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Short communication

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CYCLOOXYGENASE-2 INHIBITION AFFECTS THE EXPRESSION OF DOWN SYNDROME CELL ADHESION MOLECULE *VIA* INTERACTION WITH METABOTROPIC GLUTAMATE RECEPTOR 5

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Since we previously discovered that simultaneous inhibition of cyclooxygenase-2 (COX-2; a highly inducible enzyme, crucial for the conversion of arachidonic acid to prostaglandin G2, which plays a predominant role in the CNS) by NS398 and metabotropic glutamate receptor 5 (mGluR5) through the use of its antagonist [3-((2-methyl-4-thiazolyl)ethyl)pyridine; MTEP] alters mouse behavior (*e.g.*, affects spatial learning, and induces/intensifies the antidepressant effect), our aim was to discover the mechanism responsible for these changes. Down syndrome cell adhesion molecule (DSCAM), a member of the immunoglobulin cell adhesion molecule (Ig-CAM) superfamily, is involved in developing the central and peripheral nervous system by influencing cell adhesion mechanisms necessary for synaptic activity and plasticity. Since COX-2 has been implicated in several neuropsychiatric diseases (*e.g*., major depressive disorder) resulting from neuroplasticity disorders, and on the other hand, its expression is regulated by synaptic activity, we hypothesized that cognitive changes after administration of COX-2 inhibitor and mGluR5 antagonist might be a consequence of impaired DSCAM expression. Importantly, DSCAM deficiency leads to dysregulation of glutamatergic transmission and plasticity. In previous studies, we have demonstrated glutamatergic changes after NS398 and MTEP administration, further supporting the validity of our hypothesis. Due to the different effects observed in behavioral tests, this study used the prefrontal cortex (PFC) and hippocampus (HC) of C57BL/6J mice, which received NS398 and MTEP alone, or in combination, for 7 or 14 days. Among many properties, we also previously investigated the antidepressant potential of these compounds, so we used imipramine (a tricyclic antidepressant) as the reference drug. DSCAM mRNA expression was determined by qRT-PCR. Our results indicate that DSCAM expression after administration of MTEP and NS398 and imipramine along with NS398 is structure- and time-dependent.

Key words: *Down syndrome cell adhesion molecule, metabotropic glutamate receptor 5, cyclooxygenase-2, imipramine, mice, prefrontal cortex, hippocampus, metabotropic glutamate receptor 5 antagonist*

INTRODUCTION

Synapses contain pre- and postsynaptic parts that need to be close for physical interaction. Furthermore, the timing of the interaction between pre- and postsynaptic elements is vital for the precise communication of neurons (1, 2). The enhancement of synaptic connection known as long-term potentiation (LTP), an outcome of a brief, high-frequency stimulation observed in the hippocampus (HC) or cortex (CX), results in increased glutamate (Glu) transmission (3, 4). For the occurrence of LTP, a coordinated in-time stimulation of pre-synapse with a rise in postsynaptic Ca^{2+} concentration is necessary with the local release of BDNF in the synapse (3-5).

Down syndrome cell Adhesion molecule (DSCAM) belongs to the immunoglobulin superfamily of cell adhesion molecules (Ig-CAMs) (6). DSCAM express on dendrites and axons of neurons (7) in the CX and HC of the mouse brain (8). This protein has an established position in synaptic plasticity and is a vital factor in the process of synapse strengthening and timing of synaptic transmission (9, 10). Cell adhesion molecules (CAMs) are involved in building connections between cells and are components of the extracellular matrix with documented engagement in the modulation of synaptic connectivity (11). Notably, the role of CAMs in learning has also been confirmed in experimental studies on LTP (11). Elevated soluble neural cell adhesion molecule (NCAM) levels are generally observed immediately after LTP induction (12). The neural activity can influence the synthesis of NCAMs or their metabolism and cell adhesion (11); this can induce local changes leading to structural remodeling and long-lasting changes in synapse function. DSCAM participates in neural circuit regulation *via* homo- and heterophilic adhesion (13, 14) and initiates cellular responses *via* intracellular signal transduction cascades (11). The composition of the intracellular domain of DSCAM suggests these possibilities (for details, see (2)). It is composed of 300–400 amino acids. The DSCAM protein has a high concentration of tyrosine, which is the binding site for the SH2 domain (cytokine dedicated domains (Dock)) or postsynaptic density proteins (7,

15). It is possible that through these units, DSCAM may activate downstream mechanisms and initiate synapse remodeling.

At the same time, thanks to mGlu receptors, glutamate can regulate cell excitability and synapse plasticity and transmit a signal *via* a second messenger system (16). The mGlu receptors represent a part of the membrane-bound G-protein-coupled receptor (GPCR) family (16). Eight mGlu receptors are subdivided into three groups based on their sequence homology, pharmacological profile, and signal transduction mechanisms (17, 18). Receptors assigned to group I activate phospholipase C (PLC), while those from groups II and III inhibit adenylate cyclase (18-20). The group I mGlu receptors are localized mainly postsynaptically (18). Several hypothetical mechanisms by which Glu *via* mGluR5 or components of the arachidonic acid (AA) pathway, such as COX-2, may affect DSCAM expression. One of that hypothetical mechanisms was described by Stachowicz (15) and points to the involvement of scaffolding proteins, which can link the two signaling pathways through, *e.g.*, PSD-95/NMDA/Shank proteins (15).

Our previous studies have documented that by inhibiting COX-2, we can modulate mGlu receptor levels in the brain of mice (21). NS398 also influences mice's spatial learning capacities after using MTEP or imipramine (22-24). Looking for the mechanisms responsible for the behavioral changes induced through Glu/COX-2 pathways, we decided to check whether such an essential factor as CAMs is involved in the observed effects. The first step on this pathway is described in this short communication. We tested whether affecting COX-2, mGluR5, or both paths simultaneously would detect changes in DSCAM RNA expression in HC and PFC of mice. This question is relevant to the search for mechanisms that regulate cognition. It is well known that mGluRs ligands cannot reach clinical distribution for the treatment of mood due to side effects associated with cognitive impairment. Therefore, the question we asked is crucial. In addition, we tested whether acting directly on mGluR5 with an antagonist (MTEP) would have a similar effect to indirect modulation of the receptor with imipramine (21). Our previous studies have documented that imipramine acts through mGluR5 in mice (21). Here, we found a time-dependent increase in DSCAM gene expression in the HC or PFC of C57Bl/6J mice after treatment with MTEP or NS398. Similar effects were observed with imipramine. In addition, our hypothesis is strengthened by the results showing a decrease in the level of pro-inflammatory cytokines after the administration of the compounds studied.

MATERIALS AND METHODS

Animals and housing

The experiments were performed on group housed male C57BL/6J mice (8–10 weeks old). The animals were kept under recommended living conditions. 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 Cracow.

Drug treatment

The following drugs were used: *N*-[2-(cyclohexyloxy)-4-nitrophenyl]methanesulfonamide (NS398, Abcam Biochemicals, Cambridge, UK); 3-[(2-methyl-1,3-tiazol-4-yl)ethynyl]-pyridine (MTEP; Tocris Cookson Ltd., Bristol, UK), and imipramine (Imipramine hydrochloride, Sigma-Aldrich, Schnelldorf, Germany). MTEP (1 mg/kg) was used as 1% Tween 80 solution while imipramine (10 mg/kg) as aqua solution. NS398 (3 mg/kg) was dissolved in 10% DMSO and this solution was applied for vehicle group injections. All compounds were injected intraperitoneally (i.p.) once daily (before 11:00), for 7 or 14 consecutive days (*Fig. 1*).

Tissues collection

Twenty-four hours after the last injection, the animals were decapitated and their brains were immediately removed (*Fig. 1*). Then, the PFC and HC were dissected according to the Mouse brain atlas (26). 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 majority of the PFC. Subsequently, the brain was cut into two hemispheres in the sagittal line. Then, the whole HC was taken out from each hemisphere. The isolated brain structures were immediately frozen on dry ice and kept at –80ºC until molecular analysis began.

Real-time PCR analysis

Real-time PCR (qRT-PCR) procedure was used for measurement of DSCAM mRNA expression. In short, total cellular RNA was extracted from tissue samples by TRI Reagent (Sigma-Aldrich; St. Louis, MO, USA) in accordance with the manufacturer's protocol. RNA integrity and purity were verified by denaturing 1.5% agarose gel electrophoresis, while its concentration and also purity were evaluated spectrophotometrically using Nanodrop 2000 (Thermo Fisher Scientific, Rockfold, IL, USA). 1 μg of total RNA from each sample was first incubated with DNase I (Sigma-Aldrich; St. Louis, MO, USA) and next reverse-transcribed into complementary DNA using High Capacity cDNA Reverse Transcription Kit with RNase inhibitor and random hexamers

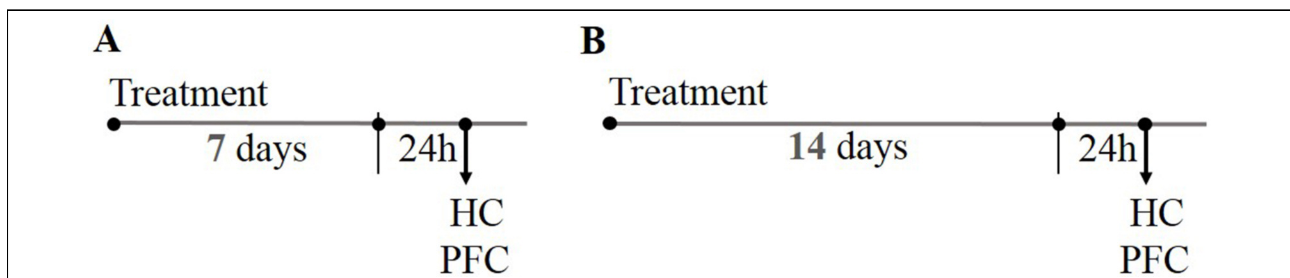

Fig. 1. A schematic diagram of injections and preparation of brain structures. Seven days of the treatment schedule is shown in (A), and fourteen days of the treatment schedule is shown in (B).

(Life Technologies; Paisley, UK). The mRNA levels were determined using the pre-designed TaqMan Gene Expression Assays (Life Technologies, Carlsbad, CA, USA) for DSCAM (RefSeq: RefSeq:NM_031174.4; -Mm00518961_m1) or Gapdh (glyceraldehyde-3-phosphate dehydrogenase; RefSeq: NM_008084.3; -Mm99999915_g1) an endogenous control (selected on the basis of literature data, and own experience and observations). qRT-PCR were 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, Des Plaines, IL, USA). Reaction mixtures included: 3 µl of cDNA sample (diluted 1:20), RNase-free water (Sigma-Aldrich, St. Louis, MO, USA), TaqMan Gene Expression Master Mix (Life Technologies, Carlsbad, CA, USA) and appropriate TaqMan Gene Expression Assay (Life Technologies, Carlsbad, CA, USA). The qRT-PCR were carried out under the following conditions: 95ºC for 10 min followed by 40 cycles at 95ºC for 15 s and 60ºC 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 DSCAM genes was normalized against the Gapdh. The Delta-Delta Comparative Threshold method was used to quantify the fold change between the samples.

Inflammation assay

The inflammatory parameters: interleukin-1beta (IL-1β), tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) were determined in the brain structures (PFC and HC) using high sensitivity commercially available ELISA kits (RayBiotech, Norcross, GA, USA, respectively) according to the manufacturer's protocols. Shortly, the appropriate volumes of standards and samples were pipetted into 96 wells coated with anti-IL-1β, TNF-α, and IL-6 antibodies and incubated. After extensive washing, specific enzyme-linked polyclonal antibodies were added. Following incubation and washing, a substrate solution was added to the wells, and color developed proportional (IL-1β, TNF-α, IL-6) to the amount of specific protein bound in the initial step. The reactions were stopped by adding a stop solution. The absorbance of each well was determined at 450 nm within max. 15 min.

Statistical analysis

The obtained data were evaluated by a one-way ANOVA, followed by Dunnett's multiple comparison test using GraphPad Prism software, ver. 8.0 (San Diego, CA, USA). The obtained data were presented as the mean ±S.E.M., P<0.05 was considered significant.

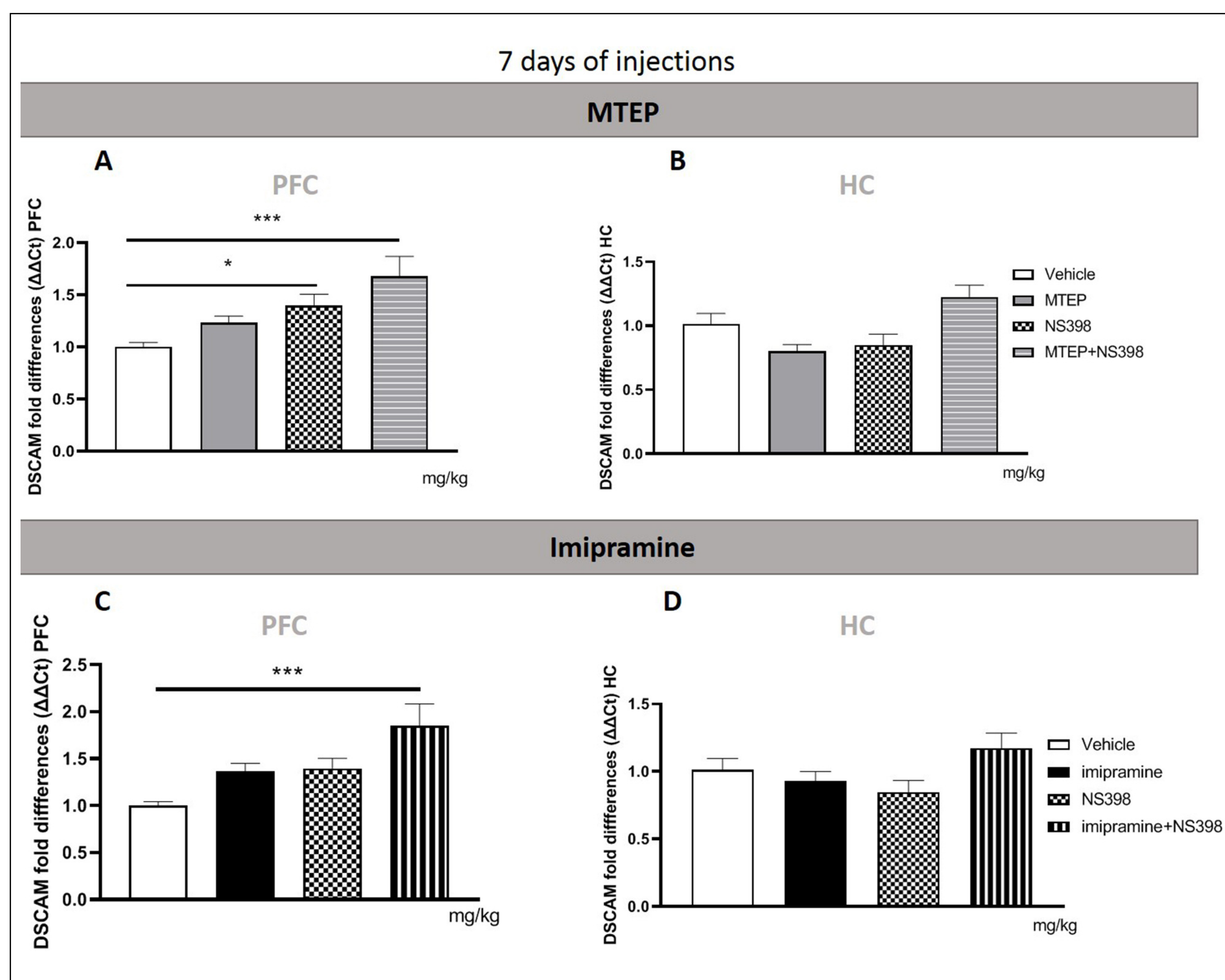

Fig. 2. The effect of treatment with MTEP (1 mg/kg) (A, B), or imipramine (10 mg/kg), (C, D), and NS398 (3 mg/kg) for seven days, on DSCAM expression in the PFC (A, C) or in HC (B, D) of C57Bl/6J mice (PCR); n=4–6. Values are expressed as the means ±S.E.M., *P<0.05, ***P<0.01 vs. vehicle group (a one-way ANOVA followed by Dunnett's multiple comparisons test).

RESULTS

Enhancement of DSCAM gene expression in the mouse brain after MTEP, NS398 or imipramine treatment for seven days

PFC: Chronic treatment with MTEP (1 mg/kg), with NS398 (3 mg/kg) or their combination for seven days resulted in an increase in DSCAM gene expression in the PFC of C57Bl/6J mice (by 23%, 39%, 68%, respectively; F(3,17)=7.373; P=0.002), (*Fig. 2A*). Significance was found in NS398 group, and in MTEP + NS398 group of mice (*Fig. 2A*). Similar effects to that observed after MTEP injections on DSCAM gene expression were found after imipramine treatment (*Fig. 2C* and *2D*). Chronic treatment with imipramine (10 mg/kg), NS398 (3 mg/kg) or their combination for seven days resulted in increase in DSCAM gene expression in the PFC (by 36%, 39%, 85%, respectively; F(3,18)=7.551; P=0.001), (*Fig. 2C*). Significance was found in imipramine + NS398 group of mice (*Fig. 2C*).

HC: In HC, the trend of increased DSCAM gene expression was found only in the group of mice treated with MTEP + NS398 for seven days (F (3,15)=5.503; P=0.009) (*Fig. 2B*). Similar effects were found in a group of mice treated with imipramine + NS398 (F(3,17)=2.385; P=0.105) (*Fig. 2D*); however the results were not significant.

In parallel, an increased spatial learning was observed in mice treated in a similar manner (23, 24).

Lack of effects of MTEP, NS398 or imipramine or suppression of DSCAM gene expression with prolonged treatment has been observed

PFC: Treatment with MTEP or with NS398 for fourteen days, resulted in slight decrease in DSCAM gene expression in the PFC of C57Bl/6J mice (*Fig. 3A*). Co-treatment of both MTEP + NS398 for fourteen days resulted in a significant decrease in gene expression (F(3,16)=6.901; P=0.003) (*Fig. 3A*). Similar effects were found in a group of mice treated with imipramine + NS398 (F(3,19)=6.238; P=0.004) (*Fig. 3C*).

HC: When injections were prolonged to fourteen days decrease in DSCAM gene expression was found in all tested groups (F(3,19)=9.750; P=0.0004) (*Fig. 3B*). Similar effects were found in imipramine groups (F(3,17)=15.12; P=0.0001) (*Fig. 3D*).

In parallel, a reduction in spatial learning was observed in mice treated in a similar manner (21-25).

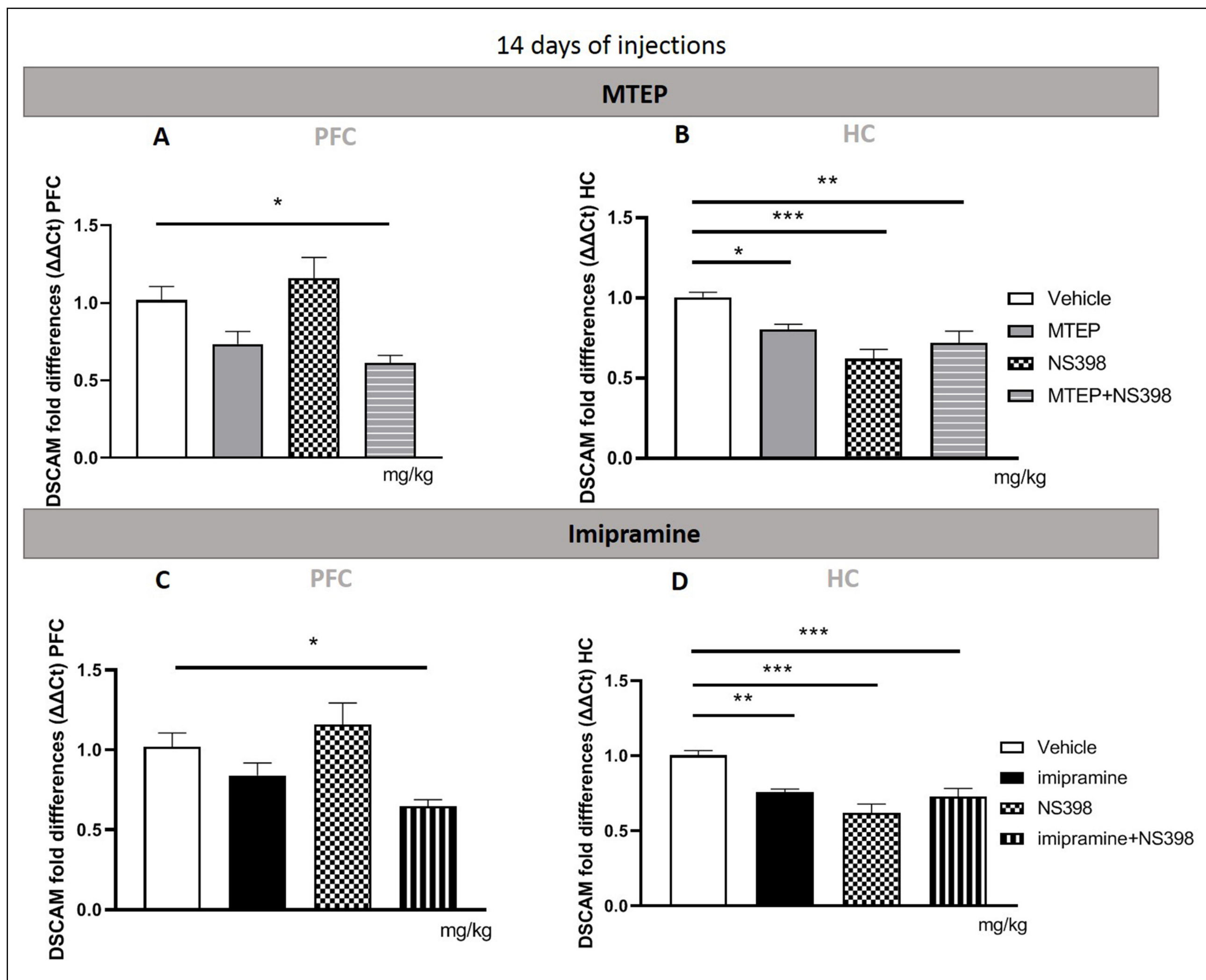

Fig. 3. The effect of treatment with MTEP (1 mg/kg) (A, B), or imipramine (10 mg/kg), (C, D), and NS398 (3 mg/kg) for fourteen days, on DSCAM expression in the PFC (A, C) or in HC (B, D) of C57Bl/6J mice (PCR); n=5–6. Values are expressed as the means ±S.E.M., *P<0.05, **P<0.01, ***P<0.01 vs. vehicle group (a one-way ANOVA followed by Dunnett's multiple comparisons test).

MTEP, like imipramine, when co-treated with NS398 for seven days, has a strong inhibitory effect on pro-inflammatory cytokines in PFC

Seven days of treatment with MTEP (1 mg/kg), NS398 (3 mg/kg) or imipramine (10 mg/kg) did not influenced significantly HC levels of tested cytokines that were: IL-1β, IL-6, TNF-α (*Fig. 4B*, *4D*, *4F*). Significant effect was detected in PFC when compounds were co-treated. Decrease in the level of IL-1β was observed when MTEP or imipramine were co-treated with NS398 for seven days (*Fig. 4A*). Similar results was found as it states to IL-6 (*Fig. 4C*). TNF-α was significantly decreased in a groups co-treated with MTEP or imipramine with NS398 (*Fig. 4E*); [F(5,34)=5.46; P=0.0009); F(5,34)=6.12; P=0.0004); F(5,33)=3.64; P=0.0098), respectively].

Suppression of brain pro-inflammatory cytokines in mice treated for fourteen days with MTEP + NS398 or imipramine + NS398

Progress was made when comparing seven days of administration of tested compounds and fourteen days, while HC was more reactive. A one-way ANOVA followed by Dunnett's, Mann Whitney detected significant decrease in the level of HC IL-1β, after treatment with MTEP + NS398 and imipramine + NS398 [F(5,32)=12.39; P<0.0001] (*Fig. 5B*). The level of IL-6 or TNF-α were not a subjects of changes (*Fig. 5D* and *5F*). In PFC tissue significant changes in IL-1β, and decrease in IL-6 and TNF-α were found in a group of mice treated for fourteen days with imipramine + NS398 [F(5,34)=4.36; P=0.0036; F(5,35)=6.83; P=0.0002; F(5,30)=3.29; P=0.0174; respectively] (*Fig. 5A*, *5C*, *5E*).

DISCUSSION

Chronic treatment of mice for seven days with either MTEP or NS398 significantly affected DSCAM RNA expression. When the compounds were injected separately, an increase in the DSCAM gene was detected in the PFC of mice. However, the effect was more pronounced when the compounds were administered together. Similar effects were found when imipramine was used. The results we presented are the first in this field, and we cannot compare them with the results of other authors. However, we can refer to intermediate results from our laboratory and others. The changes in gene expression we found here correlate with the behavioral changes we described earlier

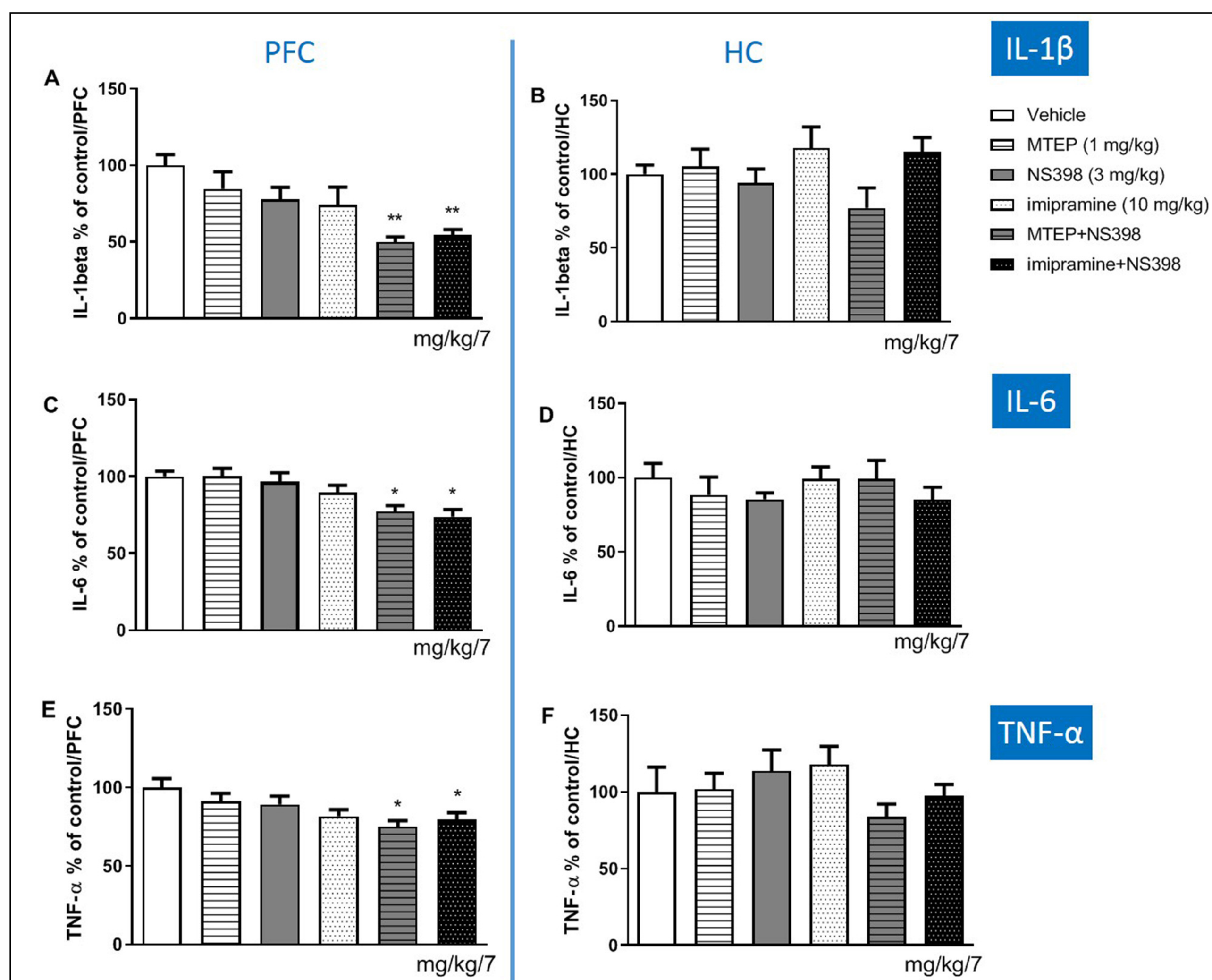

Fig. 4. The effect of chronic treatment for seven days with MTEP (1 mg/kg), NS398 (3 mg/kg), imipramine (10 mg/kg) or their combination on PFC (A, C, E), and HC (B, D, F) level of pro-inflammatory cytokines. (A) and (B) shows brain levels of IL-1β; (C) and (D) shows brain levels of IL-6; (E) and (F) shows brain levels of TNF-α. Cytokines protein level was measured using ELISA kits. Values expressed as the means ± S.E.M. were evaluated by One-way ANOVA, *P<0.05, **P<0.01, ***P<0.0001 vs. respective vehicle group; n=5–7.

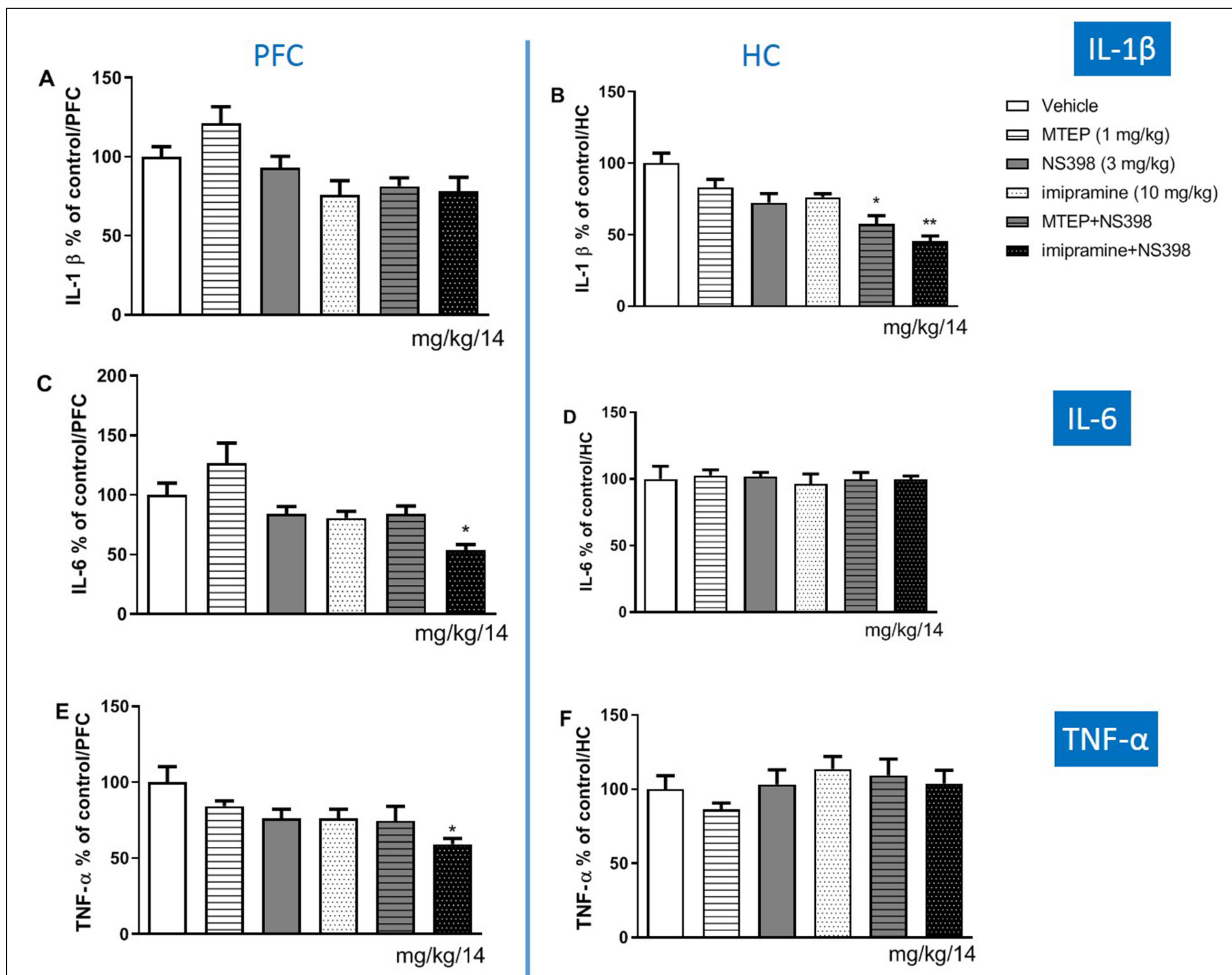

Fig. 5. The effect of chronic treatment for fourteen days with MTEP (1 mg/kg), NS398 (3 mg/kg), imipramine (10 mg/kg) or their combination on PFC (A, C, E), and HC (B, D, F) level of pro-inflammatory cytokines. (A) and (B) shows brain levels of IL-1β; (C) and (D) shows brain levels of IL-6; (E) and (F) shows brain levels of TNF-α. Cytokines protein level was measured using ELISA kits. Values expressed as the means ±S.E.M. were evaluated by one-way ANOVA, *P<0.05, **P<0.01, ***P<0.0001 vs. respective vehicle group; n=5–7.

(23-25). Mice treated with MTEP and NS398 for seven days showed improvements in spatial learning in the Barnes maze and reduced immobility time in the tail suspension test, indicating that both cognitive and depressive parameters improved after treatment with the test compounds (23-25). CAMs have been documented to be active participants in cognitive changes in rodents (2), indicating a role for DSCAM (2). However, the mechanisms of their activation in this process and their co-involvement with mGluRs and COX-2 are not known at all.

The COX-2 gene is essential for survival in vertebrates (21) and is one of the regulatory genes with a TATA box (21, 27), so it may be necessary for regulating DSCAM. Interestingly, compounds such as imipramine and MTEP injected separately did not have the power to regulate repressed genes (21, 22). Simultaneous injection of both was potent. In parallel experiments, behavioral changes were found after treatment (21-25). Ligands of mGluRs are known as powerful antidepressants. However, they have not found clinical use due to memory-related side effects. It is an open question whether we have just found the leading causes of side effects of mGluRs ligands affecting the DSCAM pathway. Such a possibility seems to be supported by enhanced spatial learning, which was observed in mice with short-term COX-2 inhibition (24). However, the opposite effects were observed with more prolonged treatment (24). The differences in results we found after a short or extended treatment period with the compounds under study may have several causes. One reason that may be mundane is the depletion of substrates in the AA pathway with long-term COX-2 inhibition. We have described the mechanism in previous publications (references 21 and 24). Subsequently, CAMs are known to affect synaptic plasticity and to participate in physical contact between cellular elements (2); hence the differences we observed between seven and fourteen days of administration of the test compounds may be due to plastic and adhesive changes. We plan to test this hypothesis.

Furthermore, considering the mechanisms, mGluR5 localizes postsynaptically (28) and associates with NMDA receptor potentiation during LTP (29). This regulation is overseen by COX-2; or stress versus an enriched environment providing a positive emotional experience (21, 22). Repeated administration of MTEP modulates Ca^{2+} and MAPK (CaM kinase II inhibitor α; Ca^{2+}/calmodulin dependent protein kinase II; CaMKIIβ) signaling (31). The mGluR5 and NMDA receptors are close to iGluRs in the postsynaptic part (21). The Homer family of proteins can functionally link mGlu5 receptors to IP3

receptors and Shank proteins in a functional form as part of the PSD-95 complex associated with the NMDA receptor (21). Similarly, Ca^{2+}/CaM-dependent CaMKII is vital in mediating COX-2 expression through CREB activation (32). Since the PDZ domain binding site (7) is a functional component of the intracellular DSCAM fragment, our experimental manipulation will likely affect DSCAM expression. Parallel to postsynaptic mechanisms, changes in DSCAM expression may be related to presynaptic changes (33). Sterne *et al.* (33) documented enlargement of presynaptic striatum in Drosophila models when DSCAM levels are deregulated. Our study seems to support this hypothesis, while changes in presynaptically localized proteins have been found after treatment with MTEP and NS398 (21-26). Here, a completely different mechanism can be postulated with the remodeling of other CAMs, *e.g.*, integrins, involved in cognition and AMPA receptor-dependent transmission (34).

Our results confirmed that translation in the HC synapse was not disturbed. At the same time, parallel experiments found both pro- and mature- forms of BDNF increased at an adequate level (in about 47% and 49%, respectively) after seven days of co-treatment with MTEP + NS398 (manuscript in preparation). The above suggests balance in a synapse metabolism. The topic was described by Ulker *et al.* (35). The mechanism responsible for BDNF changes may be connected with 5-HT receptor regulation, while imipramine acts through those receptors (34). Vaidya *et al.* (37) documented BDNF mRNA regulation in the HC, and the CX is *via* 5-HT_{2A} receptors. However, the results presented here were obtained 24 hours after the last treatment; simultaneously, a different trend of changes can be obtained at other time points, as changes at the synaptic level are dynamic.

Our hypothesis is supported by experiments indicating the involvement of pro-inflammatory cytokines in the observed changes. A characteristic time-dependent reduction in cytokine levels was observed in PFC after seven days of treatment and then in HC tissue after fourteen days of treatment. The suppression we found was most representative of IL-1β, and the results observed here are consistent with the literature. Aronica *et al.* (38) documented interactions in the regulation of mGluR5 expression in human astrocytes after exposure to IL-1β. The most representative interaction was found 48 h after exposure (38). It was shown reduced mGluR5 expression 48 h after exposure to IL-1β (38). This effect was reversed in the presence of an IL-1 receptor antagonist (38).

We showed that chronic mGluR5 antagonist treatment significantly influenced DSCAM gene expression in the PFC and HC of C57Bl/6J mice. This effect undergoes regulation by COX-2. The study findings confirm that neural activity can influence the synthesis of NCAM, as earlier postulated by Ronn *et al.* (11). The modulation of neural activity by our manipulations influenced DSCAM gene expression in the PFC and the HC. Because changes in DSCAM content in different brain structures may affect synapse plasticity, size, and behavior of dendrites, our findings may be vital in solving the problem of adhesion modifications. Thus, changes in the level of synaptic adhesions may be followed by changes in the structure of the synapse, its physical properties, and, finally, changes in signal transmission. In physics, contact geometry modulates adhesion. According to Varenberg *et al.* (39), adhesion is directly proportional to the contact perimeter but does not correlate with the contact area. In neurobiology research, buttons represent contact perimeters, the contact area being the synapse (40, 41) in the nervous system. These observations may explain the presynaptic button enlargement observed in DS and changes in synapse positions, with an accompanying shift of the inhibitory synapse position from shafts onto the neck of dendrites (42). The consequence is cognitive impairment. Our observations may be interesting new research in cognitive science. However, the obtained results are only the first step, and further research is needed in which, among others, the size of dendritic spines with a changed DSCAM level will be examined, and the strength of adhesion will be checked.

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Conflict of interest: None declared.

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