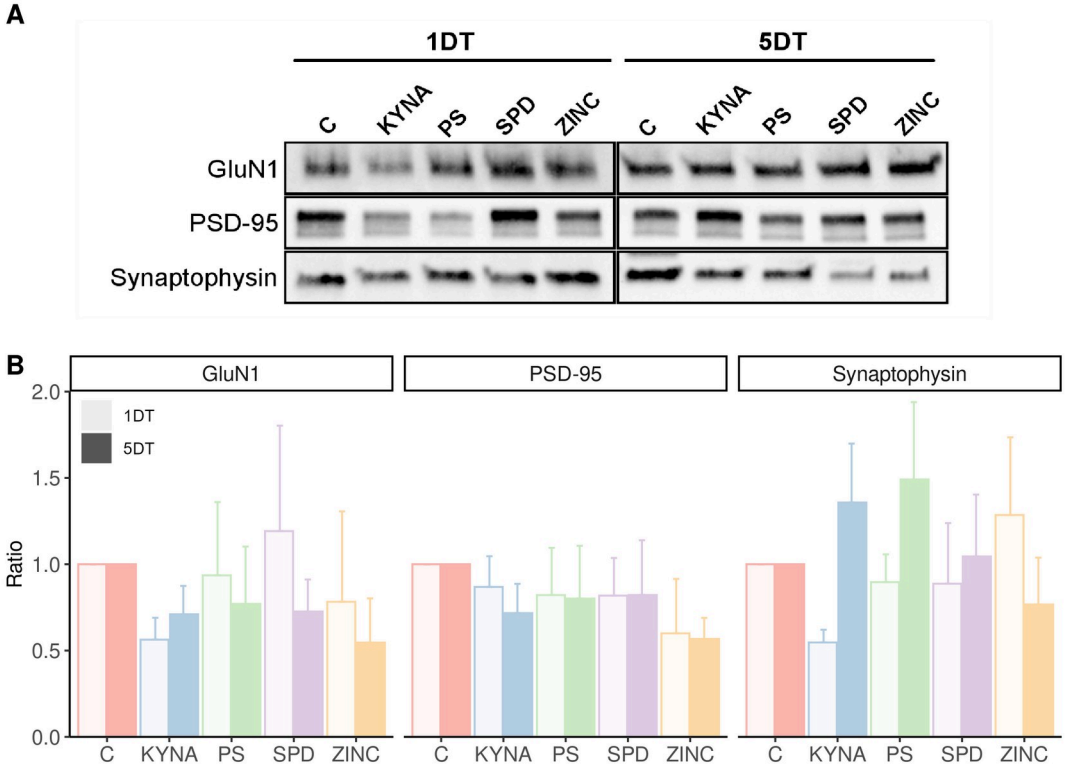

Figure 18. Analysis of western blot in cortical cultures treated with endogenous NMDAR modulators for 1 or 5 days. A Representative immunoblots of the GluN1, PSD-95 and synaptophysin for the different 1- and 5-day treatments. **B** Quantification of the corresponding proteins normalized to the total load protein of the stain-free gel (TGX Stain-Free BioRad).

Cortical cultures were stained with anti-GluN1 antibodies to measure the density of synaptic and extrasynaptic NMDAR puncta. However, these attempts were unsuccessful due to the rapid bleaching of the fluorescence signal, which was similar to the phalloidin staining.

4.2 Effect of endogenous NMDAR modulators on inhibitory neurons

4.2.1 Endogenous NMDAR modulators on inhibitory markers

To study the inhibitory function of interneurons in primary cortical cultures, we measured the levels of the predominant inhibitory neurotransmitter, gamma-aminobutyric acid (GABA), using ELISA. After 5 days of treatment, endogenous NMDAR modulators did not alter intracellular (one-way ANOVA; $F_{(1,4)} = 0.03$, $p = 0.99$) and extracellular GABA concentrations (one-way ANOVA; $F_{(1,4)} = 0.93$, $p = 0.49$) (Figure 19).

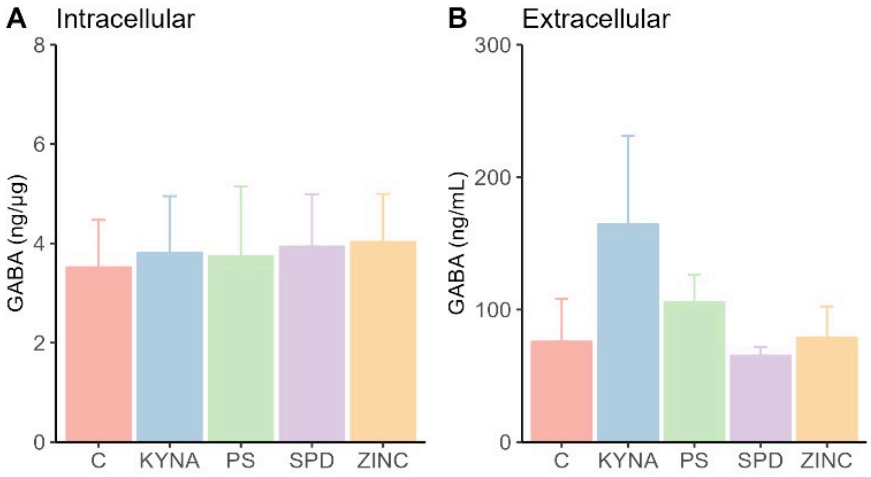

Figure 19. GABA expression after exposure to endogenous NMDAR modulators. A Intracellular and **B** extracellular levels of GABA determined by ELISA (n = 3-4 biological replicas) from cortical cultures after 5DT. Data is shown as means ± S.E.M. One-way ANOVA.

4.2.2 Endogenous NMDAR modulators do not alter inhibitory synaptic proteins

To complement the assessment of inhibitory function, we measured the expression of two proteins: gephyrin, an inhibitory postsynaptic scaffolding protein, and the vesicular GABA transporter (VGAT), an inhibitory presynaptic protein. Western blot analysis (Figure 20) revealed that the expression of these proteins was not altered by endogenous NMDAR modulators.

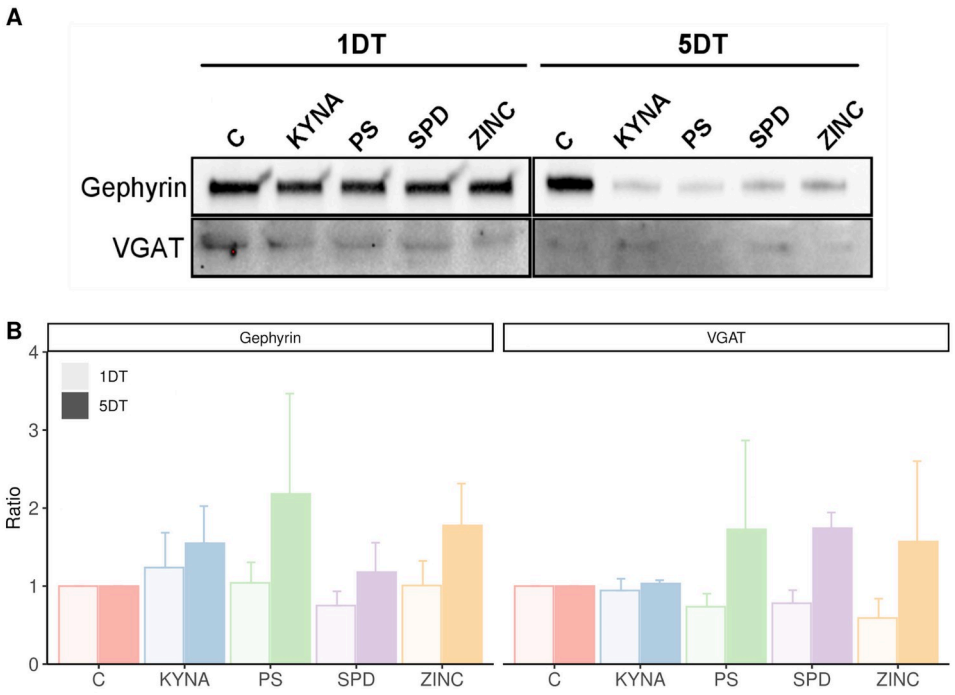

Figure 20. Analysis of western blot in cortical cultures treated with endogenous NMDAR modulators for 1 or 5 days. A Representative immunoblots of gephyrin and VGAT for the different 1- and 5-day treatments. **B** Quantification of the corresponding proteins normalized to the total load protein load of the stain-free gel (TGX Stain-Free BioRad). The data is presented as means ± S.E.M. Two-way ANOVA. n = 2-5 biological replicas.

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

At DIV 14, cortical cultures were treated with endogenous NMDAR modulators for 5 days. After treatment, parvalbumin-positive neurons, the largest class of inhibitory neocortical, were stained to measure morphological changes (Figure 21).

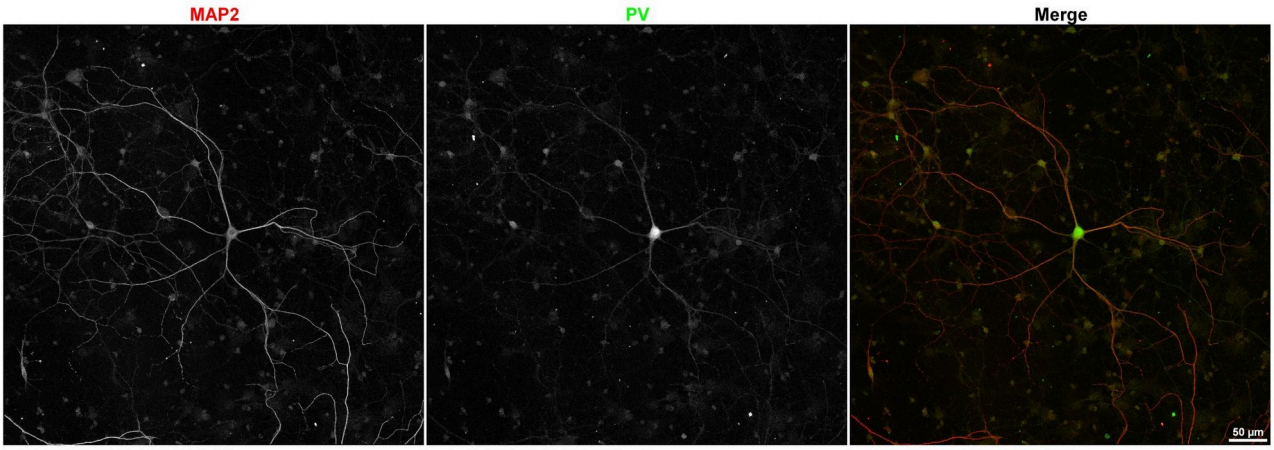

Figure 21. Representative confocal image of a parvalbumin-positive neuron. Cortical culture at DIV 19.

Endogenous modulators of NMDAR produced a decrease in total dendritic length (Figure 22B. One-way ANOVA; $F_{(1,4)} = 4.59$, $p = 0.002$. Dunnett's post hoc test; KYNA $p = 0.002$, PS $p = 0.004$, SPD $p = 0.006$), total number of dendritic branches (Figure 22C. One-way ANOVA; $F_{(1,4)} = 4.83$, $p = 0.001$. Dunnett's post hoc test; KYNA $p = 0.003$, SPD $p = 0.015$), number of branching

points (Figure 22E. One-way ANOVA; $F_{(1,4)} = 3.19$, $p = 0.017$. Dunnett's post hoc test; KYNA p = 0.040, SPD p = 0.016), without changes in the number of primary branches (Figure 22D). When performing Sholl analysis (Figure 22F), the endogenous NMDAR modulators also produced a decrease of the area under the curve (Figure 22G. One-way ANOVA; $F_{(1,4)} = 3.82$, $p = 0.007$. Dunnett's post hoc test; KYNA $p = 0.003$, PS $p = 0.012$, SPD $p = 0.017$) and the maximum number of intersections (Figure 22H. One-way ANOVA; $F_{(1,4)} = 2.86$, $p = 0.028$. Dunnett's post hoc test; KYNA $p = 0.004$).

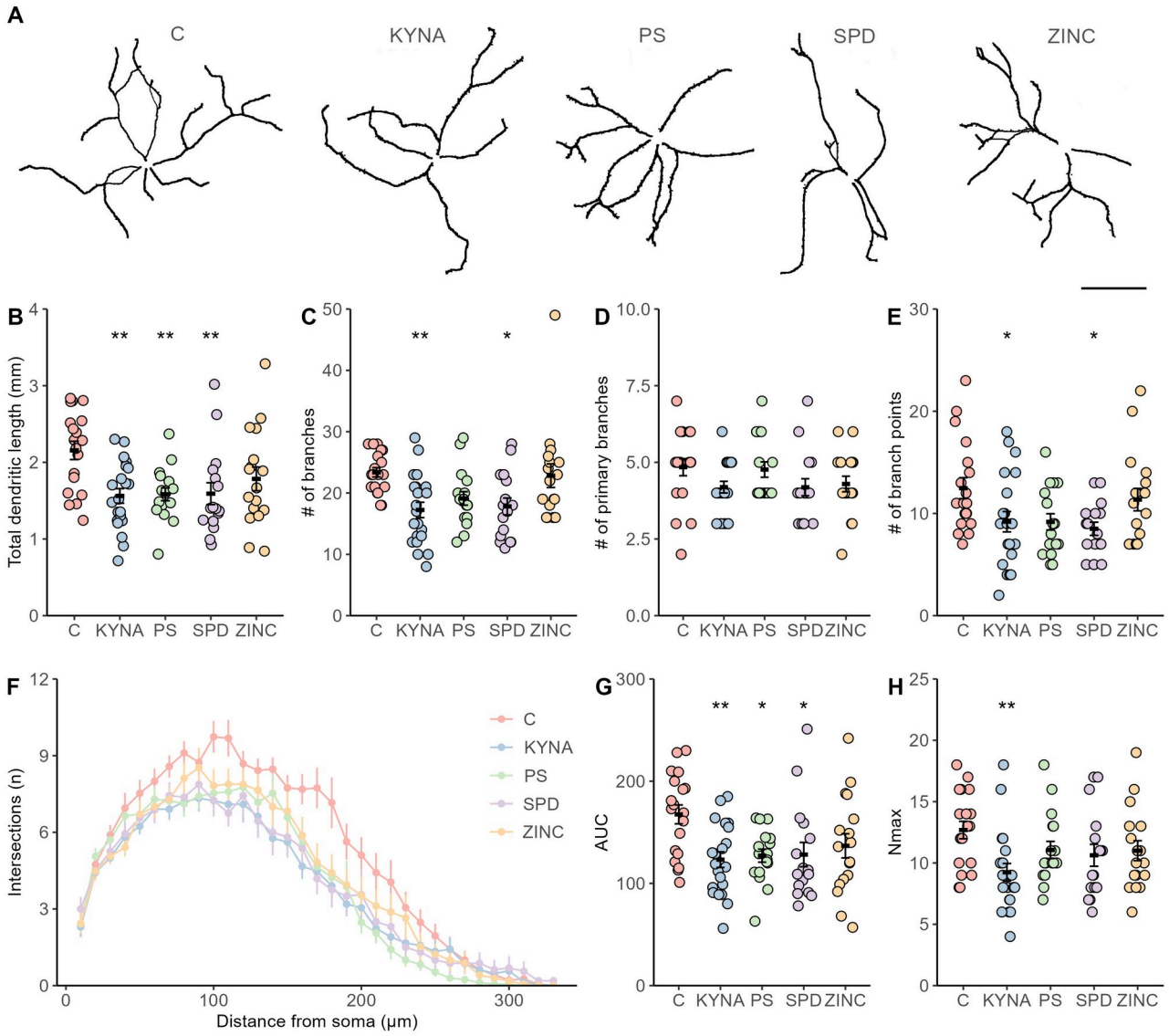

Figure 22. Analysis of dendritic branching and arbor complexity in cortical parvalbumin neurons treated for 5 days with different NMDAR modulators. **A** Representative examples of reconstructed neurons. **B–E** NMDAR modulators effects on **B** total dendritic length, **C** number of branches, **D** number of primary branches, **E** number of branch points. **F** Sholl analysis shows the number of intersections along with the distance from the soma for different treatments, **G** area under the curve and **H** number of maximum intersection. Data are represented as means ± S.E.M. n = 16–21 randomly chosen neurons per treatment from three biological replicas. One-way ANOVA followed by Dunnett's post hoc. Scale bar = 100 µm.

4.3 Effect of pregnenolone sulfate on chronic despair model in mice

To explore the antidepressant-like effects of PS, mice underwent consecutive behavioral tests following the chronic despair model (CDM) protocol. These tests included open field test (OFT) for evaluating anxiety-like behavior, three chamber test (3-CT) for evaluating social interaction and the forced swim test (FST) for evaluating despair behavior. As described in Figure 7, PS (40 mg/kg) was injected intraperitoneally 60 min before each test. As expected, CDM protocol increased the immobility time when comparing the day 5 with the day 1 ($p < 0.001$).

4.3.1 Anxiolytic-like effect of pregnenolone sulfate

OFT revealed no difference in total distance traveled (Unpaired t-test, $p = 0.17$) and number of entries to the center (Unpaired t-test, $p = 0.14$) (Figure 23B,D). However, PS administration increased the time spent in the center (42.5 ± 6.97 s) in comparison to control (34.1 ± 12.9 s) (Unpaired t-test, $p = 0.03$, Figure 23D).

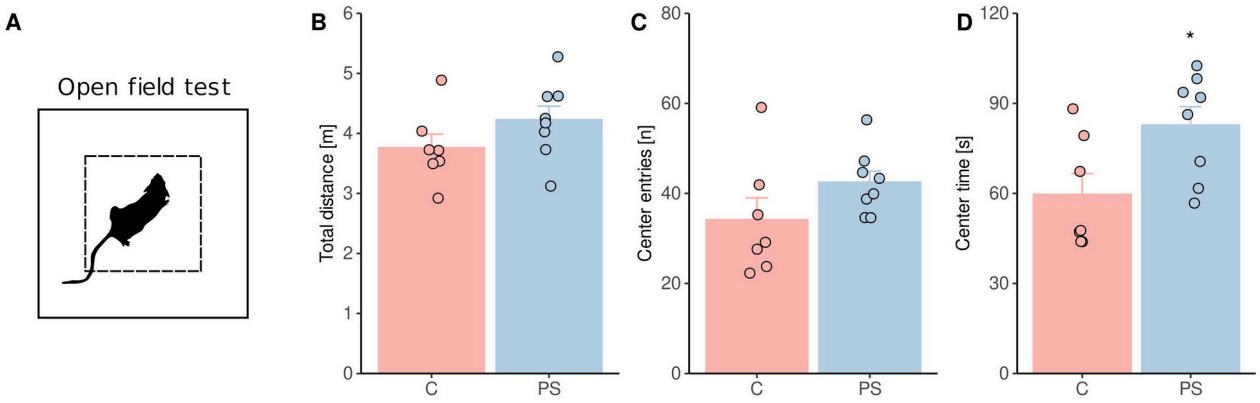

Figure 23. Decreased anxiety-like behaviors after pregnenolone sulftate administration in an open field test. A Schematic diagram of the open field test. **B** Total distance traveled and **C** number of entries to the center are unchaged between groups. **D** Pregnenolone sulfate increased the time spent in the center of the open field. Unpaired t-test. n = 7-8 per group. Data is shown as means ± S.E.M.

4.3.2 Effect of pregnenolone sulfate on chronic distress model in mice

The day after the OFT, the 3-CT was performed. First, there was 5 minutes of habituation session were subject mouse was placed in the center of the arena and allowed to explore freely. Then, tested for sociability in a socialbility session (Figure 24A) and for preference for social novelty in a social novelty session (Figure 24E). There was not difference amongst the time spent in each chamber or sniffing the glasses during the habituation session, meaning that there was not bias for visiting any chamber. During the sociability test, PS administration did no change the time spent with the mouse (Unpaired t-test, $p = 0.62$) or empty glass (Unpaired t-test, $p = 0.14$) and social index (Unpaired t-test, $p = 0.45$) (Figure 24B-D). No difference were found neither in preference for

social novelty, since there was not difference in time spent with the familiar mouse (Unpaired t-test, $p = 0.05$) or novel mouse (Unpaired t-test, $p = 0.72$) and social novel index (Unpaired t-test, $p = 0.15$) (Figure 23F-H).

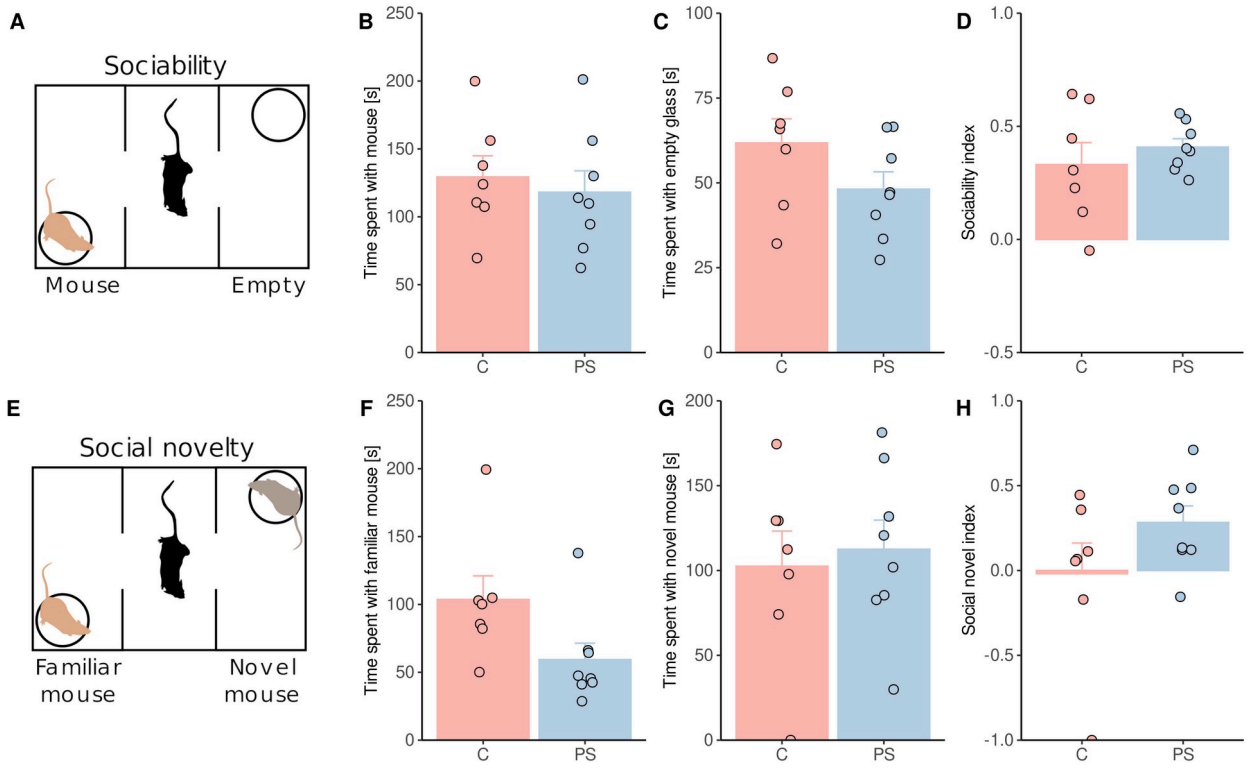

Figure 24. Pregnenolone sulftate administration does not change social interaction in the three chamber test. A Schematic diagram of the three chamber test to measure sociability. **B** Time spent with the mouse. **C** Time spent with the empty glass. **D** Social index. **E** Diagram of the three chamber test to measure social novelty. **F** Time spent with the familiar mouse. **G** Time spent with the novel mouse. **D** Social novel index. Unpaired t-test. n = 7-8 per group. Data is shown as means ± S.E.M.

4.3.3 Effect of pregnenolone sulfate on chronic distress model in mice

Finally, the FST was performed (Figure 25A). There were no change in the immobility time (Unpaired t-test, $p = 0.48$) and latency of the first episode of immobility (Unpaired t-test, $p = 0.90$) (Figure 25B-C) between the control and PS group.

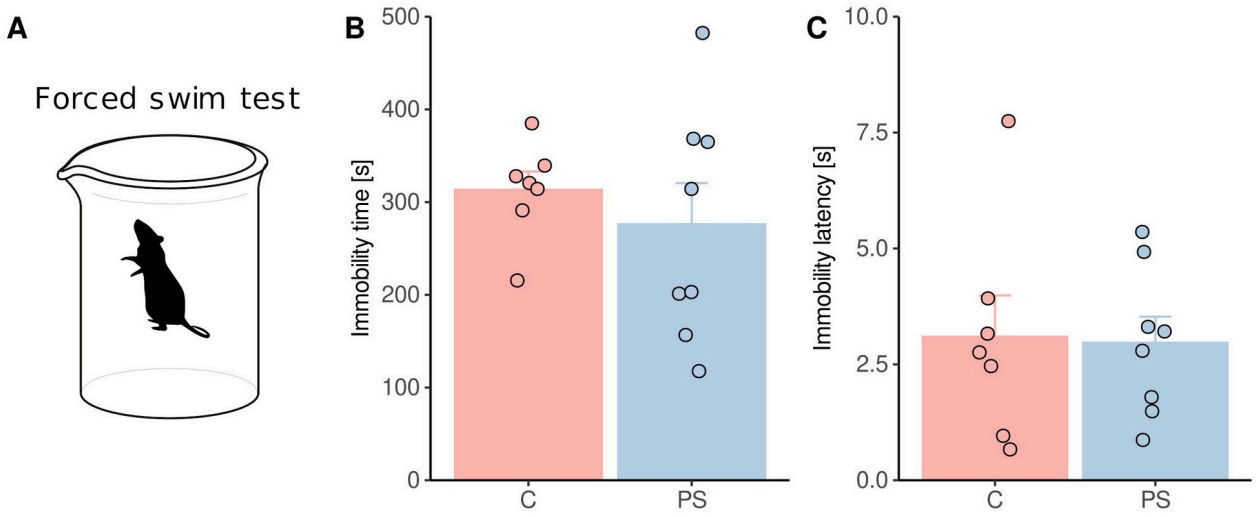

Figure 24. Pregnenolone sulftate administration does not change antidepressant-like effect in the forced swim test. A Schematic diagram of the forced swim test. **B** Immobility time and **C** immobility latency. Unpaired t-test. n = 7-8 per group. Data is shown as means ± S.E.M.

5. Discussion

In this study, we investigated the impact of brief and prolonged exposure of primary cultured neurons to the most prevalent endogenous NMDAR modulators on evoked glutamate release, excitatory/inhibitory synaptic inputs, dendritic branching and activation of BDNF/TrkB/ERK signaling pathway. Our results indicate that endogenous NMDAR modulators had no effect on evoked glutamate release. PS was observed to increase distal dendritic arborization. After one hour of treatment, PS increased the phosphorylation of the TrkB receptor and showed a tendency towards increased BDNF expression. The density of puncta of PSD-95 was decreased by PS, but the synaptic density was not altered by endogenous NMDAR modulators. The expression and release of GABA, as well as the protein expression of gephyrin and VGAT, were not affected by the endogenous NMDAR modulators. All endogenous NMDAR modulators, except for ZINC, decreased dendritic branching and arbor complexity of parvalbumin-positive neurons. The effects of PS are summarised in Figure 25.

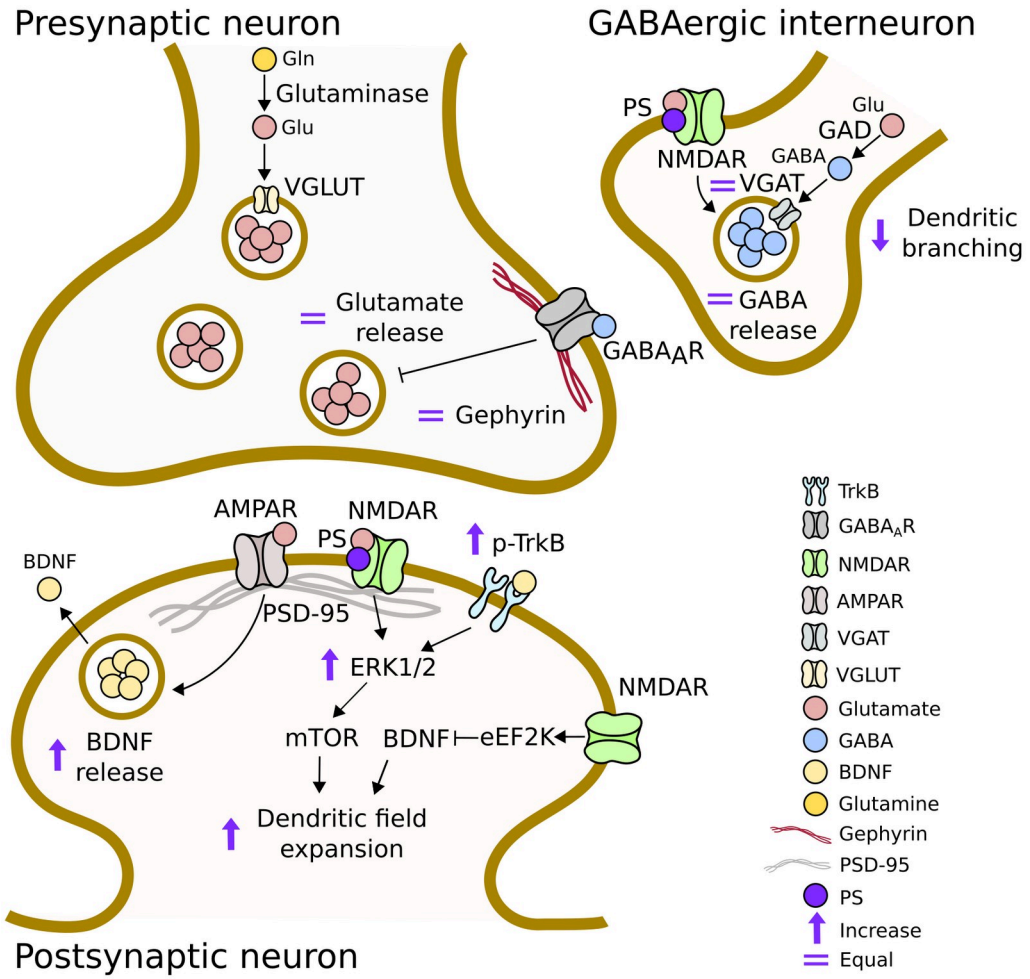

Figure 25. Summary of the effect of pregnenolone sulfate on neurons. Pregnenolone sulfate increases BDNF release, which activates TrkB receptors and increases ERK1/2 expression. This may be the mechanism that leads to dendritic field expansion. No differences were found in evoked glutamate release and gephyrin expression. In GABAergic neurons, pregnenolone sulfate did not alter VGAT and decreased dendritic branching in the parvalbumin-positive subtype of interneuron. The figure employs the following abbreviations: GAD for glutamate decarboxylase, Gln for glutamine, and Glu for glutamate.

5.1 Glutamate release in not altered by endogenous NMDAR modulators

Depolarization-induced glutamate release, measured in the presence of DL-TBOA (an inhibitor of glutamate clearance), remained unchanged after 1 and 5 days of treatment with endogenous NMDAR modulators. This suggests that the overall number of glutamatergic connections remained constant. It is important to note that presynaptic NMDAR enhances glutamate release from axons through a calcium-dependent mechanism (Berretta & Jones, 1996; Cochilla & Alford, 1999). The NMDAR antagonist ketamine rapidly inhibits glutamate release by reducing the release competence of synaptic vesicles (Guhathakurta et al., 2024; Lazarevic et al., 2021). To investigate whether endogenous NMDAR modulators affect glutamate release mediated by presynaptic NMDAR, we treated primary cortical cultures with Krebs-Ringer solution containing high potassium concentration and endogenous NMDAR modulators. The results showed that depolarization-induced glutamate release remained unchanged, indicating that presynaptic NMDAR activity was not altered. Electrophysiological techniques have reported that PS enhances glutamate release. This effect is achieved by targeting metabotropic σ1 receptors (Meyer et al., 2002), presynaptic NMDA receptors (Mameli et al., 2005), or transient receptor potential channels (K. H. Lee et al., 2010; Zamudio-Bulcock et al., 2011) to increase presynaptic calcium influx and enhance glutamate release. Given that previous experiments used a similar concentration of PS to ours, the absence of an increase in glutamate release in our findings may be attributed to the higher temporal resolution of the electrophysiological methods.

5.2 Pregnenolone sulfate increases dendritic field expansion

The data suggest that PS regulates dendritic arborization, but not branching. This is supported by an increase in the number of dendritic intersections far from the soma, but no changes in the number and length of dendritic branches. It is worth noting that spermidine had a mild effect of decreasing the number of dendritic intersections close to the soma. To the best of our knowledge, the role of endogenous NMDAR modulators in shaping the geometry and extent of dendritic projections has not been previously demonstrated. Although further analysis is required to understand the underlying processes of observed changes, there is overwhelming evidence suggesting the key role of NMDAR in regulating the complexity of the dendritic tree of neurons (Cline & Haas, 2008; Ewald et al., 2008; L.-J. Lee et al., 2005). The effects of NMDAR on morphological plasticity are mediated through changes in the activity of local structural proteins (Lisman et al., 2002), confined to postsynaptic compartments and spines. Alternatively, they may activate long-range molecular signalling mechanisms, such as mitogen-activated protein kinase (MAPK) and transcription factors, resulting in global responses (Cline & Haas, 2008). Notably, intriguing results have been observed through the activation and inhibition of NMDAR by agonists

and antagonists. While some studies have found that NMDA increases the number of dendritic branches and inhibition of NMDAR by MK801 and ketamine decreases them (Chevaleyre et al., 2002; Sepulveda et al., 2010), other studies showed that ketamine increased dendritic arbor complexity (Ly et al., 2018) and the NMDAR co-agonist D-serine administration led to more compact dendritic arbors (Chorghay et al., 2023).

Expansion of the dendritic tree without altering the total dendritic length can have at least two effects on cortical neurons. Firstly, it can change the geometry and distribution of synaptic inputs (Callan et al., 2021). Secondly, it can affect the intrinsic excitability and regenerative activity (Mainen & Sejnowski, 1996). According to a computer model, extending the dendritic surface distally from the soma results in increased responsiveness to synaptic inputs targeting soma and proximal dendrites, leading to increased firing activity (Jorratt et al., 2023). Dendritic field expansion caused by PS thus can directly impact cellular excitability. It is worth noting that this may impact synaptic integration and cortical network dynamics.

Other neurosteroids, such as DHEA and DHEAS, have been shown to stimulate axonal and dendritic growth, respectively (Compagnone & Mellon, 1998), while allopregnanolone causes neurite regression (Brinton, 1994). Additionally, *in vitro* and *in vivo* studies showed that progesterone promotes dendritic growth and dendritic spine formation of the Purkinje cell (Sakamoto et al., 2001, 2002).

5.3 Pregnenolone sulfate increases BNDF expression and TrkB activation

Numerous studies have been conducted *in vitro* and *in vivo*, which indicates that BDNF plays a crucial role in shaping neuronal architecture by activating the TrkB receptor in both glutamatergic and GABAergic neurons (Gottmann et al., 2009; Ji et al., 2005; Park & Poo, 2013). Therefore, the expansion of dendritic fields after PS treatment is consistent with the tendency to increase the expression of BNDF. It is worth noting that these BNDF levels are intracellular. This is because the amount released into the supernatant of the neuronal cultures is too low to be measured by ELISA. We were unable to obtain values above the limit of detection.

The activation of TrkB depends on its dimerization and subsequent phosphorylation of tyrosine residues within its intracellular domain (Minichiello, 2009). Therefore, assessing the phosphorylation status of specific tyrosine residues provides a method to measure TrkB activation. We observed that the ratio of p-TrkB (p-TrkB/TrkB) significantly increased after 1 hour of PS treatment, but not after 6 hours. Consistently, there was a trend towards increased expression of ERK1/2 after one hour of treatment, suggesting activation of the BDNF-TrkB-ERK pathway, which can be involved in the expansion of dendritic fields. However, after 6 hours, there was a decrease in ERK1/2 phosphorylation, which may be due to a compensatory mechanism.

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

Pregnenolone sulfate not only affects dendritic tree expansion but also reduces the density of excitatory postsynaptic marker PSD-95 puncta. This result was observed only in the proximal branches (< 100 μm), but not in the distal ones (> 100 μm). 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. Results from studies of ketamine are mixed. Some studies show that ketamine induces a loss and shortening of dendritic spines and PSD-95 expression, while others show an increase in the number of dendritic spines and PSD-95 expression (Jiang et al., 2018; N. Li et al., 2010; Piva et al., 2021; Pryazhnikov et al., 2018). Therefore, it is difficult to interpret if the decreased PSD-95 density by PS is due to a change in the NMDAR activity.

Interestingly, synaptic expression of PSD-95 restricts dendritic branching by obstructing the synaptic clustering of GluN2B-NMDARs (Bustos et al., 2014). The observed decrease in PSD-95 expression with PS may be due to a displacement of PSD-95/GluN2A-NMDARs complexes to SAP102/GluN2B-NMDARs complexes, resulting in an increase in arbor complexity. To test this hypothesis, we measured whether PS alters the ratio of synaptic GluN2A/GluN2B subunits. Confocal microscopy was used to count the puncta density. However, obtaining the images was unsuccessful due to the rapid bleaching of the fluorescence signal.

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

Fast-spiking parvalbumin-positive interneurons, the largest class of inhibitory neocortical cells (Hafner et al., 2019), are characterised by their inhibitory feedforward and feedback projections onto pyramidal neurons (Beierlein et al., 2003; Cruikshank et al., 2010; Pouille et al., 2009). These projections mediate network synchrony and regulate theta and gamma oscillations (Cardin et al., 2009; Sohal et al., 2009), which have been implicated in learning and memory processes (Donato et al., 2013; Kuhlman et al., 2013; Letzkus et al., 2011; Yazaki-Sugiyama et al., 2009).

Parvalbumin-positive interneurons express functional NMDARs (Lewis et al., 2022). We found that all the endogenous NMDAR modulators, except for zinc, decreased dendritic branching and arbor complexity in those interneurons. Parvalbumin-positive interneurons are enriched in GluN2D subunits (Garst-Orozco et al., 2020; Perszyk et al., 2016; Standaert et al., 1996), where PS exerts an inhibitory effect. 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). Also, 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. Ketamine reduces parvalbumin and GAD67 immunoreactivity in cultured interneurons (Kinney et al., 2006) as well as in cortical and hippocampal regions (Kokkinou et al., 2021). However, we did not observe any difference in the parvalbumin immunoreactivity. It is worth noting that NMDAR activity in parvalbumin-positive interneurons is crucial, as genetic ablation leads to alterations in theta and gamma oscillations, and disrupts spatial memory and associative learning (Carlén et al., 2012; Korotkova et al., 2010).

GABA release and expression were measured, along with the expression of the postsynaptic inhibitory marker gephyrin, to test the inhibitory function. No differences were found in any of these results. To better understand the effect of endogenous NMDAR modulators on inhibitory drive onto excitatory neurons, further experiments, such as measuring the miniature inhibitory postsynaptic potentials and GABA currents, would be necessary.

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

The hypothesis of depression known as the "neurotrophin hypothesis" is largely based on the observation that stress-induced depressive behaviors are correlated with decreases in hippocampal BDNF levels, and that the expression of *Bdnf* is enhanced by antidepressant treatment (Duman & Monteggia, 2006; Martinowich et al., 2007). Studies have also shown that depression is linked to a decrease in the size of brain regions that regulate mood and cognition, such as the prefrontal cortex and hippocampus, as well as a reduction in neuronal synapses in these areas (Duman & Aghajanian, 2012). 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. It was found that PS had an anxiolytic effect when injected one hour before the open field test. However, no differences were observed in social interaction or the forced swim test. Previous studies have shown that PS has an antidepressant effect by reducing immobility time in the forced swim test (Dhir & Kulkarni, 2008) and an anxiolytic effect in a mirrored chamber test (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.

Our results in cultured neurons indicate that GABAergic neurons were more sensitive to structural changes after PS treatment. As ketamine, which preferentially acts on GABAergic neurons by blocking NMDARs, has been hypothesized as the model for a fast-acting antidepressant effect, the anxiolytic effect we found could be due to an inhibitory effect on inhibitory neurons,

leading to an increase in the firing of pyramidal neurons. It is worth noting that PS enhances LTP in CA1 (Sliwinski et al., 2004). In the future, it would be interesting to measure the expression of synaptic proteins, as well as dendritic spine density, after PS treatment in mice that underwent the chronic despair model protocol.

It is important to note that the endogenous NMDAR modulators used in our experiments may interact with other receptors that are also involved in synaptic transmission and plasticity mechanisms. The effects of PS cannot be attributed to intracellular mechanisms or metabolites. This is because, unlike unconjugated steroids, PS cannot freely cross the plasma membrane due to the negatively charged sulfate group. Additionally, there is no evidence of extracellular expression of the enzyme involved in the conversion of PS.

6. Conclusion

Glutamatergic neurotransmission impairments have been associated with various neurological and psychiatric disorders, with NMDAR dysfunctions playing a crucial role. There is a significant knowledge gap regarding how molecular and functional changes in glutamatergic synaptic inputs translate into phenotypes of mood disorders, addiction, and developmental brain diseases such as autism and schizophrenia. Endogenous modulators of NMDAR, which are produced and released from neurons and glial cells, have a wide range of effects on neuronal processes and functions. Changes in their levels in the cerebrospinal fluid and peripheral circulation have been reported in patients with mental disorders.

Our findings provide new insights into the impact of endogenous NMDAR modulators on neuronal morphology and synaptic dynamics. The increase in BDNF release, TrkB activation, and dendritic field expansion observed with PS is likely to contribute to anxiolytic-like behaviour in mice. Therefore, dysregulation of endogenous PS levels could impair the BNDF/TrkB/ERK signaling cascade, which may be related to the decreased PS levels in patients with affective disorders, generalized social phobia, and anxiety disorder. Furthermore, the results demonstrate the increased vulnerability of parvalbumin-positive neurons to structural changes induced by these modulators, elucidating their role in neuronal circuitry and their potential significance in psychiatric pathophysiology.

The molecular effect of PS is similar to that of the fast-acting antidepressant ketamine, whose mechanism is proposed to be through the disinhibition of glutamatergic neurons and the enhancement of glutamatergic firing. Therefore, PS is a good candidate for treating depressive disorders.

7. Summary

7.1 Effect of endogenous NMDAR modulators on excitatory neurons

- Endogenous NMDAR modulators did not affect neuronal viability or evoked glutamate release.
- PS was observed to increase distal dendritic arborization as measured by the Sholl analysis without altering the number of branches, branch points, and total dendritic length. On the other hand, SPD had a mild effect on dendritic arborization by reducing proximal dendritic intersections.
- There was a tendency towards increased BDNF expression in cultures treated with PS.
- PS increased the phosphorylation ratio of TrkB receptor and showed a tendency towards increased expression of the signal mediator ERK1/2 after 1 hour of treatment. However, after 6 hours of treatment, it decreased the phosphorylation of ERK1/2.
- Endogenous NMDAR modulators did not alter the density of excitatory synapses. However, PS decreased the density of puncta of the postsynaptic scaffolding protein PSD-95.
- The protein expression of PSD-95 and presynaptic marker synaptophysin remained unaltered by endogenous NMDAR modulators. Additionally, the expression of the mandatory NMDAR subunit GluN1 was not affected.

7.2 Effect of endogenous NMDAR modulators on inhibitory neurons

- The expression and release of the neurotransmitter GABA were not affected by the endogenous NMDAR modulators.
- The expression of the inhibitory postsynaptic scaffolding protein gephyrin and the vesicular GABA transporter (VGAT) remained unaltered after the administration of endogenous NMDAR modulators.
- All endogenous NMDAR modulators, except for ZINC, were found to decrease dendritic branching and arbor complexity of parvalbumin-positive neurons.

7.3 Effect of pregnenolone sulfate on chronic despair model in mice

- PS reduced anxiety-like behaviour in the chronic despair model in mice by increasing the time spent in the center of the open field test.
- PS did not modify the social interaction of mice that underwent chronic despair model protocol.
- In the chronic despair model in mice, PS did not exhibit an antidepressant-like effect.

8. Shrnutí

8.1 Účinek endogenních modulátorů NMDAR na excitační neurony

- Endogenní modulátory NMDAR neovlivnily životaschopnost neuronů ani evokované uvolňování glutamátu.
- PS zvýšil distální dendritickou arborizaci podle Sholl analýzy, aniž by měnil počet větví, body větvení a celkovou délku dendritů. Naproti tomu SPD měl mírný vliv na proximální dendritickou arborizaci, jež byla snížená.
- V kulturách ošetřených PS byla pozorována tendence ke zvýšené expresi BDNF.
- PS zvýšil po 1 hodině působení fosforylaci receptoru TrkB a vykazoval tendenci ke zvýšené expresi ERK1/2. Po 6 hodinách působení pak byla fosforylace ERK1/2 snížena.
- Endogenní modulátory NMDAR neovlivnily hustotu excitačních synapsí. PS však snížil hustotu fluorescenčního barvení postsynaptického scaffold proteinu PSD-95.
- Exprese proteinu PSD-95 a presynaptického markeru synaptofysinu zůstala endogenními modulátory NMDAR nezměněna. Dále také nebyla ovlivněna exprese povinné podjednotky NMDAR GluN1.

8.2 Účinek endogenních modulátorů NMDAR na inhibiční neurony

- Exprese a uvolňování neurotransmiteru GABA nebyly endogenními modulátory NMDAR ovlivněny.
- Exprese inhibičního postsynaptického scaffold proteinu gephyrinu a vezikulárního GABA transportéru (VGAT) zůstala po podání endogenních modulátorů NMDAR nezměněna.
- Všechny endogenní modulátory NMDAR s výjimkou zinku snižují dendritické větvení a komplexitu arborů u parvalbumin-pozitivních neuronů.

8.3 Účinek pregnenolon sulfátu na model chronického zoufalství u myší

- PS snížil úzkostné chování myší v chronic despair modelu tak, že zvýšil čas strávený ve středu arény v open field testu.
- PS nezměnil sociální interakci myší.
- V chronic despair modelu nevykazoval PS u myší antidepresivní účinek.

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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. doi:10.1007/s12035-022-03147-0 **(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. doi:10.1002/alz.12244 **(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. https://doi.org/10.3390/brainsci13050794 **(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. doi:10.3389/fphar.2023.1120419 **(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. doi:10.1111/adb.13216 **(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. doi:10.3389/fnins.2022.866161 **(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. doi:10.1007/s40263-020-00764-y **(IF = 6.497).**