

THE EFFECTS OF MEMANTINE AND MK801 ON NMDA RECEPTOR SWITCHING 2B AND 2A SUBUNITS IN HIPPOCAMPAL CELL CULTURE

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Abstract

Background: Schizophrenia (SCZ) is a severe and chronic neurodevelopmental disorder whose onset begins in adolescence or early adulthood. Notwithstanding, brain dysfunction occurs before the disease onset and involves a switch in NMDA receptor subunit composition from GluN2B to GluN2A at early neonatal period. We have recently postulated memantine (MEM) as an effective experimental treatment, due to its effect on modulating NMDA receptor subunit turnover during the postnatal period, as it prevents glutamatergic hypofunction in the maternal deprivation model of SCZ.

Methods: We evaluated the turnover of pre and postsynaptic glutamatergic synaptic components by using primary mouse hippocampal neurons during the synaptic formation period. **Results:** MK801 stimulation prevented the GluN2B to GluN2A molecular switch at 11 days in vitro (DIV). Vesicular glutamate transporter 2 (VGLUT2) was also reduced at this time point. MEM treatment reverted these effects by normalizing GluN2B, and GluN2A, and over-expressing VGLUT2 expression. **Conclusion:** Our data supports a molecular mechanism by which SCZ may be prevented with MEM treatment through regulation of the glutamatergic synaptic molecular composition.

Keywords: Schizophrenia, synapses, SNARE, glutamate, development, NMDA receptor.

Introduction

Schizophrenia (SCZ) is considered a neurodevelopmental disorder,¹ that onsets in adolescence or early adulthood in 80% of cases, without showing clear signs of behavioral dysfunction during childhood.² The glutamatergic system has been widely associated with the disease,³ specifically, the N-Methyl-D-Aspartate Receptor (NMDAR), which undergoes subunit changes along the life of the mammalian brain. The most important of these changes occur in the postpartum period, when the exchange of GluN2B to GluN2A subunits takes place,⁴ generating a very low concentration of GluN2B in hippocampal tissue during early adulthood.⁵ This change of subunits produces significant variations in synaptic connections between the hippocampus and prefrontal cortex, causing a high risk to develop neuropsychiatric disorders during adulthood.⁶ In addition, the inversion of the normal ratio between GluN2A/GluN2B in the hippocampus causes poor synaptic metaplasticity, altering long-term depression and potentiation,⁷ a major cause of NMDA receptor hypofunction in schizophrenic patients.^{8,9} We have proposed that this, in turn, could cause altered patterns of hippocampal-cortical

communication,¹⁰ which may produce the clinical symptoms of the disease in early adult life, during the maturation of the prefrontal cortex.¹¹

The GluN2A subunits transcription is a key step in the neonatal brain and is enhanced by the presence of brain-derived neurotrophic factor (BDNF), as well as by the correct activation of GluN2B subunits during the switch.¹² Memantine (MEM) is a low-affinity voltage-dependent uncompetitive antagonist at NMDAR, that has a preference for GluN2B subunits and is able to induce up-regulation of BDNF throughout the modulation of glutamatergic signaling.¹³ Previous work from our laboratory showed that MEM treatment in the early postnatal period prevents brain atrophy, and electrophysiological and behavioral abnormalities induced by the maternal deprivation model of SCZ.^{14,15} To gain further insight into the molecular mechanisms potentially operating in the development of hippocampal synapses, which is difficult with the maternal deprivation model *in vivo*; we have modeled glutamatergic hypofunction by stimulating hippocampal neurons *in vitro* with the NMDAR non-competitive antagonist MK-801. NMDAR antagonists are commonly used to model SCZ as they



produce acute molecular abnormalities consistent with those observed in the disease. We show that GluN2B receptors are downregulated with a parallel decrease in VGLUT2 expression, which is prevented by MEM co-stimulation.

Methods

2.1 Hippocampal cell culture

Brains obtained from E15 mice embryos (*Mus musculus*) were dissected on ice-cold Hank's balanced salt solution (HBBS) buffer. The meninges were removed, and the brains were cut through the midline to expose the hippocampi, which were removed using fine forceps and incubated in HBSS containing 0.25% trypsin (15090-046; Gibco, Schwerte, Germany) and 60 U/mL DNase-I (D5025; Sigma) for 25 min at 37 °C. Trypsinization was stopped by the addition of 5% fetal bovine serum (FBS; Invitrogen, Waltham, MA, USA) diluted in a Neurobasal medium. The tissue was mechanically dissociated by repeated pipetting. Cells were seeded at a density of 7.5×10^4 cells/mL on coverslips coated with 50 $\mu\text{g}/\text{mL}$ polyornithine (P4957; Sigma) and 20 $\mu\text{g}/\text{mL}$ laminin-entactin (Corning, New York, NY, USA). Neurons were incubated in a Neurobasal medium containing 2% B27 (Invitrogen), glutamine (20 mM; Invitrogen), and PenStrep antibiotic mix (Invitrogen).

2.2 Drugs administration

MK801 was applied on the 8th DIV, the moment in which synaptic connections are visible at the microscope. A dose $\geq 40 \mu\text{M}$ of MK801 is cytotoxic *in vitro*, and between 10 - 20 μM ensures the NMDAR hypofunction without cytotoxicity.¹⁶ The medium was washed 24 hours later. On the 10th DIV, MEM, a neuroprotective, was applied (5 μM).¹⁷ Both, MK801 and MEM are noncompetitive antagonists, but act at different places of NMDAR with diverse purposes, for example, MEM interacts with two places, at the magnesium site and with less affinity, the extracellular vestibule of the channel, modulating calcium influx.¹⁸ Besides, MK801 binds within the ion channel vestibule, promoting closure of the ion channel gate, and physically blocking ion permeation.¹³

The study was designed with six groups: control group (CONTROL), Memantine group (MEM), MK801 at 10 μM treatment group (MK801 (10 μM)); MK801 at 10 μM and posterior MEM treatment (MK801 (10 μM) + MEM); MK801 at 20 μM treatment group (MK801 (20 μM)); MK801 at 20 μM and posterior MEM treatment (MK801 (20 μM) + MEM) (Figure 1). In addition, the time course of GluN2A and GluN2B subunits with no intervention drugs was recorded from DIV 8 to DIV 11 (Figure 2).

DIV 8	DIV 9	DIV 10	DIV 11
X	Wash	X	End
X	Wash	MEM (5 μM)	End
Mk801 (10 μM)	Wash	X	End
Mk801 (10 μM)	Wash	MEM (5 μM)	End
Mk801 (20 μM)	Wash	X	End
Mk801 (20 μM)	Wash	MEM (5 μM)	End

Figure 1. Timeline and experimental groups.

2.3 Protein extraction and western Blot

Whole-cell lysates were prepared in a buffer containing 50 mM Tris HCL pH 7.4, 150 mM NaCl, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate, plus proteinase and phosphatase inhibitors. Samples (20 μg) were run on a 10% SDS-PAGE gel, transferred to a nitrocellulose membrane, blocked with 5% milk in TBS, and incubated overnight at 4°C with primary antibodies against GluN2A (1:500, Life technologies, A-6473), GluN2B (1:500, Life technologies, A-6474), PSD95 (1:10.000, Abcam, ab18258), VGLUT1 (1:2000; Synapticssystem), ab227805), and VGLUT2 (1:1000, Synapticssystem, ab216463); syntaxin-1 (Abcam, ab272736) was used at 1:20.000 as well as B-Actin (1:1000, Santa Cruz Biotechnology H-63, USA) as a loading control. Membranes were washed in TBS, incubated with an HRP-conjugated secondary antibody (1:5000; Santa Cruz Biotechnology) for 1 hour at RT, washed, and incubated with ECL solution (Perkin Elmer) for 1 min. Blots were developed using Amersham ECL Prime Western Blotting Detection Reagent (Life Sciences, Waltham, MA, USA) and scanned using a digital enhanced chemiluminescence (ECL) detection device (Thermo Fisher).

2.4 Statistical Processing

All data points are presented below as means \pm SD. Multiple-group values were compared with the non-parametric Kruskal–Wallis (KW) test and the Dunn post-hoc test. To compare the two groups the non-parametric Mann-Whitney test was used. For nonparametric correlations, the Spearman coefficients were calculated. WB percentage was the target protein band signal normalized to the loading control (Ratio of control at Y-axis label). Four (8) WB were made (N=8).

Results

3.1. An NMDA receptor switch is observed between 8 and 10 DIV

We identified an exchange of GluN2B and GluN2A subunits between 8 and 11 DIV (Figure 2A). The levels of GluN2A receptor subunits were undetectable at 8 DIV. GluN2A expression progressively increased (9 DIV: $P=0,0003$; 10 DIV: $P=0,006$) until the end of the incubation period at 11 DIV ($P=0,0008$). Interestingly, GluN2B receptor subunits presented a maximal level of expression at 8 DIV which was progressively lower until 11 DIV.

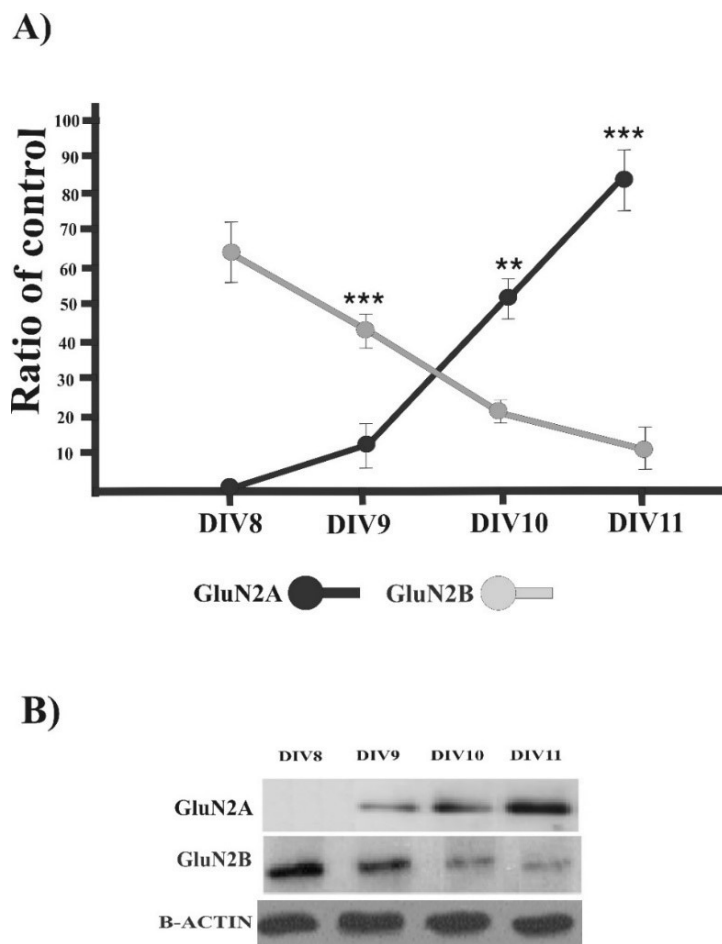


Figure 2. Time course of GluN2A and GluN2B subunits

NMDAR subunits are exchanged in vitro during the early phases of neuronal maturation. **A)** Time course evaluation of GluN2A and GluN2B expression. GluN2A subunits (black) presented a progressive increase while GluN2B subunits (grey) showed a progressive decrease during the incubation period. Significant differences with respect to the initial evaluation condition were identified from 9 to 11 DIV for both NMDAR subunits. **B)** Representative western blots. N=5 experiments analyzed in triplicate. Values are mean \pm SD and analyzed by non-parametric Mann-Whitney test. ** $P < 0.01$ and *** $P < 0.005$ for differences between pairs of groups.

3.2 MEM and MK801 have opposite effects on NMDAR subunit expression *in vitro*

NMDAR subunit composition was then evaluated at 11 DIV after incubation with MK-801 with or without MEM. MK-801 did not show any dose-dependent effect over GluN2A expression. MEM significantly increased the GluN2A expression, although this effect was dose-dependently reduced with MK-801 co-stimulation (Figure 3A). By contrast, MK-801 induced a significant increase in GluN2B expression at 11 DIV which was blocked by MEM co-stimulation (Figure 3B). This suggests an antagonist effect of MK-801 and MEM over GluN2A and GluN2B expression.

3.2.1 GluN2A

MEM increased the GluN2A expression (+34.2%) compared with control ($P=0,0021$). On the other hand, no differences were found in the expression of GluN2A after MK801 at 10 μM with respect to control ($P=0,072$); MEM treatment subsequently increased it (+28,9%) ($P=0,0042$).

No differences were found after the application of MK801 at 20 μM with respect to control ($P=0,068$) nor with the posterior administration of MEM ($P=0,091$). Moreover, no differences were found in the GluN2A expression after the application of MK801 at 10 ($P=0,071$) and 20 μM ($P=0,089$) (Figure 3A).

3.2.2 GluN2B

No differences were found after the administration of MEM compared with control ($P=0,27$). MK801 at 10 μM increased the GluN2B expression (+47,1%) with respect to control ($P=0,0003$), and the posterior administration of MEM reduced it (-37,3%) significantly ($P=0,0011$). MK801 at 20 μM induced an even higher increase (+59,6%) with respect to control ($P=0,0001$); MEM administration also reduced it (-52,9%) ($P=0,0026$). Significant differences were found in the GluN2B expression after the application of MK801 at 10 and 20 μM ($P=0,003$) (Figure 3A).

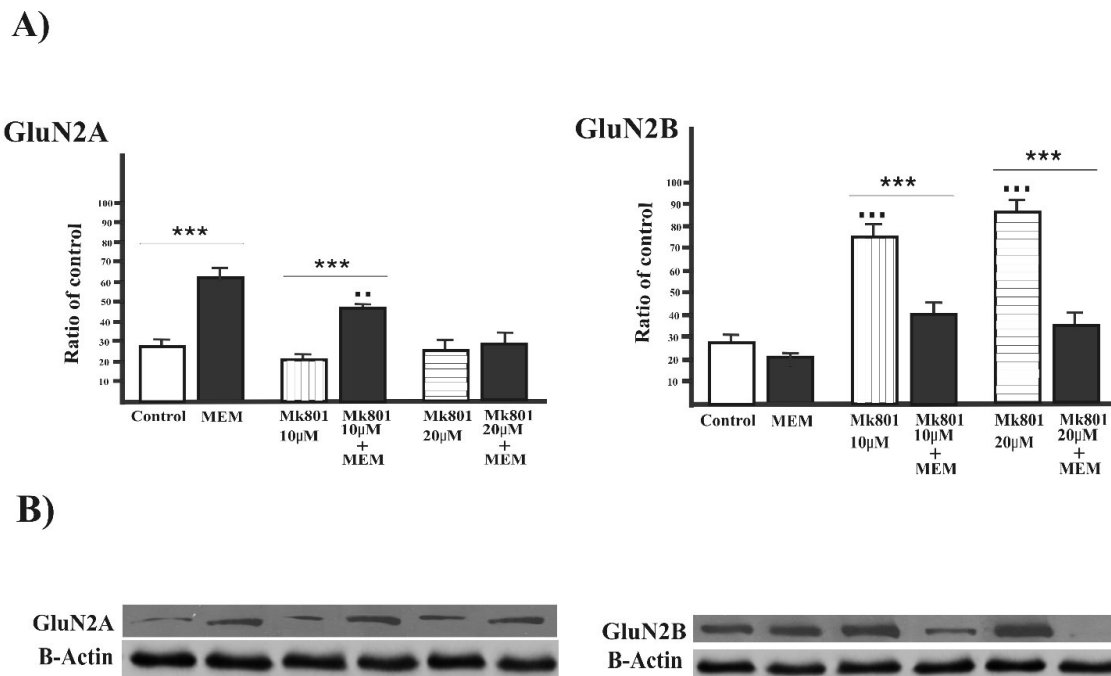


Figure 3. MK-801 and MEM have opposite effects on NMDAR subunit expression *in vitro*

A) MEM induced an increase of GluN2A subunit expression that was dose-dependently reversed by MK-801. MK801 increased GluN2B subunit that was reversed by MEM. **B)** Representative western blots. $N=3$ experiments. Data are mean values \pm SD. *** $P<0.001$ between conditions. (two squares) $P<0.01$ and (three squares) $P<0.001$ for condition vs untreated control (non-parametric Kruskal-Wallis test followed by Dunn post-hoc test).

3.3 MEM promotes the expression of glutamatergic presynaptic components altered by MK801 at 11 DIV

We hypothesized that NMDAR subunit exchange may be accompanied by alterations to other elements of the synaptic machinery. Hence, we evaluated the presynaptic proteins VGLUT1, and VGLUT2, two members of the SNARE complex, synaptotagmin and Syntaxin-1, and the NMDAR interacting component PSD95. VGLUT1 and PSD95 were unaffected by

the different treatments (Figure 4). However, MEM caused a significant reduction of VGLUT2 ($P=0,02$). MEM had no effect on VGLUT2 expression when co-incubated with MK-801 $10\mu M$ ($P=0,5$). Moreover, while MK801 $20\mu M$ significantly reduced VGLUT2 expression compared to the untreated control, co-incubation with both stimuli induced a notable increase of VGLUT2 expression compared to the MK-801 ($P=0,0003$) and untreated control conditions ($P=0,0006$) (Figure 4).

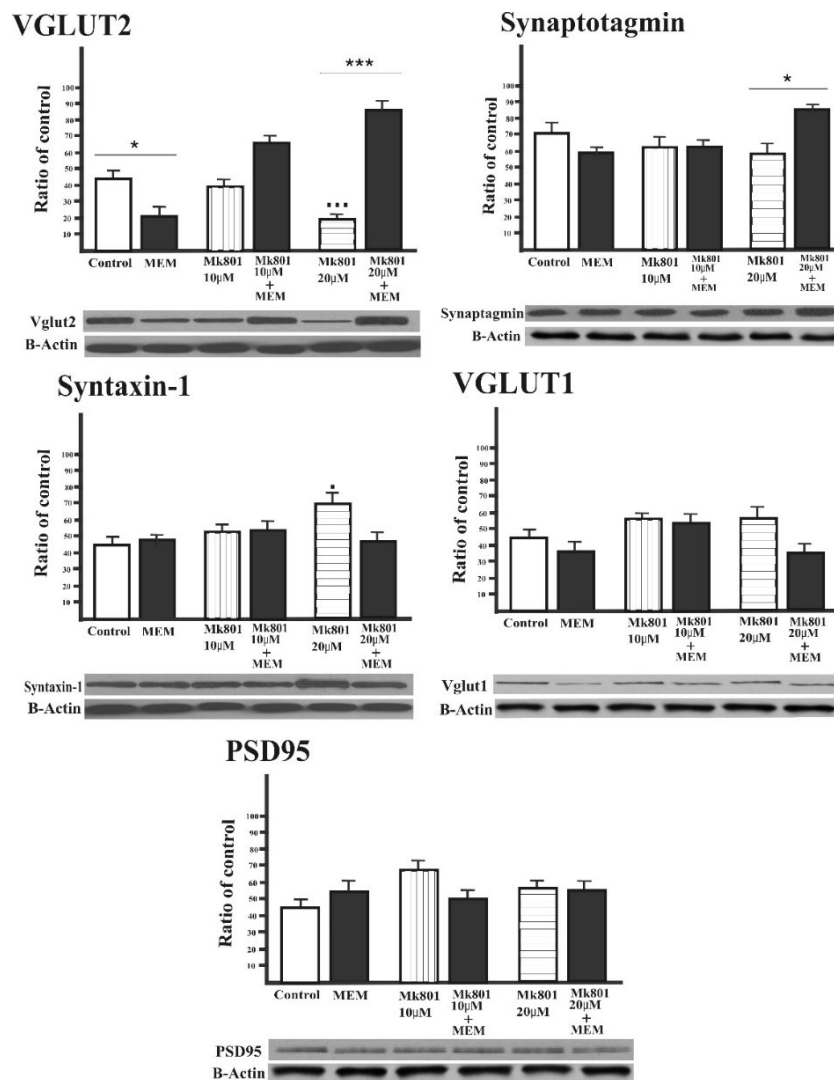


Figure 4. MK-801 treatment alters presynaptic components of the glutamatergic synapse

VGLUT1 and PSD95 were not affected by the different treatments. VGLUT2 expression was significantly reduced compared to the untreated control by MK-801 $20\mu M$. MEM reduced VGLUT2 compared to the untreated control but reversed the effect of MK-801 $20\mu M$. Syntaxin 1 was significantly increased only when MK-801 was applied. Synaptotagmin was significantly increased only under MK-801 and MEM co-incubation. * $P<0.05$, *** $P<0.005$ for differences between groups; one black point $P<0.05$, three black points $P<0.005$ for differences with Control group (non-parametric Kruskal-Wallis test followed by Dunn post-hoc test).

Also, this protein had an inverse correlation with GluN2A in the MEM group ($P = -0,031$), and a positive correlation with GluN2A in the MK801 20 μ M group ($P = 0,039$) (Table 1). Importantly, Syntaxin-1 was upregulated only by MK801 20 μ M ($P = 0,04$), while co-incubation with MEM prevented this effect ($P = 0,45$) (Figure 4). Synaptotagmin was significantly increased by MEM only when co-incubated with MK-801 20 μ M ($P = 0,03$) (Figure 4), and it had a positive correlation with GluN2B in the control group ($P = -0,029$) (Table 1). PSD95 had a positive correlation with GluN2A in the MEM group ($P = 0,048$); Synaptotagmin had an inverse correlation with GluN2B in the control group ($P = -0,029$) (Table 1).

Discussion

The NMDAR GluN2B and GluN2A subunit switch is a key event in the formation of mature synapses, which has been proposed to be disrupted in SCZ.¹⁹ The glutamatergic hypofunction may be causative of long-lasting events affecting cortical connectivity, leading to SCZ-like behavior in rodents.¹⁰ Alteration of normal subunit switch causes abnormalities in synaptic metaplasticity, including long-term depression and potentiation,⁷ which have also been identified in the schizophrenic brain.²⁰ Here we have used in vitro stimulation with MK-801 to model the glutamatergic hypofunction, which is supposed to be the premorbid stage of SCZ.¹⁰ Our results showed that at 11 DIV glutamatergic hypofunction caused an increase in GluN2B subunit expression together with a reduction of the presynaptic marker VGLUT2 and upregulation of Syntaxin-1. These effects induced by MK-801 were reversed by MEM, along with a mild upregulation of Synaptotagmin.

MK-801 has already been shown to modulate the expression of NMDAR subunits in the brain cortex.²¹ Both, MK801 and MEM have an affinity for NMDAR containing GluN2B subunits, which are highly expressed during the early stage of brain development.^{4,22} There was no alteration in absolute levels of GluN2A and PSD-95 by incubation with MK-801, whose interaction has been otherwise shown to be deficient in the schizophrenic brain.²³ Importantly, the upregulation of GluN2B subunits occurred in parallel to VGLUT2 upregulation while VGLUT1 remained unaltered. The relevance of this finding is substantiated by the observation of VGLUT2 upregulation, but the stability of VGLUT1 levels, observed in post-mortem samples from schizophrenic drug-naive patients.²⁴ On the other hand, deficits to phosphorylation of Syntaxin 1 have also been associated with schizophrenia.²⁵ Alterations to VGLUT and α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

Table 1. Correlations matrix between NR2A and NR2B and the synaptic protein

	NR2A	NR2B
CONTROL		
VGlut1	0,811	0,712
VGlut2	0,192	0,112
PSD95	0,329	0,711
Syntaxin-1	0,772	0,621
Synaptotagmin	0,45	-0,029
MEM		
VGlut1	0,992	0,081
VGlut2	-0,031	0,062
PSD95	0,048	0,099
Syntaxin-1	0,221	0,361
Synaptotagmin	0,401	0,078
MK801 (10MM)		
VGlut1	0,609	0,063
VGlut2	0,701	0,093
PSD95	0,06	0,129
Syntaxin-1	0,218	0,067
Synaptotagmin	0,072	0,213
MK801(10MM) + MEM		
VGlut1	0,228	0,91
VGlut2	0,054	0,188
PSD95	0,251	0,108
Syntaxin-1	0,061	0,083
Synaptotagmin	0,199	0,309
MK801(20MM)		
VGlut1	0,092	0,054
VGlut2	0,039	0,199
PSD95	0,991	0,081
Syntaxin-1	0,872	0,013
Synaptotagmin	0,901	0,072
MK801(20MM) + MEM		
VGlut1	0,085	0,791
VGlut2	0,681	0,078
PSD95	0,141	0,212
Syntaxin-1	0,091	0,064
Synaptotagmin	0,199	0,931

Significant correlations ($P < 0.05$) are expressed with underlined Spearman R correlation coefficient. Inverse correlations are denoted with a negative sign. Control group (CONTROL), Memantine group (MEM), MK801 at 10 μ M treatment group (MK801 (10 μ M)); MK801 at 10 μ M and posterior MEM treatment (MK801 (10 μ M) + MEM); MK801 at 20 μ M treatment group (MK801 (20 μ M)); MK801 at 20 μ M and posterior MEM treatment (MK801 (20 μ M) + MEM).

receptor (AMPA) correlated with abnormal hippocampal neuronal arborization *in vivo*, which was shown to be actively dependent *in vitro*.²⁶ Therefore, it could be speculated that the brain atrophy and abnormal electrophysiological activity previously reported on the model of maternal deprivation¹⁵ will be accompanied by decreased GluN2A, increased GluN2B expression, and compensatory effects on VGLUT2 and Syntaxin 1 levels, causing abnormalities in glutamate release, all of which could generate further structural abnormalities within the cortical and hippocampal regions.

The mechanisms by which MK-801 and MEM interact and influence these pre and postsynaptic events are not clear. The NMDAR activation requires the binding of glutamate and glycine together with voltage-dependent relief of magnesium block, resulting in membrane depolarization and calcium influx, which are critical in synaptic transmission and plasticity as well as in cellular mechanisms for learning and memory, elements affected in schizophrenia.²⁷ Both MK801 and MEM have an affinity for NMDAR containing GluN2B subunits.^{5,22} MK801 binds inside the vestibule of the ion channel of the receptor, preventing the flow of calcium and other ions, and blocking the pore in two symmetry-related postures, MEM, on the other hand, seems to block the pore primarily in a single position.¹³ Importantly, there are reduced levels of BDNF, GluN2A, and GluN2B sub-units in the hippocampus and the prefrontal cortex in the schizophrenic rats.^{28,29} In contrast, MEM was shown to increase BDNF mRNA in SIV-infected macaques³⁰ and also to reverse the loss of BDNF and TrkB mRNA in the prefrontal cortex.³¹ Accordingly, deficiencies of BDNF/TrkB signaling coincided with reduced hippocampal neuron arborization and abnormal VGLUT expression, which reduces the hippocampal neuroplasticity.²⁶

Conclusion

Our data suggests that MEM, which has recently been approved for the treatment of dementia,³³ could prevent the emergence of molecular abnormalities of SCZ, regulating pre and post-synaptic elements of the glutamatergic synapse. By understanding the different molecular stages of brain development, it will be possible to prevent the onset of neuropsychiatric disorders in people with a genetic predisposition using adequate treatment.

On the other hand, the present study has several limitations. A simple culture of hippocampal cells excludes the interactions of this group of neurons with the frontal cortex, a brain region highly involved in the development of SCZ. The use of MK801

as a preclinical model of schizophrenia has been questioned,³³ however, it is currently considered useful for reproducing symptoms and brain alterations characteristic of schizophrenia *in vivo*.³⁴ In this study, it was used as an *in vitro* model to reproduce the NMDAR hypofunction in the hippocampus of the schizophrenic brain, and later, MEM was used to reverse this state. Since both drugs act on the same receptor, this effect could be interpreted as a simple pharmacokinetics consequence in the NMDAR, rather than a solution to the molecular problem. Future research should involve NMDAR subunit knockout to evaluate the effects of MEM.

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