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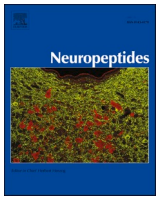

Cyclooxygenase-2 inhibition affects the ratio of GluN2A/GluN2B receptor subunits through interaction with mGluR5 in the mouse brain

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ABSTRACT

N-methyl-D-aspartic acid receptors (NMDARs) are the most studied receptors in mammalian brains. Their role in depression, cognition, schizophrenia, learning and memorization, Alzheimer's disease, and more is well documented. In the search for new drug candidates in depression, intensive studies have been conducted. Compounds that act by influencing NMDARs have been particularly intensively investigated following the success of ketamine in clinics. Unfortunately, the side effects associated with ketamine do not allow it to be useful in all cases. Therefore, it is important to learn about new unknown mechanisms related to NMDAR activation and study the impact of changes in the excitatory synapse environment on this receptor. Both direct and intermediary influence on NMDARs via mGluRs and COX-2 are effective. Our prior studies showed that both mGluRs ligands and COX-2 inhibitors are potent in depression-like and cognitive studies through mutual interactions. The side effects associated with imipramine administration, e.g., memory impairment, were improved when inhibiting COX-2. Therefore, this study is a trial that involves searching for modifications in NMDARs in mouse brains after prolonged treatment with MTEP (mGluR5 antagonist), NS398 (COX-2 inhibitor), or imipramine (tricyclic antidepressant). The prefrontal cortex (PFC) and hippocampus (HC) were selected for PCR and Western blot analyses. Altered expression of *Gin2a* or *Grin2b* genes after treatment was found. The observed effects were more potent when COX-2 was inhibited. The finding described here may be vital when searching for new drugs acting via NMDARs without the side effects related to cognition.

1. Introduction

One of the leading hypotheses of depression suggests that its pathomechanism is associated with over-activation of *N*-methyl-D-aspartic acid receptors (NMDARs), excessive release of glutamate (Glu), and the induction of a depressive phenotype (Mardsen, 2021). Released Glu acts on glutamate ionotropic (NMDARs; α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid AMPARs; or Kainate) and metabotropic (mGluRs) receptors. The mGluRs form a heterogeneous group of receptors that are linked to the secondary messenger pathways via the G protein, and their activation influences behavioral changes (Mardsen, 2021). Interestingly, mGluR5 and NMDA receptors were found in proximity at the postsynaptic cleft (Szewczyk et al., 2012), and the Homer family of proteins can functionally link mGlu5 receptors with inositol trisphosphate *receptors (*IP3Rs) and with Shank proteins functionally existing as part of NMDA receptor-associated PSD-95 complex; thus, one can hypothesize mutual regulation (Stachowicz, 2019). NMDAR activation (e.g., by stress) opens a non-selective ion channel for Ca^{2+} and Na^{+} ions that can enter the postsynaptic neuron (Mardsen, 2021). Secondly, NMDARs can be activated by voltage-dependent mechanisms, depolarization, Mg^{2+} release, and channel opening (Mardsen, 2021). The synaptically or extrasynaptically located NMDAR subunits are influenced by physiological or pathological stress (Stark and Bazan, 2011). Synaptic NMDARs have a bidirectional effect on the functionality of neurons, with low-level activation that induces long-term depression (LTD) and high-level activation that induces long-term potentiation (LTP) (Mardsen, 2021). Extrasynaptic NMDARs are associated with LTD-inducing neuronal failure (Mardsen, 2021). Xu et al. (2009) found prior activity history to be an inducer of bidirectional changes in the NMDAR (GluN2A/GluN2B) subunits ratio. However, the mechanism of reciprocal regulation between the NMDA receptor and mGluR5 in the behavioral response is not known. It is reasonable to

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assume that there is an extrinsic factor that regulates the reciprocal interaction between these receptors in stress. Given the research, this factor could be an enzyme that controls lipid metabolism in cells by affecting arachidonic acid (AA) levels, that is, cyclooxygenase-2 (COX-2) (Stachowicz, 2021). Stark and Bazan (2011) documented lipid peroxidation of AA operating as a modulator of network activity history. Synaptic NMDARs caused enhancement of neuronal COX-2 expression, but prolonged synaptic activity suppressed its activity, acting through AA. At the same time, decreased COX-2 expression mediated by extrasynaptic NMDAR challenge was found (Stark and Bazan, 2011). Xu et al. (2009) observed that pharmacologically manipulated GluN2A/GluN2B ratio can change the subsequent LTP/LTD threshold. All of these modifications have the potential to change depressive behavior or cognition (Adell, 2020).

NMDARs consist of various combinations of GluN1, GluN2 (A-D), and GluN3 (A/B) subunits (Mardsen, 2021). Their number, localization and subunit composition are strictly regulated and differ in a cell- and synapse-specific manner (Sanz-Clemente et al., 2013). Studies on depression and cognition have shown that GluN1 and GluN2 are present in neurons as a heterotetrameric combination and are linked to depression (Mardsen, 2021). GluN2A and GluN2B subunits are highly expressed in the cortex and hippocampus (HC), modulating the properties of endogenous NMDARs (Sanz-Clemente et al., 2013). NMDARs mainly occur postsynaptically but are also observed extrasynaptically (Adell, 2020). Activation of synaptic NMDARs promotes survival of synapses and cells, while overactivation of extrasynaptic NMDARs is associated with excessive Glu release and cell death (Adell, 2020). Histopathological data have shown that GluN2A subunits are primarily located at the synapse, while GluN2B are primarily localized extrasynaptically (Adell, 2020). Receptors mainly containing the GluN2A subunit may contribute to improved synaptic plasticity, while selective GluN2B antagonists may exhibit neuroprotective properties (Adell, 2020). The composition of NMDA receptor synaptic subunits changes from dominant GluN2B to GluN2A during synaptic maturation and in response to activity and stress (Sanz-Clemente et al., 2013). It was shown that the inhibition of NMDARs was connected with a decrease in extrasynaptically localized GluN2B subunits when using selective serotonin reuptake inhibitors (SSRIs) (Mardsen, 2021). Memantine and ketamine (NMDAR antagonists) act via desensitization (Glasgow et al., 2017), while ligands of mGluRs induce changes in NMDAR subunit composition (Sanz-Clemente et al., 2013). The proportion and composition of NMDAR subunits are important factors influencing mental health, cognition, memory, and anxiety (Mardsen, 2021). Human postmortem studies have found reduced levels of GluN2A and GluN2B subunits in the prefrontal cortex (PFC), perirhinal cortex, and increased levels of GluN2A subunits in the lateral amygdala in major depression (Adell, 2020). Furthermore, higher expression levels of the NMDAR subunit 2B (Grin2b) gene in the locus coeruleus of depressed patients was detected (Adell, 2020). Grin2b has been postulated as a biomarker of suicide, and polymorphism of Grin2b has been postulated to predict treatment-resistant depression (Adell, 2020). Chronic corticosterone administration was potent in increasing Grin2a and Grin2b mRNAs in the HC (Adell, 2020). Inactivation of GluN2A subunit has been shown to possess antidepressant-like effects in mice (Adell, 2020). Furthermore, GluN2B-NMDARs regulate the activation state of the protein degradation-modulating memory liability, while GluN2A-NMDARs promote cyclic AMP response element-binding protein (CREB) phosphorylation and LTP involved in memory re-encoding and maintenance (Radiske et al., 2021). However, the way in which altering the composition of NMDAR subunits affects mood parameters by interfering with memory has not been clarified, nor have the intracellular mechanisms that induce these changes been elucidated.

Here, we used MTEP, imipramine, and NS398, separately or in combination for seven and fourteen days, and verified the levels of the NMDAR subunits using PCR (Grin2a/Grin2b) and Western blot (GluN2A/GluN2B) techniques. We found a significant effect of COX-2 inhibition on changes in NMDAR subunit composition induced by the mGluR5 antagonist or imipramine. This discovery could be crucial in the search for new treatments for depression without the side effects of clinically used antidepressants.

2. Materials and methods

2.1. Animals and housing

The experiments were performed on group-housed male C57BL/6 J mice (8–10 weeks old/ 26–32 g). The animals were kept under guideline conditions (temperature of 21 ± 1 °C, relative humidity of 50 ± 5%). Each group included ten animals. Food and water were freely available. Experiments were performed during the light period (8:00–18:00). All procedures were conducted according to the guidelines of the National Institutes of Health Animal Care and Use Committee and were approved by the Ethics Committee of the Institute of Pharmacology, Polish Academy of Sciences in Krakow (Approval Number: 1099 and 178/ 2017).

2.2. Drug treatment

The following drugs were used: N-[2-(Cyclohexyloxy)-4-nitrophenyl]methanesulfonamide (NS398; 3 mg/kg; Abcam Biochemicals, UK); 3-[(2-methyl-1,3-tiazol-4-yl)ethynyl]-pyridine (MTEP; 1 mg/kg; Tocris Cookson, Bristol, UK), imipramine (Imipramine hydrochloride; 10 mg/kg; Sigma-Aldrich, Germany) and were dissolved according to the manufacturer's instructions. Drugs were administered in a volume of 10 ml. NS398 was dissolved in 10% DMSO, MTEP was used as 1% aqueous solution of Tween 80, and imipramine in H_2O. Vehicle group injections involved administering 10% DMSO. All compounds were injected intraperitoneal (*i.p.*) once daily (before 11:00), for 7 or 14 consecutive days.

2.3. Tissue collection

Twenty-four hours after the last injection, the animals were decapitated by interrupting the spinal cord and their brains were immediately removed. Then, the PFC and HC were dissected according to the mouse brain atlas (Paxinos and Franklin, 2001), frozen on dry ice, and stored at −80 °C. The PFC was taken by cutting the anterior part of the forebrain at the level of Bregma 2.20 mm. Olfactory bulbs and the anterior striatum were cut off. Therefore, the tissue taken for analysis contained most of the PFC. Subsequently, the brain was cut into two hemispheres along the sagittal line. Then, the whole HC was taken out from each hemisphere.

2.4. Western blot analysis

A Western blot procedure was used for detection and semi-quantitative analysis of GluN2A and GluN2B protein. Briefly, the tissues were homogenized in 2% aqueous sodium dodecyl sulfate (SDS; Bio-Rad; Frankfurt, Germany) solution, denatured for 10 min at 95 °C, and centrifuged for 5 min at 9000 x*g* at 4 °C. In the obtained supernatant total protein, concentration was determined using the bicinchoninic acid (BCA) method (Thermo Fisher Scientific; Rockfold, IL, USA). The samples containing 20 μg of total protein were fractionated by SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad; Frankfurt, Germany). Non-specific binding was blocked for 30 min at 4 °C with 1% blocking solution (BM Chemiluminescence Western Blotting Kit; Mouse/Rabbit; Roche; Basel, Switzerland). Next, the membranes were incubated overnight at 4 °C with the following primary antibodies: rabbit polyclonal anti-GluN2A and or anti-GluN2B antibodies (diluted 1:1000 Abcam, Cambridge, UK; cat. ab14596 and ab65783, respectively). After that, the membranes were washed in Tris-buffered saline with 1% addition of Tween 20 (TBS-T) and incubated for 30 min at room

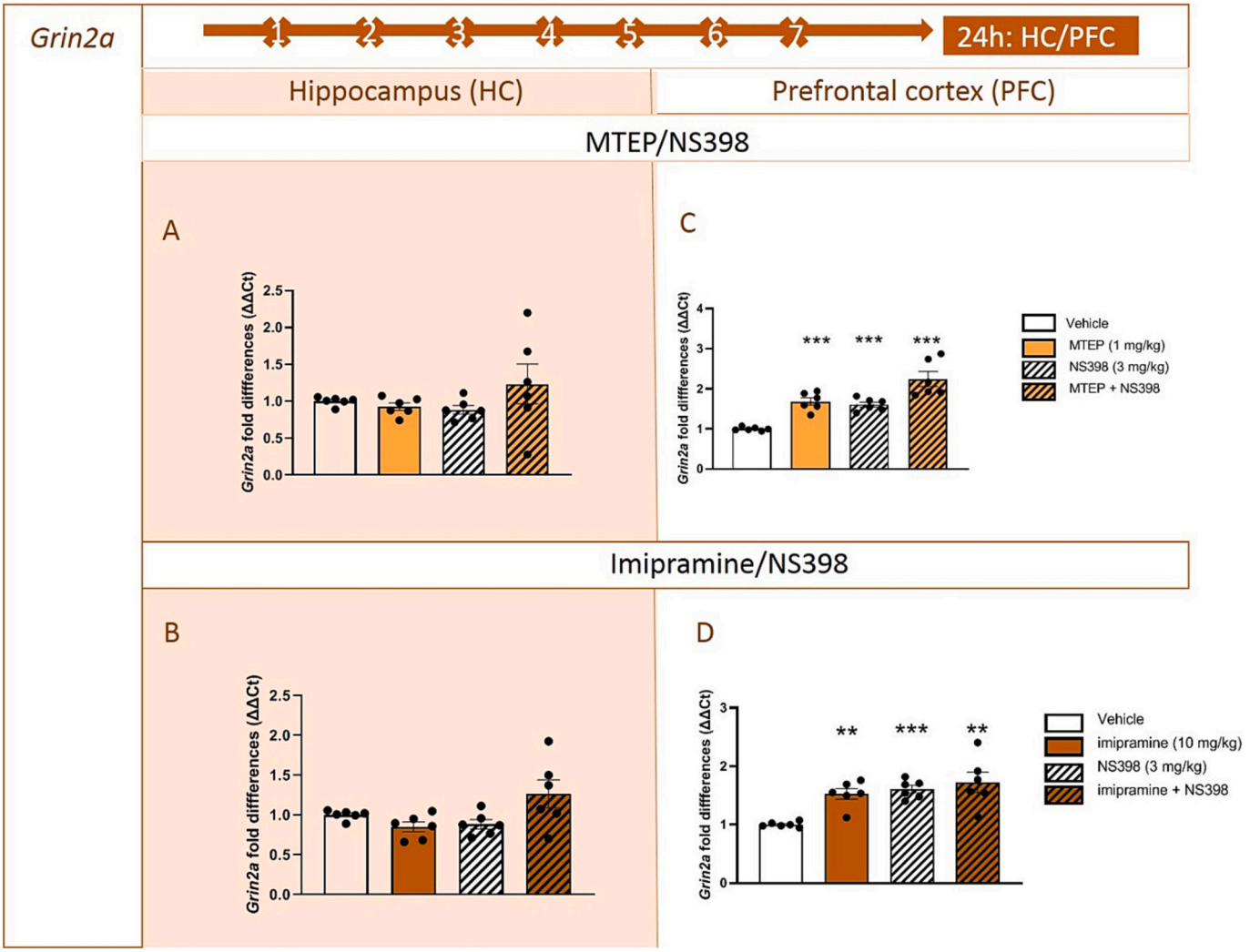

Fig. 1. Effect of treatment with MTEP (1 mg/kg) (top panel), imipramine (10 mg/kg) (bottom panel), and NS398 (3 mg/kg) for seven days, on *Grin2a* expression in the PFC (right panel) or HC (left panel) of C57Bl/6 J mice (PCR). One-way ANOVA, followed by Dunnett's, $n = 6$. The p value given is for the whole Dunnett's analysis while the stars in the figure refer to the post-multicomparison analysis against vehicle. Values are expressed as the mean ± S.E.M., $^{**}P < 0.01$, $^{***}P < 0.001$ vs. vehicle group. The same values for vehicle and NS398 are repeated in panels A, B, C, and D.

temperature with a secondary goat HRP-conjugated anti-rabbit/mouse IgG (diluted 1:25,000; Roche; Basel, Switzerland). After washing in TBS-T, the signal from the tested proteins was detected using enhanced chemiluminescence reaction (BM Chemiluminescence Western Blotting Kit; Roche; Basel, Switzerland) and Fuji-Las 1000 with Fuji Image Gauge v. 4.0 software. As a control for transfer and loading, β-actin was assessed on each blot. For this, primary mouse monoclonal anti-β-actin antibody (diluted 1:7000; Sigma-Aldrich; Darmstadt, Germany) and secondary goat HRP-conjugated anti-rabbit/mouse IgG (diluted 1:25,000; Roche; Basel, Switzerland) were used. The final results represent the ratio of the optical density of a particular protein to the optical density of β-actin present in the same sample.

2.5. Real-time PCR analysis

A real-time PCR (qRT-PCR) procedure was used for measurement of *Grin2a* and *Grin2b* gene expression, i.e., the genes encoding the GluN2A and GluN2B protein, respectively. Briefly, total cellular RNA was extracted from tissue samples by TRI Reagent (Sigma-Aldrich; Saint Louis, MO, USA) according to the manufacturer's instructions. RNA integrity was verified by electrophoresis in denaturing agarose gel and observation under UV light, while its concentration and purity were evaluated spectrophotometrically using Nanodrop 2000 (Thermo Fisher Scientific, Rockfold, IL, USA). First, 1 μg of total RNA from each sample was incubated with DNase I (Sigma-Aldrich; Saint Louis, MO, USA) and, next, reverse-transcribed into complementary DNA using a High Capacity cDNA Reverse Transcription Kit with RNase inhibitor and random hexamers (Life Technologies; Paisley, UK). The mRNA levels were determined using the following pre-designed TaqMan Gene Expression Assays (Life Technologies, Carlsbad, CA, USA): *Grin2a*—Mm00433802 (RefSeq: NM_008170.2); *Grin2b*—Mm00433820_m1 (RefSeq: NM_008171.3). The *Gapdh* (glyceraldehyde-3-phosphate dehydrogenase; Mm99999915_g1; RefSeq: NM_008084.3) was used as endogenous control. qRT-PCR was carried out on 96-well optical plates in a final volume of 10 μl using a CFX96 Real-Time System and C1000 Touch Thermal Cycler (Bio-Rad, USA). Reaction mixtures included: 3 μl of cDNA sample (diluted 1:20), RNase-free water (Sigma-Aldrich), TaqMan Gene Expression Master Mix (Life Technologies, Carlsbad, CA, USA), and appropriate TaqMan Gene Expression Assay (Life Technologies, Carlsbad, CA, USA). qRT-PCR was carried out under the following conditions: 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Each cDNA sample was run in triplicate and no template control wells were included on each plate to check for contamination. The results for the examined genes were normalized against *Gapdh*. The

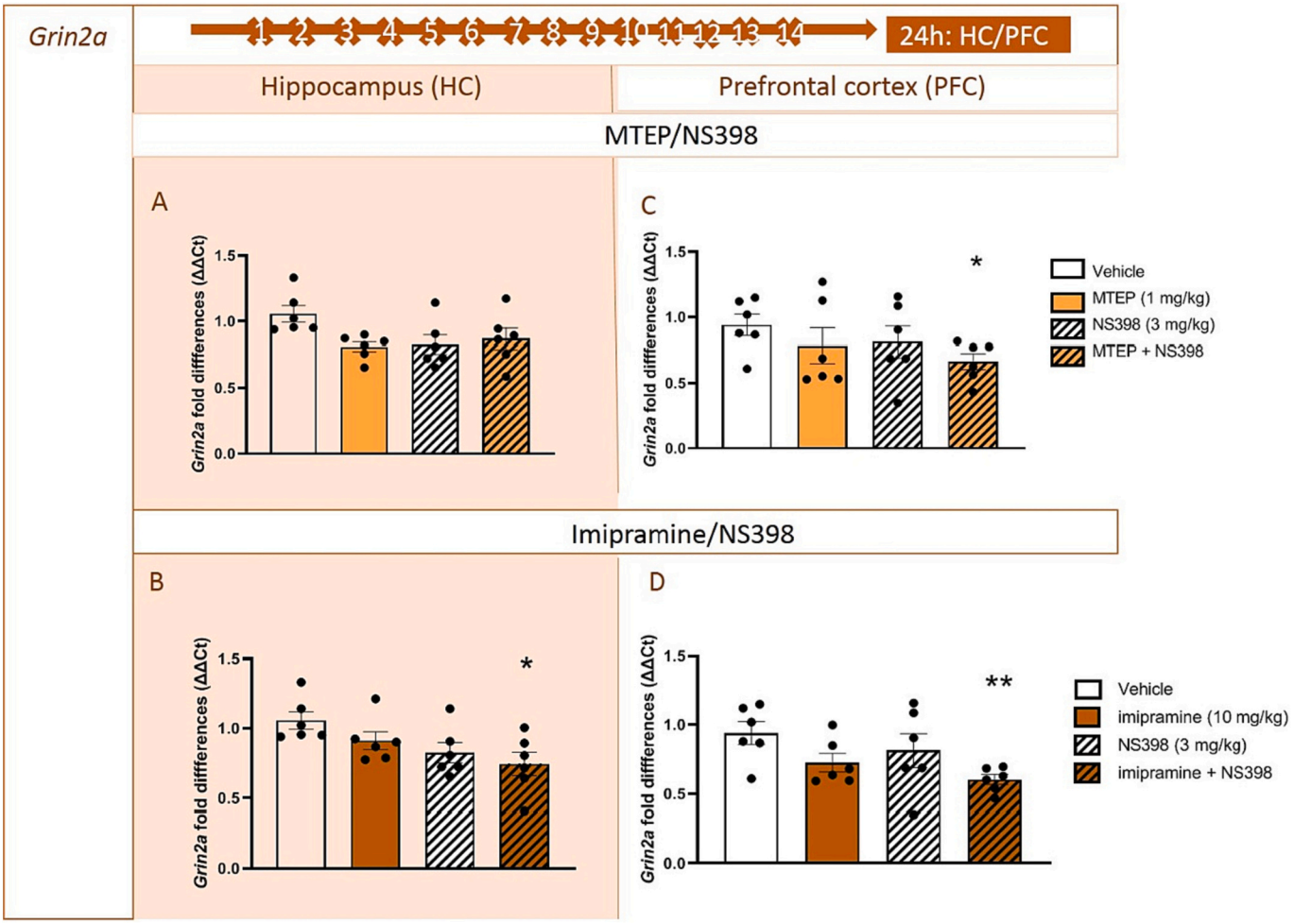

Fig. 2. Effect of treatment with MTEP (1 mg/kg) (top panel), imipramine (10 mg/kg) (bottom panel), and NS398 (3 mg/kg) for fourteen days on *Grin2a* expression in the PFC (right panel) or HC (left panel) of C57Bl/6 J mice (PCR). One-way ANOVA, followed by Dunnett's, $n = 6$. The p value given is for the whole Dunnett's analysis, while the stars in the figure refer to the post-multicomparison analysis against vehicle. Values are expressed as the mean ± S.E.M., $^{*}P < 0.05$, $^{**}P < 0.01$ vs. vehicle group. The same values for vehicle and NS398 are repeated in panels A, B, C, and D.

Delta–Delta Comparative Threshold method was used to quantify the fold change between the samples.

2.6. Statistical analysis

The obtained data were evaluated with one-way ANOVA, using the non-parametric Kruskal–Wallis method followed by multiple comparisons against the vehicle value, using GraphPad Prism software, ver. 8.0 (San Diego, CA, USA). The p value given is for the whole Dunnett's analysis (PCR) and Kruskal–Wallis, while the stars in the figures refer to the post-multicomparison analysis against vehicle. Furthermore, two-way ANOVA analysis (followed by Tukey) was used to determine whether there was a significant difference between the effect of different subunits and different brain areas, and interactions between groups of compounds. The obtained data are presented as the mean ± SEM, and $P < 0.05$ was considered significant.

3. Results

3.1. Long-term treatment for seven days with MTEP, NS398, or imipramine enhances Grin2a expression in the prefrontal cortex of mice

HC: Treatment for seven days with MTEP (1 mg/kg), NS398 (3 mg/kg), or imipramine (10 mg/kg) had no effect on *Grin2a* in the HC of C57Bl/6 J mice. The same co-treatment of tested compounds for seven days did not change the level of *Grin2a* gene in the HC. One-way ANOVA followed by Dunnett's showed the following results: [F(1,199, 5995) = 0.256; $P = 0.673$] (Fig. 1A) and [F(1,151, 5757) = 2.59; $P = 0.161$] (Fig. 1B).

PFC: Significant increase in *Grin2a* expression was found in the PFC of mice treated for seven days with MTEP (1 mg/kg), NS398 (3 mg/kg), imipramine (10 mg/kg), or a combination of compounds. One-way ANOVA followed by Dunnett's showed the following results: [F(1,988, 9,94) = 32,55; $P < 0.0001$] (Fig. 1C) and [F(1,370, 6850) = 11.03; $P = 0.0101$] (Fig. 1D).

3.2. Repeated treatment for fourteen days with both MTEP, NS398, or imipramine suppresses Grin2a expression in the prefrontal cortex and hippocampus of mice

HC: Treatment for fourteen days with MTEP (1 mg/kg), NS398 (3 mg/kg), or imipramine (10 mg/kg) resulted in no significant changes in *Grin2a* in the HC of C57Bl/6 J mice. Similar results were observed after co-treatment, however post-multicomparison test found significant decrease in imipramine+NS398 group (Fig. 2B). One-way ANOVA followed by Dunnett's showed the following results: [F(2,642, 13,21) = 2637; $P = 0.0981$] (Fig. 2A) and [F(1,830, 9150) = 3959; P = 0,0603] (Fig. 2B).

PFC: No significant changes in *Grin2a* expression was found in the PFC of mice treated for seven days with MTEP (1 mg/kg), NS398 (3 mg/

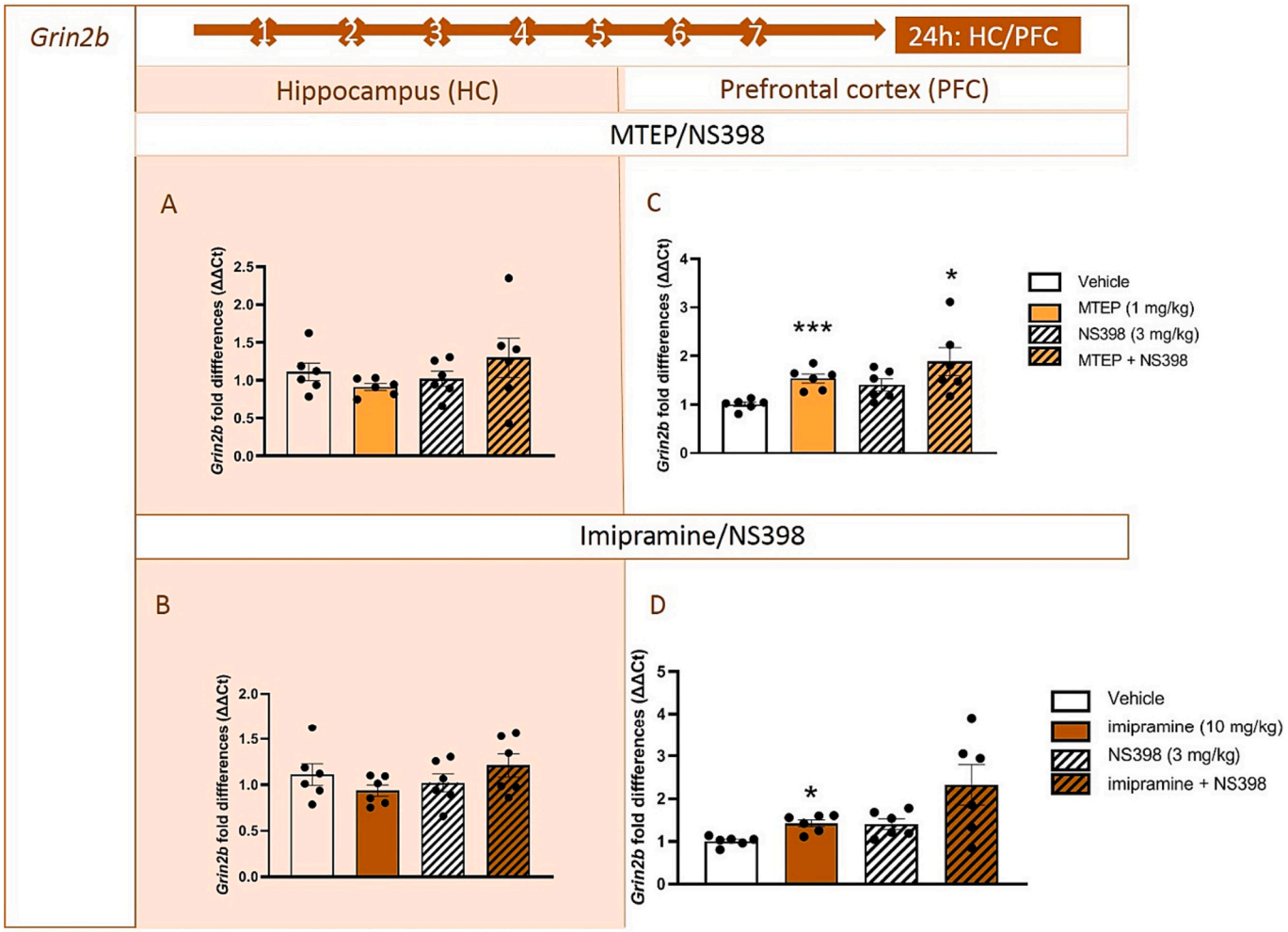

Fig. 3. Effect of treatment with MTEP (1 mg/kg) (top panel), imipramine (10 mg/kg) (bottom panel), and NS398 (3 mg/kg) for seven days, on *Grin2b* expression in the PFC (right panel) or HC (left panel) of C57Bl/6 J mice (PCR). One-way ANOVA, followed by Dunnett's, $n = 6$. The p value given is for the whole Dunnett's analysis, while the stars in the figure refer to the post-multicomparison analysis against vehicle. Values are expressed as the mean ± S.E.M., $^{*}P < 0.05$, $^{***}P < 0.001$ vs. vehicle group. The same values for vehicle and NS398 are repeated in panels A, B, C, and D.

kg), imipramine (10 mg/kg), or a combination of compounds. One-way ANOVA followed by Dunnett's showed the following results: [F(1,779, 8897) = 1.150; $P = 0.3526$] (Fig. 2C) and [F(1,395, 6975) = 2933; $P = 0.1263$] (Fig. 2D). There was a significant decrease in Grin2a in the MTEP + NS398 group (Fig. 2C), and in the imipramine group, imipramine + NS398 group (Fig. 2D), as showed by post-multicomparison test.

3.3. Long-term treatment for seven days with MTEP, NS398, or imipramine enhances Grin2b expression in the prefrontal cortex of mice

HC: Treatment for seven days with MTEP (1 mg/kg), NS398 (3 mg/kg), or imipramine (10 mg/kg) had no effect on *Grin2b* in the HC of C57Bl/6 J mice. The same co-treatment of tested compounds for seven days did not change the level of *Grin2b* gene in the HC. One-way ANOVA followed by Dunnett's showed the following results: [F(1,439, 7194) = 0.5738; $P = 0.5336$] (Fig. 3A) and [F(1,654, 8272) = 1082; $P = 0.3687$] (Fig. 3B).

PFC: A significant increase in *Grin2b* expression was found in the PFC of mice treated for seven days with MTEP (1 mg/kg), imipramine (10 mg/kg), and MTEP+NS398. One-way ANOVA followed by Dunnett's showed the following results: [F(1,914, 9570) = 5970; $P = 0.0216$] (Fig. 3C) and [F(1,269, 6346) = 4399; $P = 0.0734$] (Fig. 3D).

3.4. Repeated treatment for fourteen days with MTEP, NS398, or imipramine suppresses Grin2b expression in the prefrontal cortex and hippocampus of mice

HC: Treatment for fourteen days with MTEP (1 mg/kg), NS398 (3 mg/kg), or imipramine (10 mg/kg) resulted in a significant decrease in *Grin2b* in the HC of C57Bl/6 J mice. Similar results were observed after co-treatment. One-way ANOVA followed by Dunnett's showed following results: [F(2,051, 10,25) = 5.389; $P = 0.0246$] (Fig. 4A) and [F(1,940, 9702) = 5.325; $P = 0.0282$] (Fig. 4B).

PFC: A significant decrease in *Grin2b* expression was found in the PFC of mice treated for fourteen days with imipramine+NS398 (post-multicomparison). One-way ANOVA followed by Dunnett's showed the following results: [F(1,832, 9162) = 1.792; $P = 0.2204$] (Fig. 4C) and [F (1,556, 7778) = 3.053; $P = 0.1114$] (Fig. 4D).

3.5. Long-term treatment for seven days with NS398 or imipramine but not MTEP enhances GluN2A in the hippocampus of mice

HC: Treatment for seven days with MTEP (1 mg/kg) or NS398 (3 mg/kg) had no effect on GluN2A in the HC of C57Bl/6 J mice. Imipramine administered for seven days resulted in increased GluN2A in the HC. One-way ANOVA followed by Kruskal–Wallis showed the following results: [F(4,36) = 12.61; $P = 0.006$] (Fig. 5B).

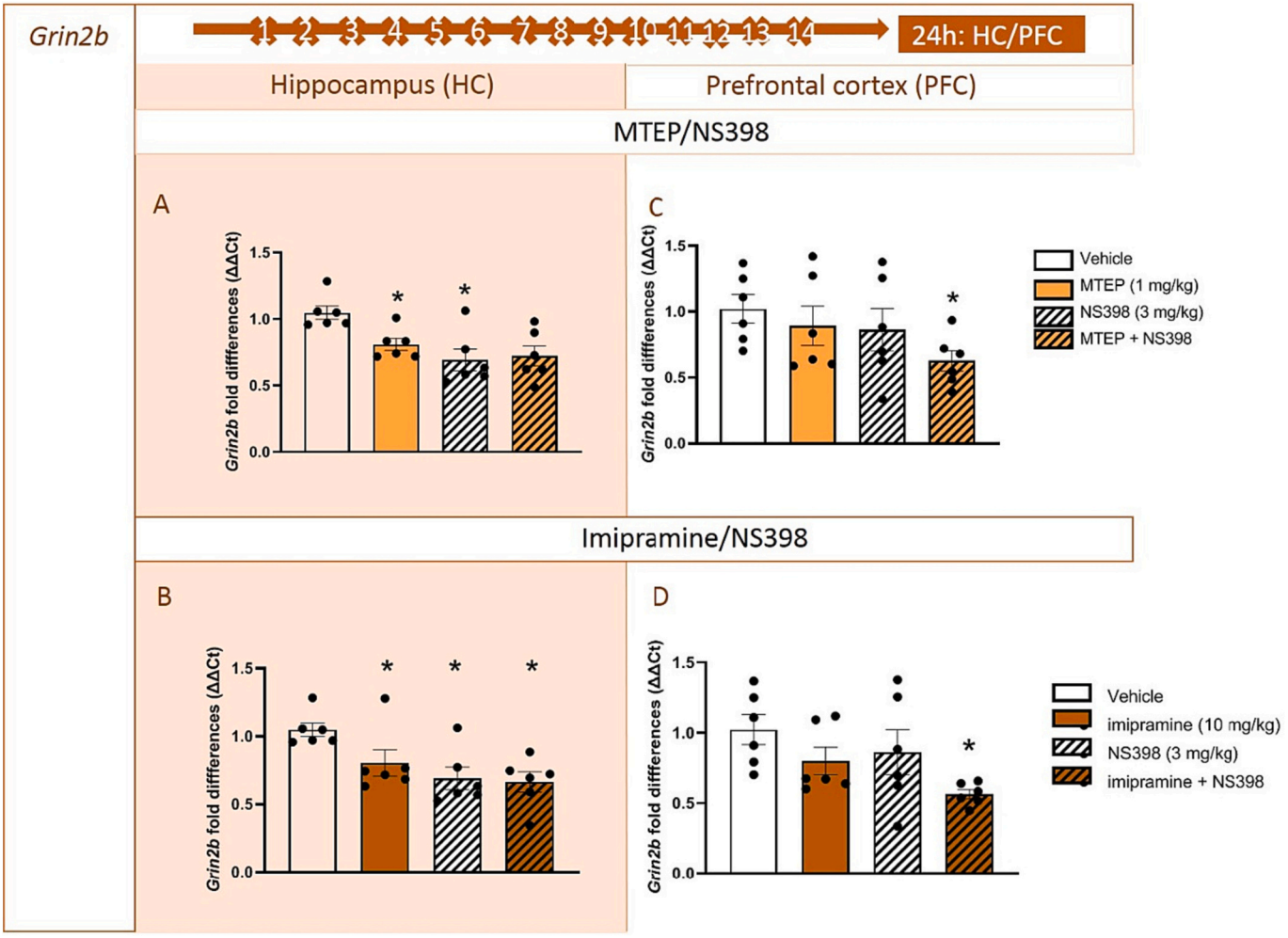

Fig. 4. Effect of treatment with MTEP (1 mg/kg) (top panel), imipramine (10 mg/kg) (bottom panel), or NS398 (3 mg/kg) for fourteen days on *Grin2b* expression in the PFC (right panel) or HC (left panel) of C57Bl/6 J mice (PCR). One-way ANOVA, followed by Dunnett's, n = 6. The *p* value given is for the whole Dunnett's analysis, while the stars in the figure refer to the post-multicomparison analysis against vehicle. Values are expressed as the mean ± S.E.M., $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. vehicle group. The same values for vehicle and NS398 are repeated in panels A, B, C, and D.

In the same time, the GluN2B protein level was not changed significantly (Fig. 6). One-way ANOVA followed by Kruskal–Wallis showed the following results: [F(4,34) = 10.19; $P = 0.0170$] (Fig. 6A); [F(4,37) = 6.99; $P = 0.007$] (Fig. 6B); [F(4,38) = 8.92; $P = 0.030$] (Fig. 6C); [F(4,38) = 4.99; $P = 0.172$] (Fig. 6D).

3.6. Repeated treatment for fourteen days with MTEP, imipramine or NS398 did not changed GluN2A or GluN2B in mice

After 14 days of the treatment, there were no changes in protein levels (GluN2A and GluN2B) in the PFC or HC of C57Bl/6 J mice (Fig. 7, Fig. 8 respectively). The one-way ANOVA showed significance only for the group of mice treated with MTEP and NS398 in HC, Fig. 7A: [F(3,18) = 3.234; $P = 0.047$]. There were no significances for other groups, Fig. 7B: [F(3,17) = 3.149; $P = 0.052$]; Fig. 7C: [F(3,20) = 0.5948; $P = 0.626$]; Fig. 7D: F(3,20) = 1.531; $P = 0.237$].

The one-way ANOVA found significance only for group of mice treated with MTEP and NS398 in HC, Fig. 8A: [F(3,25) = 3.113; $P = 0.044$]. There were no significances for other groups, Fig. 8B: [F(3,18) = 0.8970; $P = 0.4619$]; Fig. 8C: [F(3,24) = 2.624; $P = 0.074$]; Fig. 8D: F(3,20) = 1.001; $P = 0.413$].

One-way ANOVA did not detected significance in ratio GluN2A/GluN2B in HC or PFC.

3.7. Changes in Grin2a/Grin2b ratio in the HC or PFC of mice after prolonged treatment with MTEP, NS398, imipramine, or their combinations

HC: For the *Grin2a/Grin2b* ratio, seven days of administration of tested compounds had no effect on the parameter when considering MTEP (1 mg/kg) treatment: two-way ANOVA showed no effect on MTEP treatment [F(1,17) = 1.229; $P = 0.283$], no effect on NS398 treatment [F(1,17) = 1.291; $P = 0.272$], and no interaction [F(1,17) = 0.8135; $P = 0.380$] (Fig. 9A, left diagram). Treatment with imipramine with NS398 for seven days induced changes in the *Grin2a/Grin2b* ratio in the HC of mice, while significant decrease was detected. Two-way ANOVA showed no effect on imipramine [F(1,16) = 2.126; $P = 0.164$], no effect on NS398 [F(1,16) = 0.1068; $P = 0.748$], and significant interaction [F(1,16) = 12.41; $P = 0.003$] (Fig. 9B, left diagram), indicating that co-treatment with MTEP and NS398 induce increased *Grin2b* expression. Fourteen days of treatment with tested compounds induced more effects on the *Grin2a/Grin2b* ratio. Two-way ANOVA showed no effect of MTEP treatment [F(1,13) = 3.594; $P = 0.080$], significant effect of NS398 [F(1,13) = 37.42; $P < 0.0001$], and no interaction [F(1,13) = 0.218; $P = 0.648$] (Fig. 9A, right diagram). Significant interaction was detected in the group treated with imipramine and NS398 for fourteen days [F(1,16) = 6.262; $P = 0.024$] (Fig. 9B, right diagram). It was detected significant increase in *Grin2a/Grin2b* ratio in imipramine+NS398 group

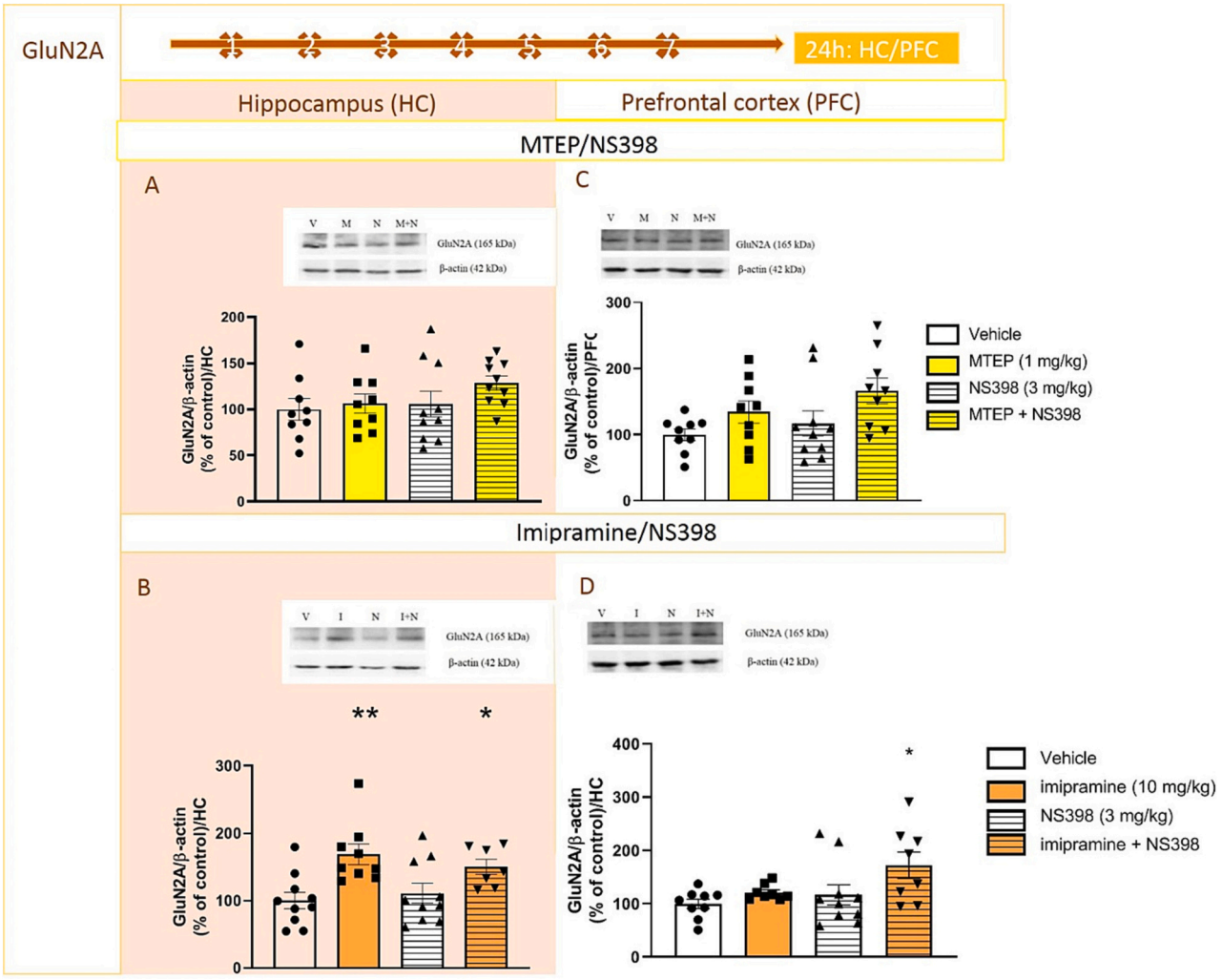

Fig. 5. Effect of treatment with MTEP (1 mg/kg) (top panel), imipramine (10 mg/kg) (bottom panel), or NS398 (3 mg/kg) for seven days on GluN2A in the PFC (right panel) or HC (left panel) of C57Bl/6 J mice (Western blot). Compounds were injected *i.p.* Values are expressed as the mean ± S.E.M. of normalized densitometry measurements from Western blots of GluN2A receptor compared with β-actin (n = 7–10); one-way ANOVA, followed by Kruskal–Wallis: *P < 0.05, **P < 0.01 vs. vehicle group. The p value given is for the whole Kruskal–Wallis analysis, while the stars in the figure refer to the post-multicomparison analysis against vehicle. The same values for vehicle and NS398 are repeated in panels A, B, C, and D.

after fourteen days of administration (Fig. 9B).

PFC: Seven days of treatment resulted in no significant changes in the *Grin2a/Grin2b* ratio in the PFC of mice, indicating that neither MTEP nor imipramine was effective in modulating the ratio (Fig. 9C and D, left diagrams). Two-way ANOVA found significant interaction in the imipramine+NS398 group [F(1,17) = 7.339; P = 0.015] (Fig. 9D, left diagram). Fourteen days of treatment resulted in changes in the *Grin2a/Grin2b* ratio, while two-way ANOVA detected significant interaction in the MTEP+NS398 group [F(1,13) = 5.064; P = 0.042] (Fig. 9C, right diagram).

Regarding changes between structures in the ratio of *Grin2a/Grin2b*, two-way ANOVA found significant increase only in the group treated with imipramine (10 mg/kg) for seven days: [F(1,14) = 4.685; P = 0.048] (Fig. 10). Significant interaction was detected between imipramine (10 mg/kg) and NS398 (3 mg/kg) [F(1,14) = 14,42; P = 0.002] (Fig. 10), indicating positive effects of NS398 treatment on the *Grin2a/Grin2b* ratio.

3.8. Changes in GluN2A/GluN2B ratio in the HC or PFC of mice after prolonged treatment with MTEP, NS398, imipramine, or their combinations

Regarding the ratio of protein levels of GluN2A/GluN2B, changes are similar to those observed in gene expression.

HC: Two-way ANOVA detected significant effects of treatment with MTEP on the GluN2A/GluN2B ratio in the HC of mice [F(1,20) = 4.702; P = 0.0424], no significant effect of NS398 [F(1,20) = 1.464; P = 0.240], and no significant interaction [F(1,20) = 1.669; P = 0.211] (Fig. 11A). Similar results were found in the imipramine group [F(1,19) = 8.707; P = 0.008] (Fig. 11B).

PFC: Two-way ANOVA detected significant effects of treatment with MTEP on the GluN2A/GluN2B ratio in the PFC of mice [F(1,23) = 4.531; P = 0.044] (Fig. 11C) and a significant effect of imipramine [F(1,22) = 5.479; P = 0.029] (Fig. 11D). The effect was influenced by NS398, while two-way ANOVA detected significant interaction [F(1,22) = 7.821; P = 0.011].

Regarding changes between structures in the ratio of GluN2A/GluN2B, a two-way ANOVA found no significant effect of imipramine [F(1,15) = 2.546; P = 0.131] (while increase in HC/PFC ratio was detected), a significant effect of NS398 [F(1,15) = 6.579; P = 0.022],

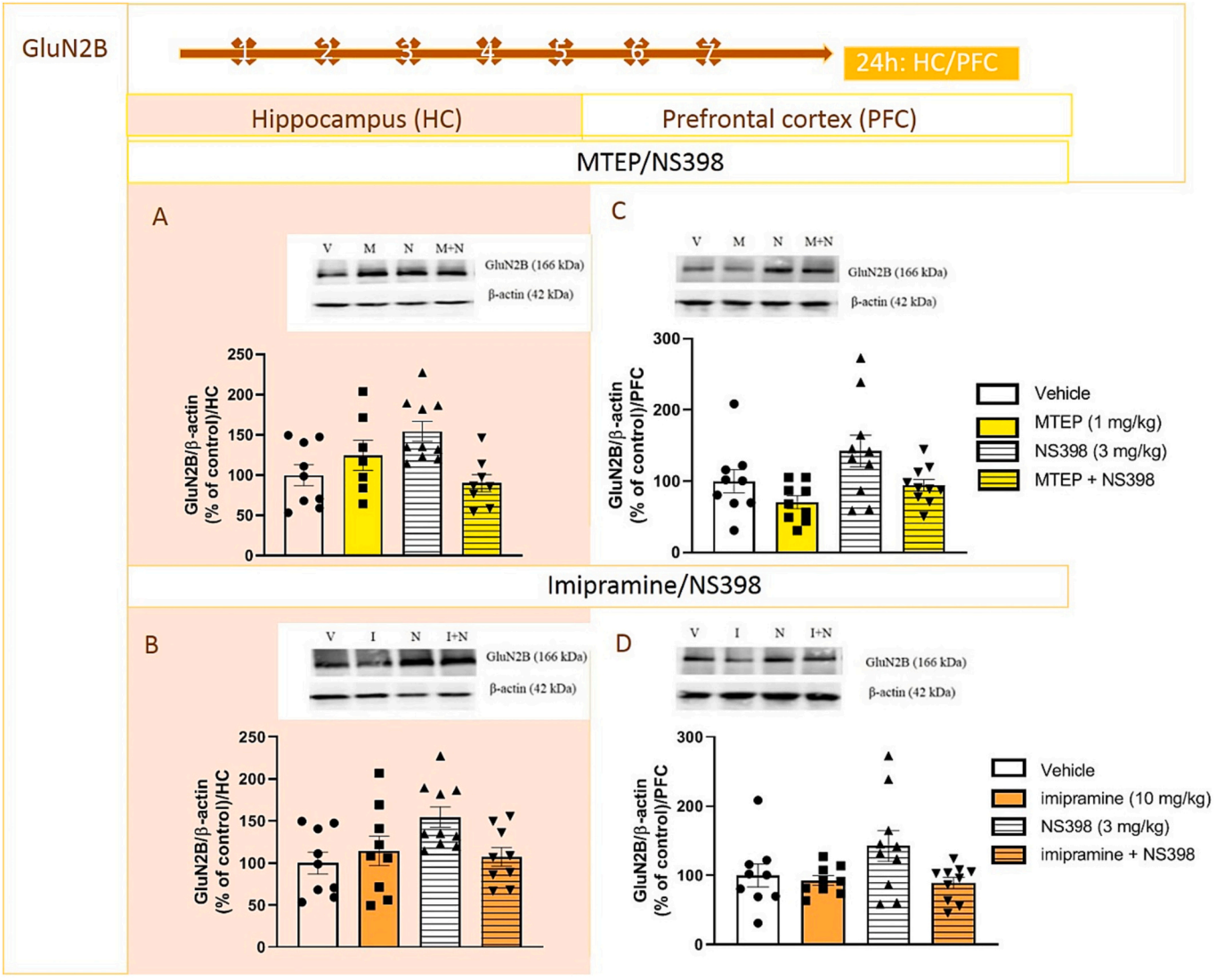

Fig. 6. Effect of treatment with MTEP (1 mg/kg) (top panel), imipramine (10 mg/kg) (bottom panel), or NS398 (3 mg/kg) for seven days on GluN2B in the PFC (right panel) or in HC (left panel) of C57Bl/6 J mice (Western blot). Compounds were injected *i.p.* Values are expressed as the mean ± S.E.M. of normalized densitometry measurements from Western blots of GluN2B receptor compared with β-actin (n = 7–10); one-way ANOVA, followed by Kruskal–Wallis. The p value given is for the whole Kruskal–Wallis analysis, while the stars in the figure refer to the post-multicomparison analysis against vehicle. The same values for vehicle and NS398 are repeated in panels A, B, C, and D.

and no interaction [$F(1,15) = 1.689$; $P = 0.213$] (Fig. 12), indicating that the most significant changes occur after application of NS398 to the PFC.

4. Discussion

Our results show that NMDARs are sensitive to MTEP (mGluR5 antagonist) and imipramine, as found in the subunit composition: GluN2A and GluN2B. At the same time, we noticed that the most significant changes in the composition of the NMDAR subunits could be observed in the long-term inhibition of COX-2. The changes we found were associated with altered *Grin2a* and *Grin2b* gene expression, and the proteins were not as sensitive to the compounds used as mRNA. When considering proportions of changes, *Grin2b* seems to be more sensitive to the treatment modification (see Fig. 1D and Fig. 3D). Furthermore, the most significant changes in protein ratio between GluN2A and GluN2B was detected in the PFC of mice.

Firstly, the results presented here are in agreement with the in vitro results described by Stark and Bazan (2011), indicating mutual regulation between NMDARs and COX-2, and are valuable because they are in vivo. Additionally, the results presented here are consistent with the results of other laboratories, where imipramine was potent in influencing NMDARs and rodent behavior via changes in NMDAR binding and modifications of gamma amino-butyric acid (GABA) levels in rodents' brains (Harvey et al., 2002). Following Stark and Bazan (2011), the increase in COX-2 is mediated by extrasynaptically localized subunits of NMDARs (Szewczyk et al., 2012), which correlates with our results.

As a consequence of modifications in subunit composition in NMDARs, changes in NMDAR-mediated excitatory postsynaptic currents (NMDA-EPSCs) affecting synaptic transmission are observed (Elmasri et al., 2022). The GluN2A to GluN2B switch mediates changes to NMDAR functions, altering the amount of calcium influx through the pore and the types of proteins interacting with the intracellular domain of the receptor, influencing LTP and LTD (Matta et al., 2011). LTD in the frontal cortex of C57Bl/6 J mice treated with imipramine (10 mg/kg) for seven days has been documented (Stachowicz et al., 2020). This effect was reversed by administration of NS398 (Stachowicz et al., 2020). Changes in the composition of NMDA receptor subunits are dynamic, so additional studies at other time points would need to be performed to

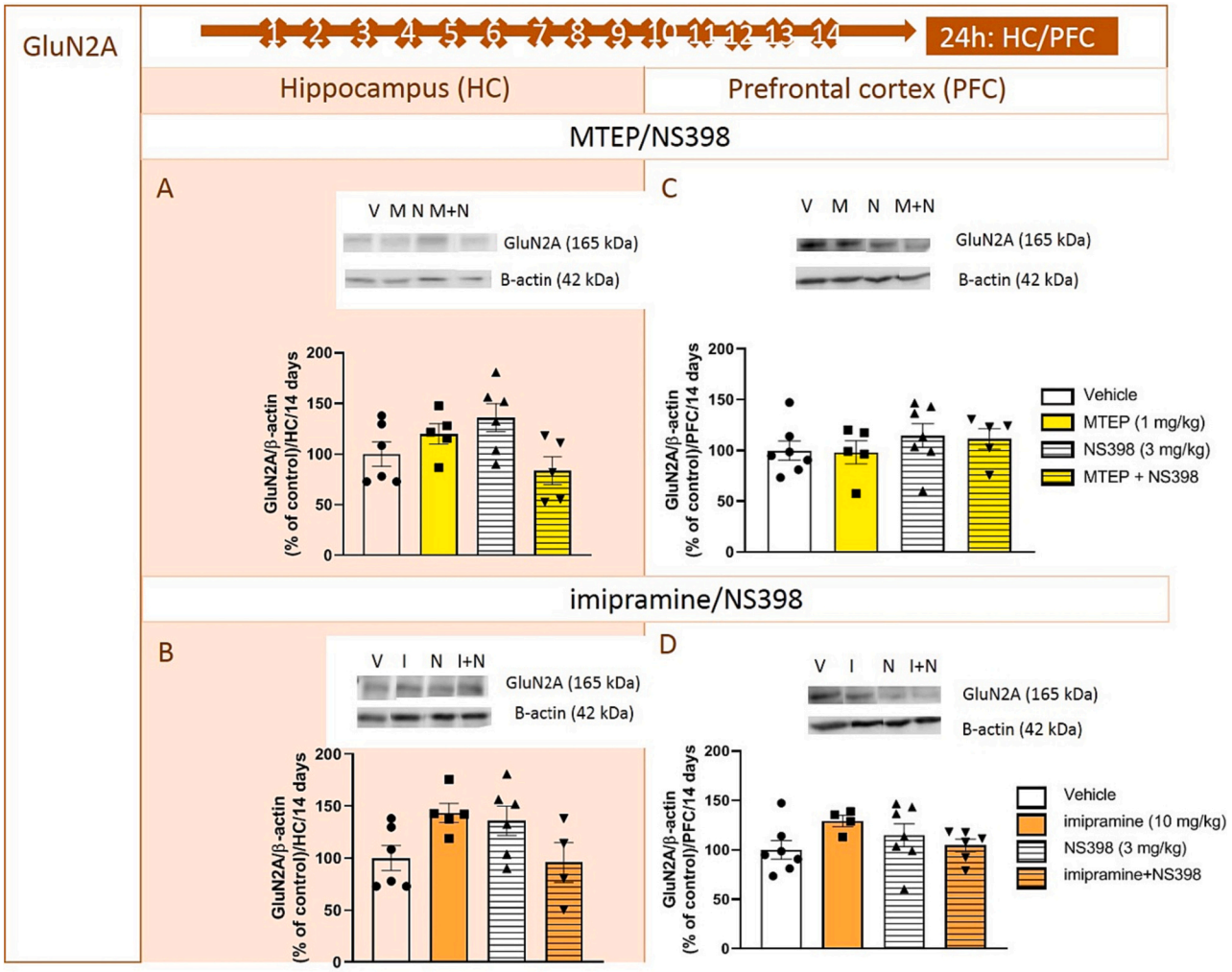

Fig. 7. Effect of treatment with MTEP (1 mg/kg), (top panel), imipramine (10 mg/kg), (bottom panel), and NS398 (3 mg/kg) for fourteen days, on GluN2A in the HC (left panel) or in the PFC (right panel) of C57Bl/6 J mice (Western blot). Compounds were injected *i.p.* Values are expressed as the means ± S.E.M. of normalized densitometry measurements from western blots of GluN2A receptor compared with β-actin ($n = 4–7$). The p value given is for the whole one Kruskal-Wallis analysis while the stars in the figures refer to the post-multicomparison analysis against vehicle. The same values for vehicle and NS398 are repeated in panels A, B, C and D. The membranes have been trimmed for the figure. The original membranes were added to the manuscript and sent to the publisher.

reach any conclusion. However, LTP and LTD in NMDA-mediated current are calcium-dependent, affecting neurotransmission through calcineurin-mediated mechanisms, so this mechanism may be suggested (Sachser et al., 2016). Secondly, the mGluR5 localizes in a synapse postsynaptically near NMDARs (Sanz-Clemente et al., 2013; Boyer et al., 1998). The way that mGluRs may connect with NMDARs is interaction via postsynaptic density protein 95 (PSD-95) (Sanz-Clemente et al., 2013; Boyer et al., 1998; Stachowicz, 2022) influencing behavior.

It is also worth asking how the observed changes in the composition of NMDA receptor subunits will affect animal behavior. Based on the literature, it can be assumed that genetic ablation of *Grin2b* or pharmacological inhibition was connected with decreased memory formation and consolidation (Jiang et al., 2017). In addition, transgenic animals with GluN2B overexpression showed better learning and memory performance compared to wild types (Jiang et al., 2017). Conversely, overexpression of *Grin2b* correlated with depression (Adell, 2020). Experiments from our laboratory documented faster antidepressant effects observed when MTEP or imipramine was co-treated with NS398 for seven days (Stachowicz, 2019). Furthermore, improvement in spatial memory was found (Stachowicz, 2019). However, the most interesting finding in this context is that LTP improvement was observed by long-term imipramine treatment when COX-2 was inhibited at the same time (Stachowicz et al., 2020). The results presented here seem to clarify improvement in long-term memory observed in mice when NS398 was applied. While an increase in the level of GluN2B or its gene may be toxic for the cells of the HC or PFC, inhibition of COX-2 seems to interfere with this increase by balancing subunit levels in favor of GluN2A. Furthermore, the most significant changes in the GluN2A/GluN2B ratio were observed in the PFC of mice co-treated with imipramine and NS398 (Fig. 11), which may be related to improvement in memory of mice (Jiang et al., 2017). It was documented that improved transmission between structures (HC and PFC) results in behavioral changes (Stachowicz et al., 2021). Importantly, we also demonstrated the activation of protective mechanisms in the HC and PFC neuronal milieu after prolonged administrations of MTEP or imipramine together with NS398, increasing cell adhesion protein level and BDNF (Stachowicz et al., 2022), which may translate into changes in mood and memory parameters, via mechanisms described by Troca-Marin et al. (2011) with Ca^{2+}/calmodulin-dependent protein kinase II (CaMKIIβ).

Limitations: Our study shows some mismatches between changes in transcript expression and protein level. For example, we observed a significant increase in GluN2A protein in the HC in the group treated with imipramine for seven days but no change in mRNA for *Grin2a*. This is undoubtedly influenced by multiple factors. First, it should be borne in mind that gene expression is regulated at several levels (Vologodskii,

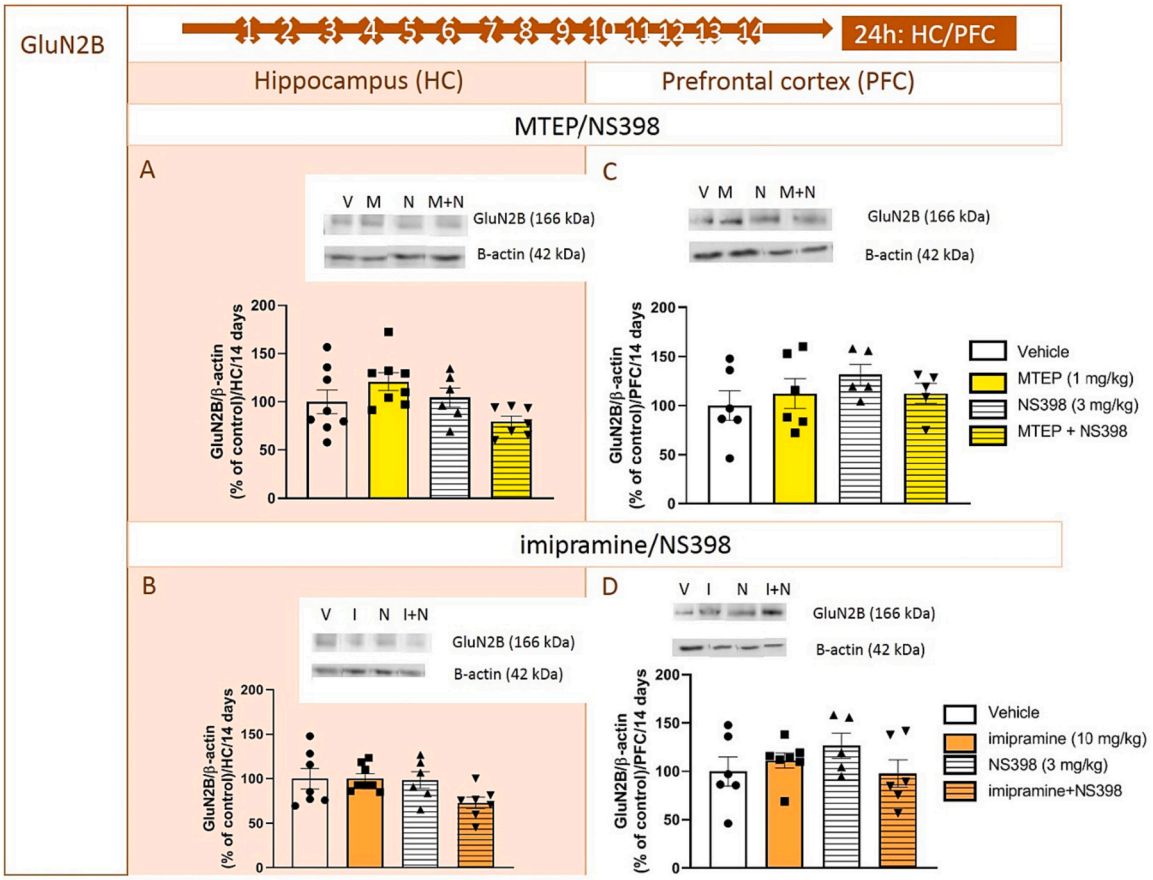

Fig. 8. The effect of treatment with MTEP (1 mg/kg), (top panel), imipramine (10 mg/kg), (bottom panel), and NS398 (3 mg/kg) for fourteen days, on GluN2B in the HC (left panel) or in the PFC (right panel) of C57Bl/6 J mice (Western blot). Compounds were injected *i.p.* Values are expressed as the means ± S.E.M. of normalized densitometry measurements from western blots of GluN2B receptor compared with β-actin ($n = 5–8$). The p value given is for the whole one Kruskal-Wallis analysis while the stars in the figures refer to the post-multicomparision analysis against vehicle. The same values for vehicle and NS398 are repeated in panels A, B, C and D. The membranes have been trimmed for the figure. The original membranes were added to the manuscript and sent to the publisher.

2022). The expressed proteins are the result of various post-transcriptional mechanisms (regulations) that can cause significant changes in the quantity and quality of the protein produced in the process of mRNA translation. Very important post-transcription regulators that we have not yet studied (in this experimental setup) are micro (*mi*)RNAs, i.e., small non-coding RNAs. It is estimated that miRNAs regulate the expression of approximately 60% of mammalian genes by affecting the stability and/or translation of target mRNAs by binding to the 3′UTR sequence of target mRNAs. It can therefore be said that miRNAs fine-tune the expression of various genes and coordinate different functions in living organisms, including the nervous system. Of the many miRNAs present in the brain, the mechanism of action is known only to a few. Some of them are known to be directly related to the NMDAR pathway, and they act either on NMDARs or on targets upstream or downstream. For example, miR-223 has been shown to act directly on *Grin2b* (Harraz et al., 2012), while miR-137 regulates GluN2A (Zhao et al., 2013). Other miRNAs that target NMDARs are also known, such as miR-129-2, miR-148a, and miR-296 (Gunasekaran and Omkumar, 2022). Therefore, we strongly believe that miRNAs may be a major contributor to the discrepancies between protein and mRNA levels we have observed, and we intend to investigate this issue further in the near future. Regardless, it is also important which cellular system is examined. For receptors, the correlations depend on reduced receptor turnover. Moreover, the biochemical diversity of proteins and differences in the detection limits of used molecular methods (real-time PCR vs. Western blot) can explain the obtained results. The small size of the combined group, due to the limited amount of single tissue, is another limitation, as are the single injections in the control group. At the same time, the fact that different group sizes in the WB analysis may have affected the final result of the statistical analysis is a limitation.

5. Conclusions

For the first time, we documented the effect of COX-2 inhibition on the composition of NMDAR subunits. The mechanism under study was detected involving mGluR5, with the simultaneous use of a selective MTEP antagonist, and a broader-spectrum compound such as imipramine. Based on our study, it can be concluded that COX-2 inhibition affects the composition of NMDARs through interaction with mGluR5. However, the observed modifications may be adaptive to the changing environment inside the synapse, so further results covering other time points than just 24 h after treatment are needed to more accurately trace the dynamics of the changes and their mechanism.

Declarations of competing interest

This study was partially supported by grant No.: UMO-2014/13/D/NZ7/00292, assigned by the National Science Centre, Poland, to K.S. and by the statutory fund of the Maj Institute of Pharmacology, PAS, Poland.

CRediT authorship contribution statement

Katarzyna Stachowicz: Writing – review & editing, Writing –

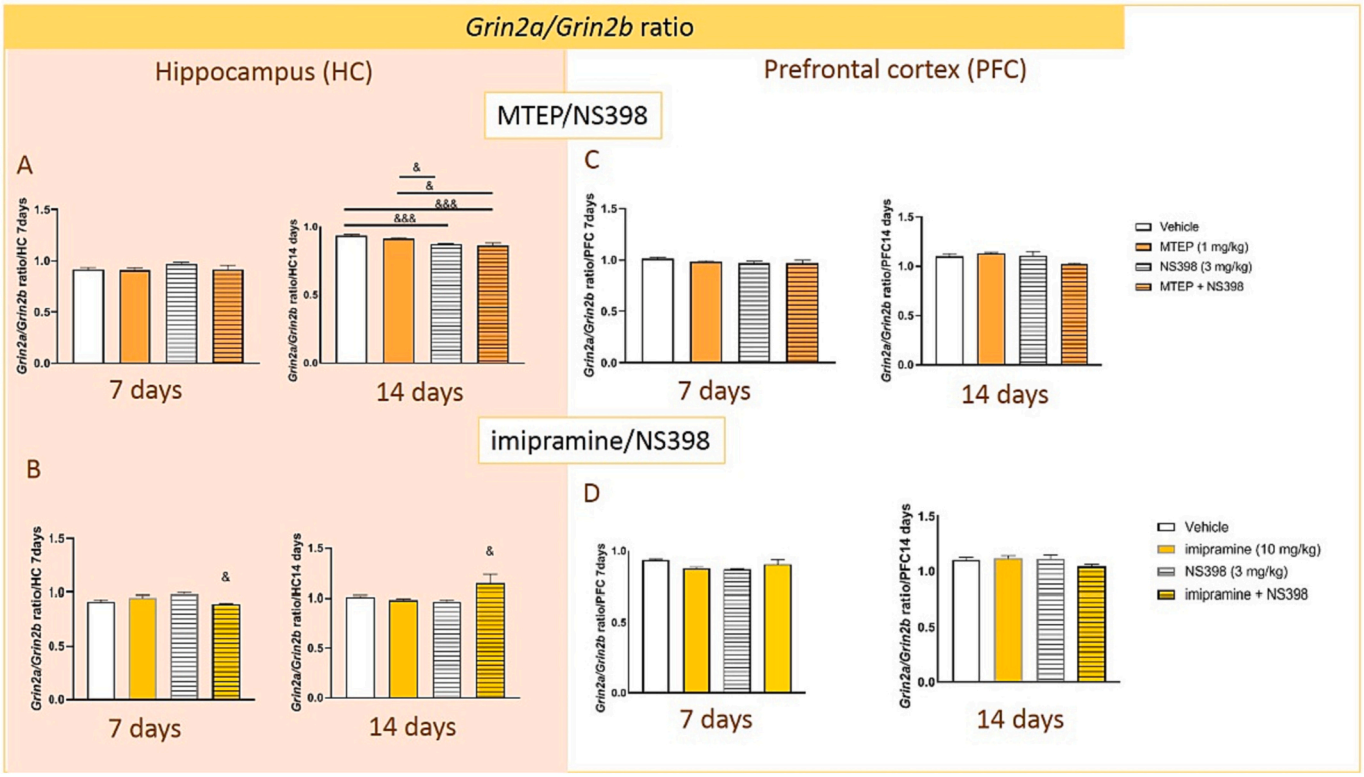

Fig. 9. Changes in *Grin2a/Grin2b* ratio after the treatment with MTEP (1 mg/kg), imipramine (10 mg/kg), NS398 (3 mg/kg), or their combinations. Groups treated with MTEP are shown in the top panel, while groups treated with imipramine are presented in the bottom panel. Data collected from the HC appear on the left side, and those from the PFC appear on the right side of the diagram. Values are expressed as the mean ± S.E.M., two-way ANOVA followed by Tukey test, $^{\&}P < 0.05$, $^{\&\&\&}P < 0.001$ vs. imipramine group. The *p* value given is for the whole Tukey analysis, while the stars in the figure refer to the post-multicomparison analysis against MTEP (top panel) or imipramine (bottom panel).

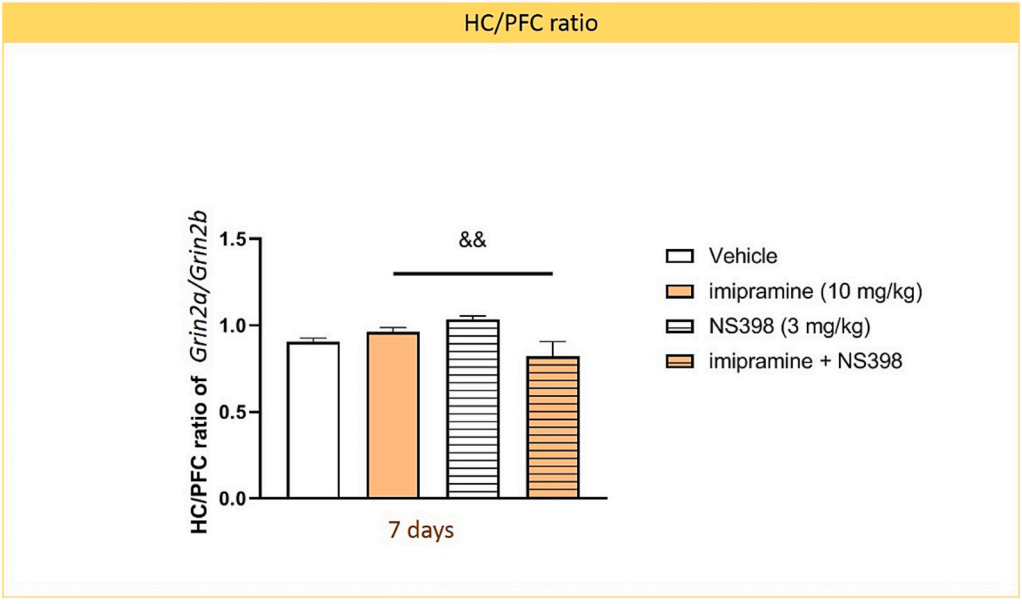

Fig. 10. Changes in *Grin2a/Grin2b* ratio between the HC and PFC calculated as the HC/PFC ratio. Values are expressed as the mean ± S.E.M., two-way ANOVA followed by Tukey test, $^{\&\&}P < 0.01$ vs. imipramine group. The *p* value given is for the whole Tukey analysis, while the stars in the figure refer to the post-multicomparison analysis against imipramine.

original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Patrycja Pańczyszyn-Trzewik:** Writing – review & editing, Visualization, Validation, Software, Methodology, Investigation, Formal analysis. **Paulina Misztak:** Software, Methodology, Investigation, Data curation. **Szymon Rzeźniczek:** Visualization, Methodology, Investigation, Data curation. **Magdalena Sowa-Kućma:** Writing – review & editing,

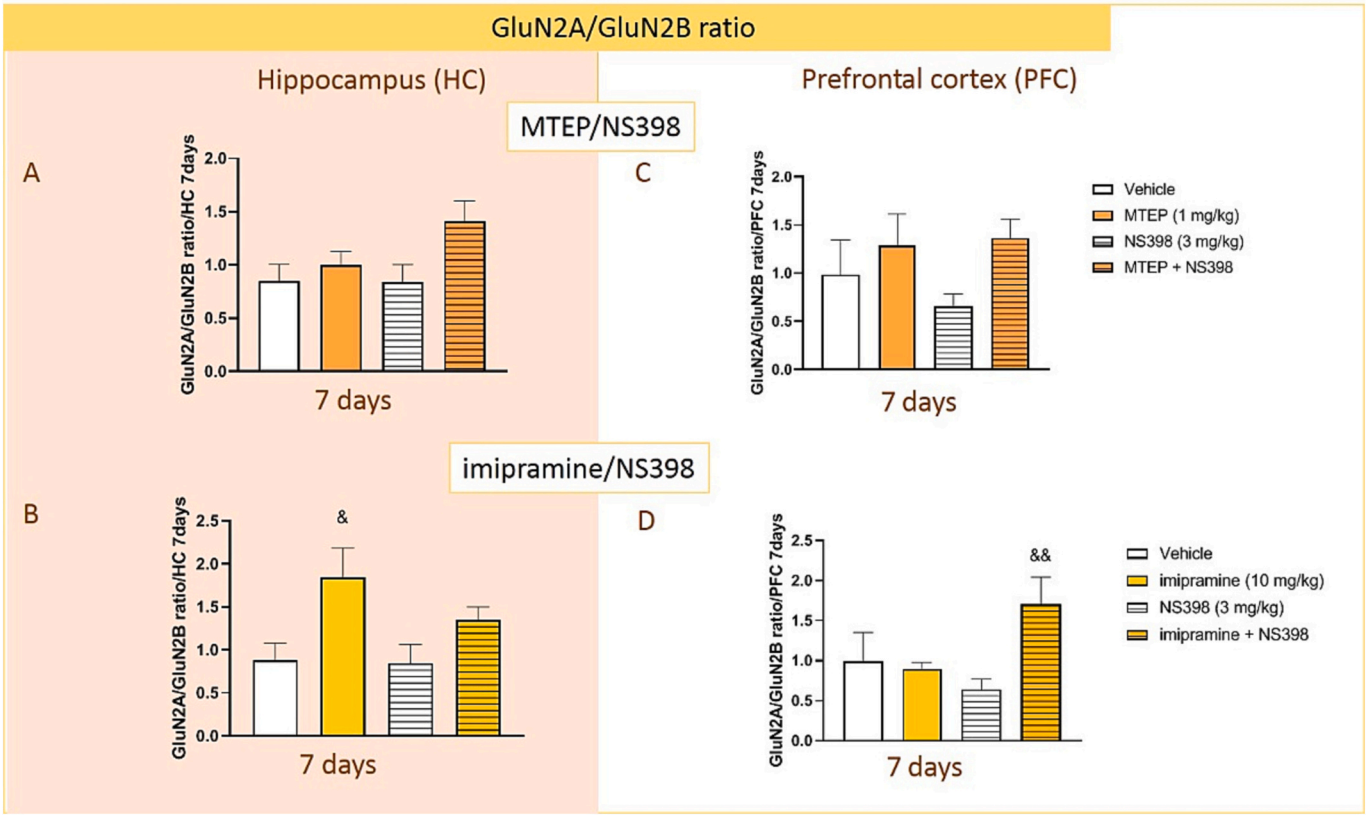

Fig. 11. Changes in the GluN2A/GluN2B ratio after treatment with MTEP (1 mg/kg), imipramine (10 mg/kg), NS398 (3 mg/kg), or their combinations. Groups treated with MTEP are shown in the top panel, while groups treated with imipramine are presented in the bottom panel. Data collected from the HC appear on the left side, and those from the PFC appear on the right side of the diagram. Values are expressed as the mean ± S.E.M., two-way ANOVA followed by Tukey test, $^{\&}P < 0.05$, $^{\&\&}P < 0.01$ vs. imipramine group. The p value given is for the whole Tukey analysis, while the stars in the figure refer to the post-multicomparison analysis against MTEP (top panel) or imipramine (bottom panel).

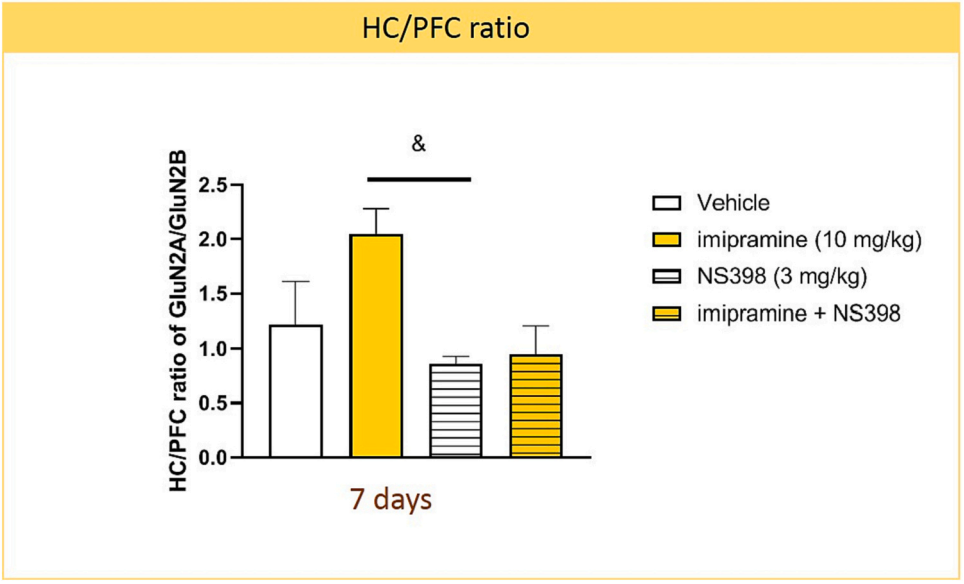

Fig. 12. Changes in the GluN2A/GluN2B ratio between the HC and PFC calculated as the HC/PFC ratio. Values are expressed as the means ± S.E.M., two-way ANOVA followed by Tukey test, $^{\&}P < 0.05$ vs. imipramine group. The p value given is for the whole Tukey analysis, while the stars in the figure refer to the post-multicomparison analysis against imipramine.

Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation.

Data availability

No data was used for the research described in the article.

Acknowledgments

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References

Adell, A., 2020. Brain NMDA receptors in schizophrenia and depression. Biomol. 10, 947.
Boyer, P.A., Skolnick, P., Fossom, L., 1998. Chronic administration of imipramine and citalopram alters the expression of NMDA receptor subunit mRNAs in mouse brain. J. Mol. Neurosci. 10, 219–233.
Elmasri, M., Lotti, J.S., Aziz, W., Steele, O.G., Karachaliou, E., Sakimura, K., Hansen, K. B., Penn, A.C., 2022. Synaptic dysfunction by mutations in GRIN2B: influence of triheteromeric NMDA receptors on gain-of-function and loss-of-function mutant classification. Brain Sci. 12, 789.
Glasgow, N.G., Povysheva, N.V., Azofeifa, A.M., Johnson, J.W., 2017. Memantine and ketamine differentially Alter NMDA receptor desensitization. J. Neurosci. 37, 9686–9704.
Gunasekaran, S., Omkumar, R.V., 2022. miR-146a and miR-200b alter cognition by targeting NMDA receptor subunits. iScience 25, 105515. https://doi.org/10.1016/j.isci.2022.105515.
Harraz, M.M., Eacker, S.M., Wang, X., Dawson, T.M., Dawson, V.L., 2012. MicroRNA-223 is neuroprotective by targeting glutamate receptors. Proc. Natl. Acad. Sci. U. S. A. 109, 18962–18967.
Harvey, B.H., Jonker, L.P., Brand, L., Heenop, M., Stein, D.J., 2002. NMDA receptor involvement in imipramine withdrawal-associated effects on swim stress, GABA levels and NMDA receptor binding in rat hippocampus. Life Sci. 71, 43–54.
Jiang, Y., Lin, M.K., Jicha, G.A., Ding, X., Mcllwarth, S.L., Fardo, D.W., Broster, L.S., Schmitt, F.A., Kryscio, R., Lipsky, R.H., 2017. Functional human GRIN2B promoter polymorphism and variation of mental processing speed in older adults. Aging 9, 1293–1305.
Mardsen, W.N., 2021. Stressor-induced NMDAR dysfunction as a unifying hypothesis for the etiology, pathogenesis and comorbidity of clinical depression. Med. Hypotheses 17, 508–528.
Matta, J.A., Ashby, M.C., Sanz-Clemente, A., Roche, K.W., Isaac, J.T.R., 2011. mGluR5 and NMDA receptors drive the experience- and activity-dependent NMDA receptor NR2B to NR2A subunit switch. Neuron 70, 339–351.
Paxinos, G., Franklin, K.B.J., 2001. The Mouse Brain in Stereotaxic Coordinates, 2nd ed. Academic press, San Diego.
Radiske, A., Gonzalez, M.C., Noga, D.A., Rossato, J.I., Bevilaqua, L.R.M., Cammarota, M., 2021. GluN2B and GluN2A-containing NMDAR are differentially involved in extinction memory destabilization and restabilization during reconsolidation. Sci. Rep. 11, 186.
Sachser, R.M., Santana, F., Crestani, A.P., Lunardi, P., Pedraza, L.K., Quillfeldt, J.A., Hardt, O., Alvares, L.O., 2016. Forgetting of long-term memory requires activation of NMDA receptors, L-type voltage-dependent Ca2+ channels, and calcineurin. Sci. Rep. 6, 22771.
Sanz-Clemente, A., Nicoll, R.A., Roche, K.W., 2013. Diversity in NMDA receptor composition: many regulators, many consequences. Neuroscientist. 19, 62–75.
Stachowicz, K., 2019. Behavioral consequences of the co-administration of MTEP and the COX-2 inhibitor, NS398 in mice. Part 1. Behav. Brain Res. 370, 111961.
Stachowicz, K., 2021. Deciphering the mechanisms of regulation of an excitatory synapse via cyclooxygenase-2, A review. Biochem. Pharmacol. 192, 114729.
Stachowicz, K., 2022. Is PSD-95 entangled in the side effects of antidepressants? Neurochem. Int. 159, 105391.
Stachowicz, K., Bobula, B., Tokarski, K., 2020. NS398, a cyclooxygenase-2 inhibitor, reverses memory performance disrupted by imipramine in C57Bl/6J mice. Brain Res. 1734, 146741.
Stachowicz, K., Misztak, P., Panczyszyn-Trzewik, P., Lenda, T., Rzezniczek, S., Sowa-Kucma, M., 2021. Upregulation of the mGlu5 receptor and COX-2 protein in the mouse brain after imipramine and NS398, searching for mechanisms of regulation. Neurochem. Int. 150, 105193.
Stachowicz, K., Panczyszyn-Trzewik, P., Rzezniczek, S., Sowa-Kucma, M., 2022. Cyclooxygenase-2 inhibition affects the expression of down syndrom cell adhesion molecule via interaction with metabotropic glutamate receptor 5. J. Physiol. Pharmacol. 73 (2), 319–326.
Stark, D.T., Bazan, N.G., 2011. Synaptic and extrasynaptic NMDA receptors differentially modulate neuronal cyclooxygenase-2 function, lipid peroxidation, and neuroprotection. J. Neurosci. 31, 13710–13721.
Szewczyk, B., Pałucha-Poniewiera, A., Poleszak, E., Pilc, A., Nowak, G., 2012. Investigational NMDA receptor modulators for depresion. Expert Opin. Investig. Drugs 21, 91–102 review.
Troca-Marin, J., Alves-Sampaio, A., Montesinos, M.L., 2011. An increase in basal BDNF provokes hyperactivation of the Akt-mammalian target of rapamycin pathway and deregulation of local dendritic translation in a mouse model of Down's syndrome. Neurobiol. Dis. 31, 9445–9455.
Vologodskii, A., 2022. Regulation of gene expression and protein activity. In book: The Basics of Molecular Biology. https://doi.org/10.1007/978-3-031-19404-7_5.
Xu, Z., Chen, R.Q., Gu, Q.H., Yan, J.Z., Wang, S.H., Liu, S.Y., Lu, W., 2009. Metaplastic regulation of long-term potentiation/long-term depression threshold by activity-dependent changes of NR2A/NR2B ratio. J. Neurosci. 29, 8764–8873.
Zhao, L., Li, H., Guo, R., Ma, T., Hou, R., Ma, X., Du, Y., 2013. miR-137, a new target for post-stroke depression. Neural Regen. Res. 8, 2441–2448.