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The impact of mGlu2 or mGlu5 receptor activators on the production of L-arginine derivatives and the expression of PRMT5 or DDAH1 enzymes in animal models of cognitive decline

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ABSTRACT

L-arginine derivatives (ADMA, SDMA, NMMA) are endogenous inhibitors of nitric oxide (NO) production, which is essential in critical brain processes including blood-brain barrier (BBB) integrity and long-term potentiation (LTP). ADMA and NMMA are degraded by dimethylarginine dimethylaminohydrolase 1 (DDAH1) and protein arginine methyltransferase 5 (PRMT5) is an emerging epigenetic enzyme that mainly represses transcription of target genes via symmetric dimethylation of arginine residues. There is no data concerning the impact of metabotropic glutamate receptors (mGlu) ligands on this aspect of brain physiology.

In the present studies the impact of positive allosteric modulators (PAM) of mGlu5 (CDPPB) and mGlu2 (LY487379) receptors on L-arginine derivatives, DDAH1 and PRMT5 expression in mouse models of cognitive dysfunction induced with MK-801(0.3 mg/kg) or scopolamine (1 mg/kg), was investigated. Experiments were performed both after acute and chronic (14 days) administration of the compounds, which were administered at the doses 0.1–5 mg/kg (CDBBB) and 0.1–1 mg/kg (LY487379).

The chronic administration of both compounds normalized the level of L-arginine derivatives in MK-801 model (in brain and plasma) and only low dose of CDPPB prevented scopolamine-induced changes. The expression of DDAH1 and PRMT5 was modulated by CDPPB and LY487379, both in MK-801 and scopolamine models.

In the novel object recognition (NOR) test low doses of the compounds, inactive after single administration, prevented cognitive decline after chronic injections.

Our findings highlight the potential of mGlu receptor modulators in treating schizophrenia and possibly dementia by normalizing L-arginine derivatives production, preventing from nitric oxide synthases uncoupling.

1. Introduction

Glutamate is the most important excitatory neurotransmitter in the brain and is essential in learning and memory processes. Glutamatergic transmission regulates long-term potentiation (LTP), one of the most important processes that strengthens connection between neurons, promotes synaptic plasticity and the formation of new neuronal networks. This synaptic plasticity involves pre-synaptic neurons releasing neurotransmitters, which bind to post-synaptic neurons, opening channels for ions to flow in Refs. [1,2].

Glutamate regulates the activity of the other neurotransmitters, in particular GABAergic inhibitory neurotransmission, ensuring

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Table 1
The levels of L-citrulline (L-Cit), L-glutamate(L-Glu), L-glutamine (L-Gln), L-ornithine (L-Orn) measured after acute (A) and chronic (B) administration of CDPPB and LY487379 in scopolamine or MK-801-treated mice. The measurements were performed both in frontal cortex (FC) and hippocampus (H). Al least **P < 0.01, ***P < 0.001 and ****P < 0.0001 comparing to control and P < 0.001, P < 0.001 and P < 0.0001 comparing to scopolamine o MK-801-treated mice. Doses are indicated in parentases. Red color = decrease, green = increase, blue = normalization.

A-acute		control	Scop (1)	Scop/CDPPB	Scop/LY487379
l-citruline	C	2.42±0.12	2.82±0.2**	2.74±0.12	2.76±0.12^
	H	2.68±0.18	3.73±0.24**	3.26±0.41	2.97±0.18^
l-glutamate	C	1038±25.22	1178±31.57**	919±22.9^^^^	1099±27.45
	H	845.8±61.03	850.7±35.63	866.4±31.35	945.4±30.54
l-glutamine	C	602.5±23.48	610.4±19.06	443.2±16.3^^^^	537.1.8±13.52^
	H	559.6±30.22	503.1±18.82	460.3±19.56	517.7±14.86
l-ornithine	C	2.0±0.12	2.56±0.34	2.71±0.16	2.89±0.14
	H	2.52±0.14	2.69±0.15	2.69±0.14	3.19±0.14^
		control	MK-801 (0.3)	MK-801/CDPPB	MK-801/LY487379
l-citruline	C	2.22±0.2	1.64±0.09	*3.03±0.16*^^^^	*2.32±0.13*^
	H	1.8±0.11	1.67±0.2	4.01±0.2^^^^	2.69±0.2^^
l-glutamate	C	1327±134.7	*1047±25.96**	1180±31.44	1239±44.33
	H	697.7±19.94	805.7±41.2	1132.4±38.24^^^^	901.4±45.81^^
l-glutamine	C	689.5±24.2	685.4±16.63	666.2±28.47	698.8±19.28
	H	519.6±15.15	551.7±33.21	691.9±20.17^^^	584.5±34.4
l-ornithine	C	10.18±0.57	10.56±0.2	1.85±0.12^^^^	1.76±0.09^^^^
	H	10.02±0.2	10.31±0.2	2.24±0.16^^^^	1.9±0.07^^^^

B-chronic		control	Scop (1)	Scop/CDPPB	Scop/CDPPB	Scop/LY487379 (0.1)	Scop/LY487379 (1)
l-citruline	C	3.,34± 0.23	4.26±0.33*	2.63±0.10^^^	2.98±0.181 ^^	2.76±0.14 ^^^	2.74±0.18^^^
	H	3.88±0.17	3.391±0.3	2.79±0,1**	2.89±0.16**	3.04±0.19	3.06±0.24
l-glutamate	C	314.5±15.5	299.9±12.37	236±5.5^^^	239±4.7^^	236±7.2^^	233±5.11^^^
	H	282±9.92	229.9±10.41***	193±8.4^	194±6.02^	204±6.54***	200±7.5***
l-glutamine	C	751.6±38.8	814.2±46.31	665±23^	616±26^^	648±18^^	631±15^^
	H	762.5±35.1	639±35.44****	604±26**	547±22****	601±16**	585±19***
l-ornithine	C	7.185±0.22	7.649±0.15	7.25±0.065	7.25±0.13	6.97±0.09	7.09±0.09
	H	7.68±0.39	7.8±0.32	7.05±0.14	7.24±0.21	7.03±0.12	7.02±0.12
		control	MK-801 (0.3)	MK-801/CDPPB	MK-801CDPPB	MK-801/LY487379	MK-801/ LY487379
l-citruline	C	2.65 ±0.2	14.97±0.82****	2.58±0,12^^^^	2.15±0.17^^^^	2.5±0.15 ^^^^	2.35±0.27 ^^^^
	H	2.9±0.11	1.2±0.17****	2.93±0.2^^^^	2.84±0.15^^^^	2.76±0.14^^^^	2.66±0.22 ^^^^
l-glutamate	C	1285±32.7	921±18.75****	1367±22.53^^^^	1363±43.8^^^^	1313±30.63 ^^^^	1398±36.27 ^^^^
	H	1115±33.2	751.7±28.23***	1201±35.28 ^^^^	1210±35.64 ^^^^	1098±28.31^^^^	1147±26.28 ^^^^
l-glutamine	C	878.7±26.6	624.7±20.91***	1049±24.84^^^^	920±25.55 ^^^^	919±35.3 ^^^^	929±39 ^^^^
	H	789.7±31.2	550±17.84****	987.3±29.27 ^^^^	879±34.14 ^^^^	823.8±27.43 ^^^^	1147±26.28 ^^^^
l-ornithine	C	1.85±0.04	10.78±0.23****	1.97±0,07 ^^^^	2.01±0.09 ^^^^	1.74±0.1 ^^^^	2.02±0.09 ^^^^
	H	2.04±0.08	10.60±0.27****	2.58±0.27 ^^^^	2.22±0.09 ^^^^	2.14±0.12^^^^	1.83±0.13 ^^^^

physiological balance and proper functioning of the remaining neurotransmitters and central nervous system (CNS) stability. Excessive activation of the glutamatergic system is observed in different pathologies, including Alzheimer's disease or schizophrenia [3,4].

Glutamatergic receptors are basic mechanisms through which glutamate exerts its effects. The receptors may mediate both fast synaptic neurotransmission (ion channels, such as NMDA, AMPA, or KA) and slow synaptic transmission (metabotropic glutamate receptors, mGlu) [5].

Since the early 90s, metabotropic receptors for glutamate (mGlu) have been considered therapeutic targets. This family consists of 8 subtypes divided into three groups according to sequence homology, pharmacology, and second messenger system they activate [6]. The receptors may be expressed postsynaptically and mediate slow synaptic currents (mGlu5 and mGlu1) or maybe expressed presynaptically regulating glutamatergic neurotransmission (i.e mGlu2 or mGlu4 receptors) [5].

Among all these subtypes, mGlu5 or mGlu2 receptor activators have been well described as putative antipsychotic agents [7–12], and to a lesser extend as anti-neurodegenerative agents, including anti--Alzheimer's activity [13–15]. Pharmacotherapy based on mGlu receptors has opened new possibilities in neuropsychopharmacology, but despite a large number of attempts, there has been no success in introducing mGlu-based drugs into regular use. Lack of efficacy compared to a placebo or the incidence of adverse effects are among the main limitations [16,17]. Expanding the knowledge of the mGlu-mediated molecular mechanisms is essential when considering their use in humans.

Nitric oxide-dependent pathways are essential in the regulation of brain functioning. Nitric-oxide synthases (NOS) catalyze the production of NO˙, a key chemical molecule involved in normal neurovascular unit function or LTP process. L-arginine serves as the substrate for NO˙ production, and its limited availability may lead to so-called NOS uncoupling, which results in the production of reactive oxygen species (ROS) instead of NO˙ [18]. Endogenous compounds that may interfere with L-arginine in the production of NO˙ are L-arginine derivatives, such as asymmetric dimethylarginine (ADMA), symmetric dimethylarginine (SDMA) or N-methyl-L-arginine (NMMA) [19], which are created as a results of formation of methylated residues on histones and non-histone proteins catalyzed by protein arginine methyltransferases family (PRMT), including PRMT5 member, which plays the role in different pathologies [21,22]. Methylarginines are degraded by dimethylarginine

dimethylaminohydrolase (DDAH) [20].

In this set of studies, we investigated L-arginine derivatives, DDAH1 and PRMT5 expression in animal models of cognitive dysfunction and the impact of mGlu5 positive allosteric modulator, CDPPB, or mGlu2 PAM, LY487379 on these parameters after acute and prolonged administration.

2. Materials and methods

2.1. Animals

Male Albino Swiss mice (Charles River Laboratory, Sulzfeld, Germany) weighing between 20 and 25 g were used throughout this study. The animals were kept in standard laboratory conditions at room temperature 22 ± 1 °C under a 12/12 light/dark cycle in accordance with EU Directive 2010/63/EU and subsequent regulations of the Polish Ministry of Agriculture and Rural Development. Food and water were freely available. Each experimental group consisted of at least 7-8 animals. The compounds were administered intraperitoneally at a volume of 10 ml/kg.

2.2. Drugs

MK-801 and LY487379 were purchased from Tocris, Bristol, UK, while scopolamine and CDPPB were obtained from Abcam, Cambridge, UK. The doses of the compounds are indicated under each graph and were adjusted according to previous studies [23–26]. MK-801 and scopolamine were dissolved in 0.9 % NaCl, LY487379 was dissolved firstly in 100 μl of DMSO and then was adjusted with 0.9 % saline to appropriate volume and CDPPB was dissolved in 10 % Tween 80. Control animals received vehicles instead of drug injections (0.9 % NaCl and 10 % Tween 80).

The Table with the catalog number and provider for every chemical used in this study was added in suplementary files (Table 1, Suppl).

2.3. Biochemical studies

Drug regimens.

Acute administration at active doses:

LY487379 -1 mg/kg or CDPPB -5 mg/kg with MK-801-0.3 mg/kg; LY487379 -1 mg/kg or CDPPB -2 mg/kg with scopolamine- 1 mg/kg.

The frontal cortex (FC) and hippocampus from each animal were dissected 30 min after administration.

Chronic administration for 14 days at low and top doses.

LY487379—0.1 or 1 mg/kg; CDPPB—0.1 and 5 mg/kg with MK-801 -0.3 mg/kg; LY487379—0.1 or 1 mg/kg; CDPPB—0.5 and 2 mg/kg with scopolamine −1 mg/kg.

The FC and hippocampus from each animal were dissected 24 h after the last administration.

The frontal cortex and hippocampus from each animal were dissected, frozen on dry ice, and ground in liquid nitrogen to a powder.

2.3.1. Western blotting

Ground samples were transferred to an ice-cold RIPA lysis buffer (Cell Signaling Technology, Leiden, The Netherlands) supplemented with PMSF (1 mM) and Protease Inhibitor Cocktail (Sigma-Aldrich, Darmstadt, Germany). The samples were vortexed and incubated on ice for 15 min. After the incubation period, the samples were centrifuged at 4 °C (12000×*g* for 15 min), and the supernatants were collected. The protein concentration in the obtained supernatants was measured with the DC™ Protein Assay Kit II (Bio-Rad, Basel, Switzerland).

The samples were then diluted with Laemmli buffer (Bio-Rad, Basel, Switzerland) containing 2.5 % β-mercaptoethanol. Then, 10 μL of each sample, containing 40 μg of total protein, was loaded onto 4–15 % polyacrylamide gels (Bio-Rad, Basel, Switzerland) along with 3-Colour Prestained Protein Marker (10–245 kDa) and submerged in ice-cold running buffer (Bio-Rad, Basel, Switzerland). Subsequently, low-temperature SDS-PAGE was performed in an ice bath.

In the next step, the gels were imaged under UV light to enable the measurement of the total protein levels for loading control. The proteins were transferred to PVDF membranes (Bio-Rad, Basel, Switzerland) using the Trans-Blot® Turbo™ Transfer System (Bio-Rad, Basel, Switzerland). The membranes were blocked with 5 % BSA (Sigma-Aldrich, Darmstadt, Germany) for 30 min and cut into parts to determine the level of DDAH1 and PRMT5 proteins from one blot. Membranes were incubated with primary rabbit, DDAH1 (1:10000 dilution, Abcam, Cambridge, UK) or PRMT5 (1:10000 dilution, Abcam, Cambridge, UK) antibodies at 4 °C overnight. The next day, after extensive washing, the membranes were incubated with a goat anti-rabbit IgG HRP-conjugated secondary antibody (1:2000 dilution, Cell Signaling Technology, Leiden, The Netherlands) at room temperature for 1 h. After incubation with VisiGlo™ HRP chemiluminescent substrate (VWR, Radnor, PA, USA), the membranes were visualized using the ChemiDoc Imaging System (Bio-Rad, Basel, Switzerland).

Densitometric measurements of the PRMT5 and DDAH1 expression and total protein level were performed using Image Lab software v. 4.1 (BioRad, Basel, Switzerland). The densities of the obtained bands of PRMT5 and DDAH1 were first normalized to the total protein levels established from the gels, and then the ratio was calculated.

2.3.2. Mass spectrometry analysis

The plasma extraction (50 μL) was performed using a protein precipitation method, using three volumes of ice-cold acetonitrile containing an internal standard (IS) (L-Arg-C13, 4 μM). The extract was vortexed in a thermomixer for 15 min at 4 °C, followed by centrifugation at 14000 rpm for 20 min at 4 °C. The supernatant was transferred to a new Eppendorf tube and evaporated to dryness in a vacuum evaporator at 45 °C for 1 h. Samples were reconstituted (50 μL) in 0.1 % formic acid (FA) solution in water, then centrifuged at 14000 rpm for 15 min at 4 °C and analyzed by means of LC/MS/MS for ADMA, SDMA, and NMMA. A total of 5 μL of each supernatant was diluted to 50 μL with 0.1 % FA in water containing 4 μM IS and analyzed for L-Arg, L-Cit, L-Glu, L-Gln, and L-Orn. A QTRAP 4500 (AB Sciex) mass spectrometer in positive ion mode in Multiple Reaction Monitoring (MRM screening) coupled with UHPLC (NEXERA XR, Shimadzu) was used. Chromatographic separation was achieved on an Amino Acid 3u 50 × 2mm (Intrada) column at a flow rate of 0.3 mL/min at 50 °C. The gradient program was as follows: 15–100 % B (3 min), 100 % B (0.5 min), 100–15 % B (0.1 min), 15 % B (3.4 min), solvent A (84 % acetonitrile with 0.3 % formic acid in 25 mM ammonium formate) and solvent B (20 % acetonitrile in 200 mM ammonium formate). The Analyst software was used to control the instrument and collect data, and Multiquant 3.0.2 software was used for quantification analysis. The electrospray ionization source was fitted with a stainless-steel capillary (100 μm i.d.). The ion transfer tube temperature was 300 °C. The spray voltage, collision energy, declustering potential, and gas parameters were optimized to achieve the maximum response using the test compounds. Selected reaction monitoring was carried out using the transition parameters, as previously described [25]. The calibration curves were constructed, and a linear response was obtained in the ranges of 0.05–10 μM (ADMA, SDMA, NMMA), 3–100 μM (L-Arg, L-Cit), 6–200 μM (L-Glu, L-Orn) and 0, 1–1000 μM (L-Gln) by spiking known amounts of each compound in control plasma.

Brain samples were ground to powder in liquid nitrogen, suspended in a solution of 10 % perchloric acid and acetonitrile (1:1 ratio) in a ratio of 200 μL buffer per 50 mg tissue. Samples were incubated in a thermomixer at 4 °C for 15 min and centrifuged at 12000×*g* at 4 °C for 15 min. Samples were kept frozen at −80 °C for LC/MS/MS analysis.

2.4. Novel object recognition

The NOR test was performed according to the previously described

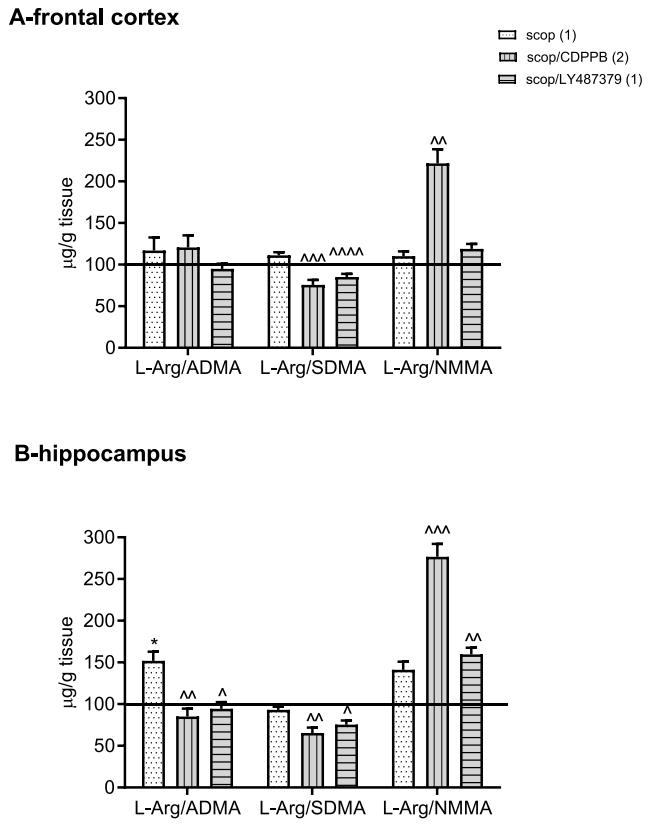

Fig. 1. The impact of CDPPB or LY487379 administration on L-Arg/ADMA, L-Arg/SDMA, and L-Arg/NMMA in the frontal cortex (A) and hippocampus (B) of scopolamine-driven animals after acute administration. The doses of the compounds used are indicated in parentheses and expressed in mg/kg b.w. The data are presented as a percentage of control ±SEM. The differences are significant according to one-way ANOVA followed by Tukey's post-hoc test. At least * $P < 0.04$ compared to the control group, and at least ^ $P < 0.04$, ^ $P < 0.009$, ^ $P < 0.0001$ compared to the scopolamine group.

method [25,27]. Briefly, a black, plastic, rectangular arena illuminated with a 355-lux bulb situated in a dark room was used to perform the habituation, training, and test trials. During the two-day-long habituation trial, the mice were allowed to explore the arena in the absence of objects for 10 min per day. The next day, the training (T1) and test (T2) trials were performed. In both T1 and T2, the animals were allowed to explore objects freely for 5 min. Throughout T1, two identical objects were used. In T2 (1 h later), one of the objects was replaced with a new one. The time spent exploring (i.e., sniffing or touching) the familiar (TF) or novel object (TN) was measured by a trained observer, and then the recognition index was calculated for each mouse $[(TN - TF)/(TF + TN)] \times 100$.

2.5. Statistics

The data are presented as the percentage of the control, except NOR results that are presented as means ± S.E.M. All statistical analysis was performed on the raw data with GraphPad Prism 10, using one-way ANOVA (or nonparametric analysis when the normal distribution was not met), followed by Tukey's post hoc comparison. A P value < 0.05 was considered to be statistically significant.

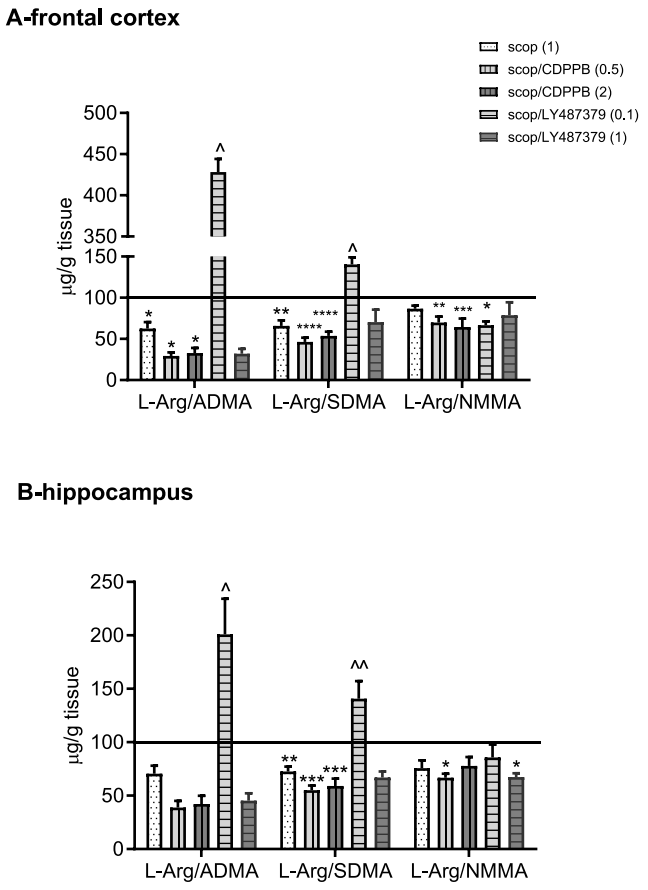

Fig. 2. The impact of CDPPB or LY487379 administration on L-Arg/ADMA, L-Arg/SDMA, and L-Arg/NMMA in the cortex (A) and hippocampus (B) of scopolamine-driven animals after 14 days of administration. The doses of the compounds used are indicated in parentheses and expressed in mg/kg b.w. The data are presented as a percentage of control ± SEM. The differences are significant according to one-way ANOVA followed by Tukey's post-hoc test. At least * $P < 0.03$, **$P < 0.007$, and ***$P < 0.0005$ compared to the control group, and at least ^ $P < 0.03$, ^ $P < 0.005$ compared to the scopolamine group.

3. Result

3.1. L-arginine derivatives

Acute scopolamine administration induced no statistically significant changes in L-Arg/ADMA, L-Arg/SDMA, and L-Arg/NMMA in the cortex. One-way ANOVA followed by Tukey's post hoc comparison revealed that L-Arg/NMMA was increased after LY487379 at the dose 1 mg/kg ($F_{(2.22)} = 7.9$, $P = 0.002$) (Fig. 1A).

In the hippocampus, an L-Arg/ADMA ratio was observed, and the effect was reversed both after CDPPB and LY487379 administration ($F_{(2.24)} = 5.05$, $P = 0.05$ and $F_{(2.24)} = 6.13$, $P = 0.007$). Decreases in L-Arg/SDMA after CDPPB and LY487379 were statistically significant, ($F_{(2.22)} = 8.62$, $P = 0.001$ and $F_{(2.23)} = 17.24$, $P = 0.0001$), respectively. Both compounds induced an increase in the L-Arg/NMMA ratio ($F_{(2.23)} = 6.74$, $P = 0.04$ and $F_{(2.23)} = 11.72$, $P = 0.0003$) (Fig. 1B).

Chronic scopolamine administration induced a statistically significant decrease in L-Arg/ADMA and L-Arg/SDMA in the cortex. The decrease in L-Arg/NMMA was not statistically significant. One-way ANOVA followed by Tukey's post hoc comparison revealed that L-Arg/ADMA and L-Arg/SDMA were reversed after CDPPB administration at the dose 0.5 mg/kg ($F_{(3.33)} = 18.56$, $P < 0.0001$ and $F_{(3.29)} = 15.69$, $P < 0.0001$). The administration of LY487379 deepened scopolamine-

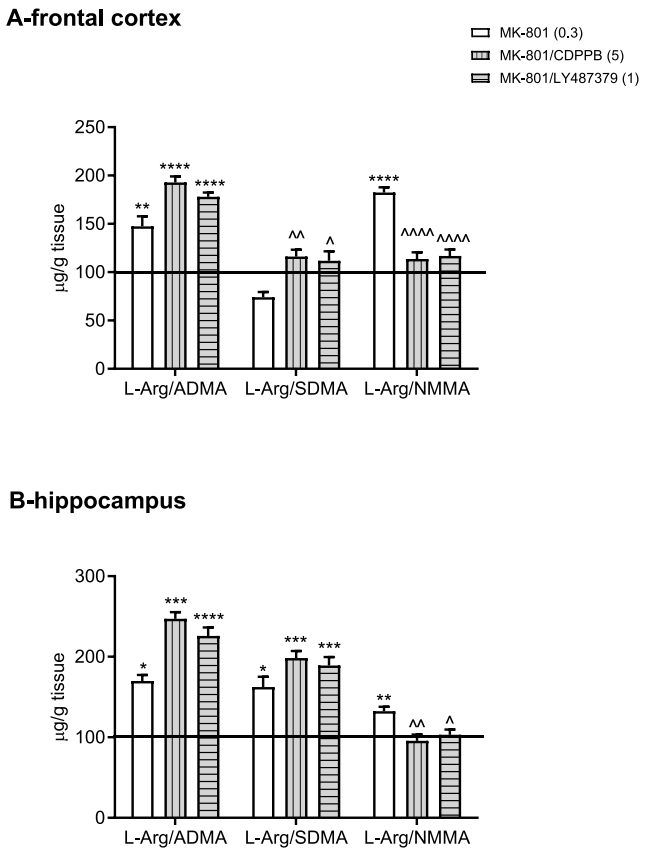

Fig. 3. The impact of CDPPB or LY487379 administration on L-Arg/ADMA, L-Arg/SDMA, and L-Arg/NMMA in the cortex (A) and hippocampus (B) of MK-801-driven animals after acute administration. The doses of the compounds used are indicated in parentheses and expressed in mg/kg b.w. The data are presented as a percentage of control ± SEM. The differences are significant according to one-way ANOVA followed by Tukey's post-hoc test. At least * $P < 0.04$, ** $P < 0.009$, *** $P < 0.0005$, and **** $P < 0.0001$ comparing to control group, and at least ^ $P < 0.01$, ^ $P < 0.003$, and ^ $P < 0.0001$ comparing to MK-801 group.

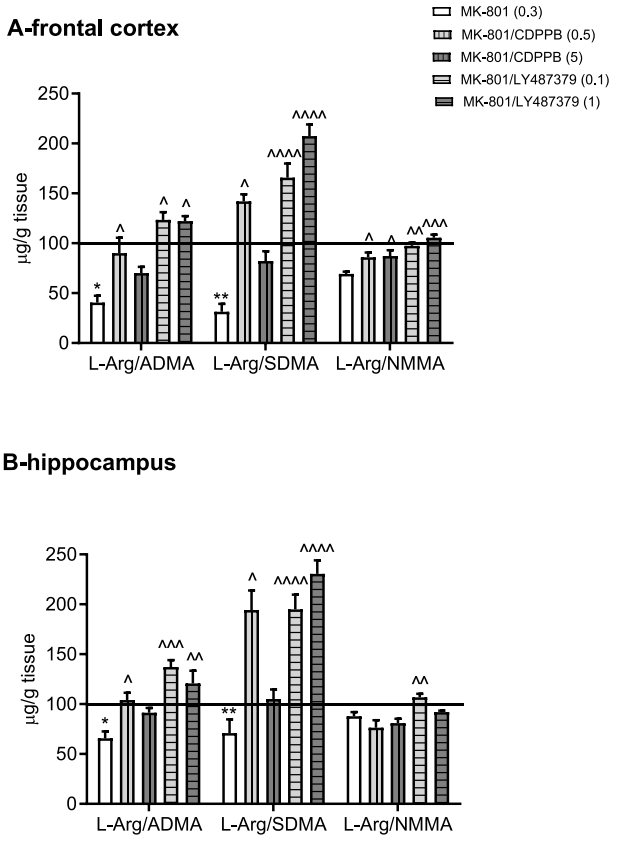

Fig. 4. The impact of CDPPB or LY487379 administration on L-Arg/ADMA, L-Arg/SDMA, and L-Arg/NMMA in the cortex (A) and hippocampus (B) of MK-801-driven animals after 14 days of administration. The doses of the compounds used are indicated in parentheses and expressed in mg/kg b.w. The data are presented as a percentage of control ± SEM. The differences are significant according to one-way ANOVA followed by Tukey's post-hoc test. At least * $P < 0.01$, ** $P < 0.002$ compared to control groups, and at least ^ $P < 0.02$, ^ $P < 0.007$, ^ $P < 0.0005$, and ^ $P < 0.0001$ comparing to the MK-801 group.

induced effect on L-Arg/ADMA, L-Arg/SDMA, and L-Arg/NMMA ($F_{(3.30)} = 25.21$, $P < 0.001$, $F_{(3.31)} = 11.99$, $P < 0.0001$ and $F_{(3.30)} = 3.6$, $P = 0.02$) (Fig. 2A).

In the hippocampus, no changes in L-Arg/ADMA were observed, while decreases in L-Arg/SDMA and L-Arg/NMMA were statistically significant. CDPPB at the dose 0.5 mg/kg increased L-Arg/ADMA and L-Arg/SDMA ratios ($F_{(3.29)} = 13.94$, $P < 0.0001$ and $F_{(3.29)} = 19.23$, $P < 0.0001$), while LY487379 decreased L-Arg/ADMA ratio ($F_{(3.28)} = 4.75$, $P = 0.008$) and had no effect on scopolamine-induced deficit in L-Arg/SDMA ($F_{(3.31)} = 6.15$, $P = 0.002$). L-Arg/NMMA ratio was decreased in the hippocampus, and both compounds aggravated this effect ($F_{(3.30)} = 4.56$, $P = 0.009$ and $F_{(3.31)} = 3.39$, $P = 0.03$) (Fig. 2B).

Acute MK-801 administration induced an increase in L-Arg/ADMA and L-Arg/NMMA in the cortex and no statistically significant decrease in L-Arg/SDMA. One-way ANOVA followed by Tukey's post hoc comparison revealed that the administration of CDPPB (5 mg/kg) and LY487379 (1 mg/kg) increased L-Arg/ADMA ($F_{(2.25)} = 15.08$, $P < 0.0001$ and $F_{(2.24)} = 15.56$, $P < 0.0001$) and L-Arg/SDMA ($F_{(2.22)} = 4.58$, $P < 0.02$ and $F_{(2.21)} = 8.74$, $P < 0.001$). Both compounds prevented MK-801-induced effect on L-Arg/NMMA ($F_{(2.23)} = 21.16$, $P < 0.0001$ and $F_{(2.21)} = 6.9$, $P = 0.004$) (Fig. 3A).

In the hippocampus, increases in L-Arg/ADMA, L-Arg/SDMA, and L-Arg/NMMA were observed. CDPPB and LY487379 administration aggravated the effect ($F_{(2.24)} = 15.64$, $P = 0.0001$, $F_{(2.25)} = 23.96$, $P < 0.0001$ and $F_{(2.22)} = 6.76$, $P < 0.005$, $F_{(2.24)} = 8.7$, $P = 0.001$). Both compounds prevented MK-801-induced effect on L-Arg/NMMA ($F_{(2.22)} = 22.26$, $P < 0.0001$ and $F_{(2.22)} = 8.01$, $P < 0.002$) (Fig. 3B).

Chronic MK-801 administration induced a statistically significant decrease in L-Arg/ADMA, L-Arg/SDMA, and L-Arg/NMMA ratios in the cortex. One-way ANOVA followed by Tukey's post hoc comparison revealed that CDPPB prevented this MK-801-induced effect on all L-arginine derivatives ($F_{(3.33)} = 18.6$, $P < 0.0001$; $F_{(3.29)} = 15.69$, $P < 0.0001$; $F_{(3.30)} = 7.84$, $P = 0.0005$) and the effect of LY487379 was clear at the dose of 0.1 mg/kg ($F_{(3.30)} = 5.22$, $P = 0.005$; $F_{(3.31)} = 11.99$, $P < 0.0001$; $F_{(3.30)} = 3.69$, $P = 0.02$) (Fig. 4A).

In the hippocampus, decrease in L-Arg/ADMA and L-Arg/SDMA were statistically significant, while L-Arg/NMMA decrease did not reach statistical significance. CDPPB reversed this MK-801-induced effect ($F_{(3.29)} = 13.94$, $P < 0.0001$; $F_{(3.29)} = 19.23$, $P < 0.0001$; $F_{(3.30)} = 4.56$, $P = 0.0009$). The effect of LY487379 reversed L-Arg/ADMA and L-Arg/SDMA deficits at the dose of 0.1 mg/kg ($F_{(3.30)} = 11.52$, $P = 0.0001$; $F_{(3.31)} = 6.15$, $P < 0.002$). L-Arg/NMMA ratio was not influenced by the compound (Fig. 4B).

The levels of remaining amino acid tested, L-citruline, L-glutamate, L-glutamine, and L-ornithine, were impacted by the scopolamine and MK-801 administration, and the MK-801-induced effect was more evident. Both investigated compounds normalized the investigated amino acid levels in the MK-801-driven model after chronic administration. The

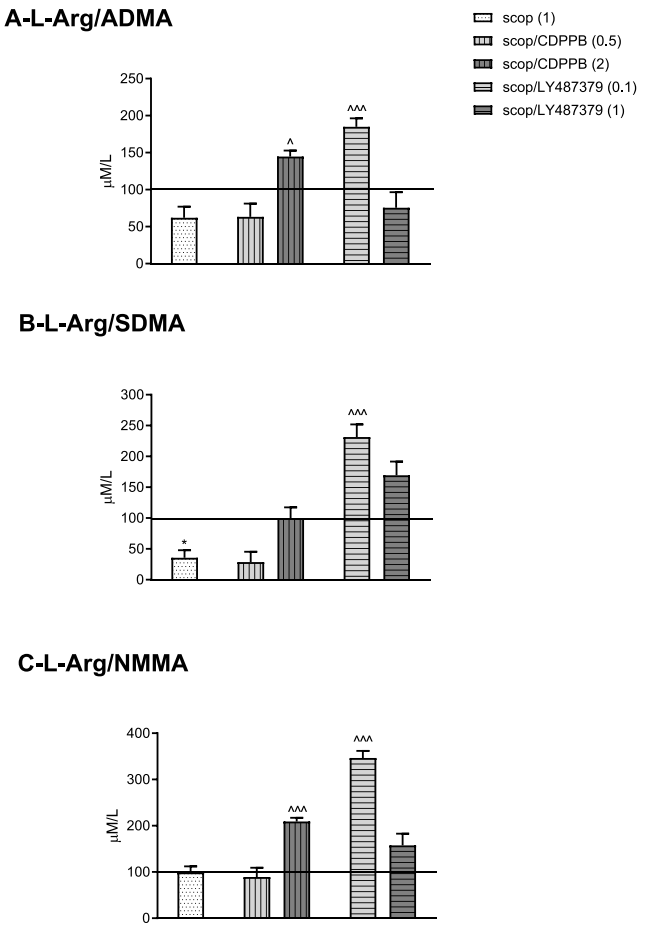

Fig. 5. The impact of CDPPB or LY487379 administration on L-Arg/ADMA (A), L-Arg/SDMA (B), and L-Arg/NMMA (C) content in the blood plasma of scopolamine-treated animals after 14 days administration. The doses of the compounds used are indicated in parentheses and expressed in mg/kg b.w. The data are presented as a percentage of control ± SEM. The differences are significant according to one-way ANOVA followed by Tukey's post-hoc test. At least * $P < 0.01$ compared to the control group, and at least $P < 0.02$, and^$P < 0.0002$ compared to the scopolamine-treated group.

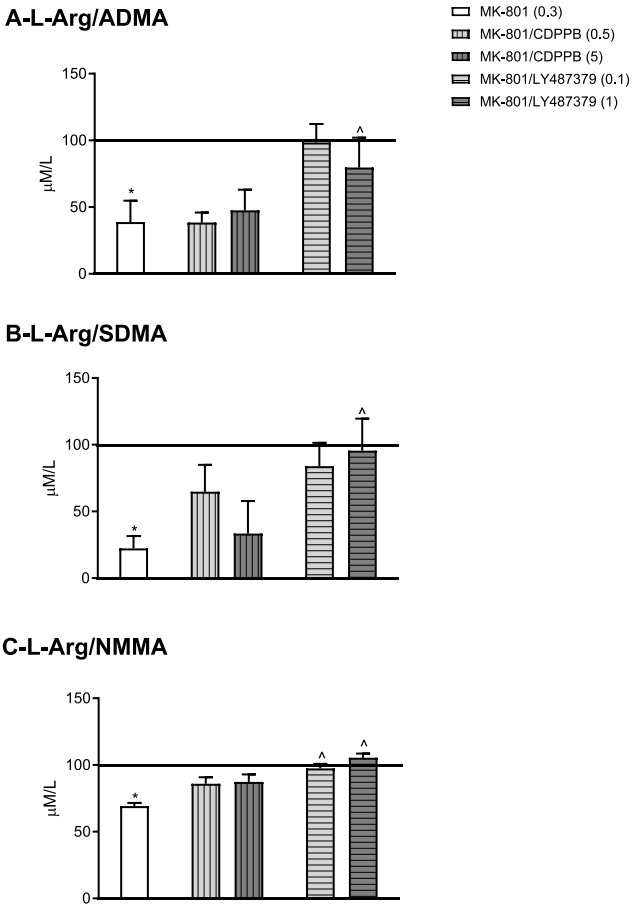

Fig. 6. The impact of CDPPB or LY487379 administration on L-Arg/ADMA (A), L-Arg/SDMA (B), and L-Arg/NMMA (C) content in the blood plasma of MK-801-treated animals after 14 days administration. The doses of the compounds used are indicated in parentheses and expressed in mg/kg b.w. The data are presented as a percentage of control ± SEM. The differences are significant according to one-way ANOVA followed by Tukey's post-hoc test. At least * $P < 0.05$ compared to the control group and at least $P < 0.02$ compared to the MK-801-treated group.

results are presented in Table 1.

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3.2. L-arginine derivatives in plasma

Chronic scopolamine administration decreased L-Arg/ADMA ratio in non-statistically significant manner. CDPPB at the dose 2 mg/kg elevated L-Arg/ADMA ratio above scopolamine and the control level, $F_{(3.30)} = 4.67$, $P = 0.008$ and similar effect was observed after LY487379 administration (1 mg/kg) $F_{(3.30)} = 7.86$, $P = 0.0005$ (Fig. 5A).

Chronic administration of scopolamine decreased the L-Arg/SDMA ratio. One-way ANOVA followed by Tukey's post hoc comparisons revealed that CDPPB at the dose of 2 mg/kg increased L-Arg/SDMA ($F_{(3.33)} = 3.47$, $P = 0.02$), similarly as LY487379 at the dose 0.1 mg/kg ($F_{(3.25)} = 7.67$, $P = 0.0008$ (Fig. 5B).

L-Arg/NMMA ratio was not changed after scopolamine administration, CDPPB at the dose of 2 mg/kg and LY487379 at the dose of 0.1 mg/kg increased the L-Arg/NMMA ratio compared to both control and scopolamine groups ($F_{(3.30)} = 10.29$, $P < 0.0001$ and $F_{(3.30)} = 10.63$, $P < 0.0001$, respectively) (Fig. 5C).

Chronic administration of MK-801 decreased L-Arg/ADMA ratio and CDPPB at both doses (0.5 and 5 mg/kg) had no influence on this parameter ($F_{(3.31)} = 8.38$, $P = 0.0003$). LY487379 administration at the dose 1 mg/kg prevented this effect, the effect of the lower dose was not statistically significant ($F_{(3.27)} = 5.7$, $P = 0.003$) (Fig. 6A).

Chronic administration of MK-801 decreased L-Arg/SDMA ratio. One-way ANOVA followed by Tukey's post hoc comparisons revealed that CDPPB was not active, and LY487379 reversed this effect at a dose 1 mg/kg only ($F_{(3.30)} = 4.29$, $P = 0.01$ and $F_{(3.28)} = 5.11$, $P = 0.006$, respectively) (Fig. 6B).

Chronic administration of MK-801 decreased L-Arg/NMMA ratio, and CDPPB had no effect on this parameter. LY487379 at both doses 0.1 and 1 mg/kg increased the L-Arg/NMMA ratio ($F_{(3.25)} = 6.97$, $P = 0.001$) (Fig. 6C).

3.3. DDAH1 and PRMT5 expression

Acute scopolamine administration decreased DDAH1 expression both in the cortex and hippocampi ($F_{(3.27)} = 5.1$, $P = 0.01$ and $F_{(3.27)} = 7.05$, $P < 0.003$). One-way ANOVA followed by Tukey's post hoc comparison revealed that neither of the investigated compounds reversed this scopolamine-induced effect (Fig. 7A).

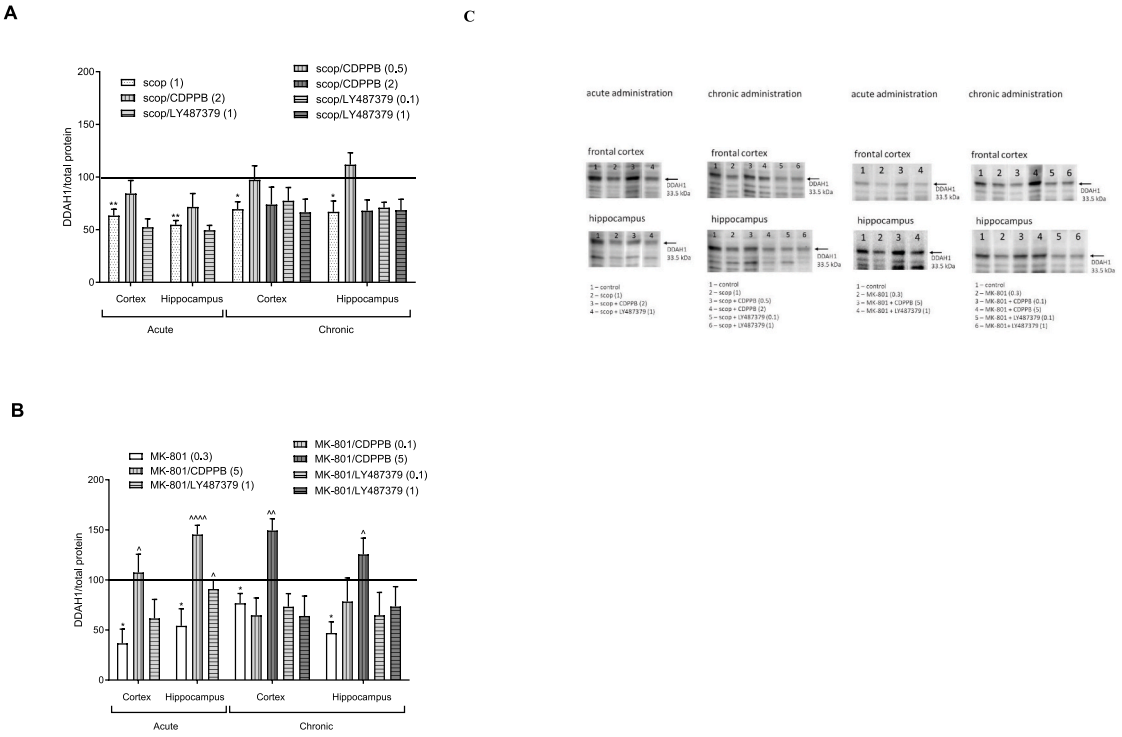

Fig. 7. The impact of CDPPB or LY487379 administration on DDAH1 expression in cortex and hippocampus of scopolamine- (A) or MK-801-treated (B) animals, after acute or chronic administration. The doses of the compounds used are indicated in parentheses and expressed in mg/kg b.w. The data are presented as a percentage of control ± SEM. The differences are significant according to one-way ANOVA followed by Tukey's post-hoc test. At least * $P < 0.03$, and **$P < 0.002$ compared to control groups, and at least ^$P < 0.04$, ^$P < 0.001$, and ^$P < 0.0001$ compared to MK-801 treated groups. Representative blots (C).

Chronic administration of scopolamine decreased DDAH1 expression both in the cortex and hippocampus ($F_{(3.29)} = 4.59$, $P = 0.008$ and $F_{(3.29)} = 6.71$, $P = 0.001$). Neither CDPPB nor LY487379 reversed this effect (Fig. 7A).

Acute MK-801 administration decreased DDAH1 expression both in the cortex and hippocampus. One-way ANOVA followed by Tukey's post hoc comparison revealed that CDPPB reversed this effect at the dose 5 mg/kg ($F_{(2.21)} = 3.91$, $P = 0.03$ and $F_{(2.24)} = 15.35$, $P < 0.0001$). LY487379 was effectively reversed MK-801-induced effect in the hippocampus only ($F_{(2.24)} = 6.43$, $P = 0.005$) (Fig. 7B).

Chronic administration of MK-801 decreased DDAH1 expression both in the cortex and hippocampus. One-way ANOVA revealed that only CDPPB at the dose of 5 mg/kg reversed this effect in both structures ($F_{(3.30)} = 97$, $P = 0.00018$ and $F_{(3.30)} = 3.75$, $P = 0.02$), and LY487379 was not effective (Fig. 7B). Representative blots of each group are presented on Fig. 7C.

Scopolamine decreased PRMT5 administration expression in the cortex after acute administration ($F_{(3.27)} = 41.69$, $P = 0.0001$), and the effect was deepened after CDPPB or LY487379 administration. No changes in the hippocampus were observed (Fig. 8A).

Fourteen days of scopolamine administration induced a decrease in PRMT5 both in the cortex and hippocampus. CDPPB at the dose of 0.5 mg/kg reversed this effect in the cortex ($F_{(3.28)} = 5.8$, $P = 0.003$) and at both investigated doses in the hippocampus ($F_{(3.28)} = 8.3$, $P = 0.0004$). One-way ANOVA revealed no changes after LY487379 administration in the cortex and reversal of the scopolamine-induced effect at the dose 0.1 mg/kg in the hippocampus ($F_{(3.29)} = 9.9$, $P = 0.0001$) (Fig. 8A).

Acute MK-801 administration decreased PRMT5 expression in the cortex and hippocampus ($F_{(3.22)} = 6.53$, $P = 0.005$ and $F_{(3.23)} = 14.34$, $P < 0.0001$). CDPPB at the dose of 5 mg/kg reversed this effect in both structures, and LY487379 was not effective (Fig. 8B).

Fourteen days of MK-801 administration, PRMT5 expression both in the cortex and hippocampus. CDPPB at the dose of 5 mg/kg reversed this effect in both structures ($F_{(3.29)} = 6.53$, $P = 0.001$ and $F_{(3.29)} = 4.8$, $P = 0.007$). LY487379 was not effective (Fig. 8B). Representative blots of each group are presented on Fig. 8C.

3.4. Novel object recognition

One-way ANOVA followed by Tukey's post-hoc comparison revealed that chronic administration of scopolamine significantly reduced the RI (to the level as observed after single administration). CDPPB was administered at both investigated doses, 0.5 and 2 mg/kg, for.

14 days of LY487379 administration prevented scopolamine-induced disruptions also at both investigated doses, 0.1 and 1 mg/kg ($F_{(3.20)} = 14$, $P < 0.0001$). After single administration also, only a higher dose of 1 mg/kg was effective ($F_{(3.23)} = 20.04$, $P < 0.0001$).

Chronic administration of MK-801 decreased the recognition index in a similar way as its single administration. Fourteen days of CDPPB injections prevented this effect in both investigated doses 0.1 and 5 mg/kg ($F_{(3.32)} = 7.99$, $P = 0.0004$). Single administration of the compound was effective only at the dose 5 mg/kg ($F_{(3.32)} = 25.35$, $P < 0.0001$). 14 days of LY487379 administration prevented MK-801-induced disruptions at the dose 0.1 and 1 mg/kg ($F_{(3.31)} = 7.83$, $P = 0.005$). Single administration of the compound was active only at the dose of 1 mg/kg ($F_{(3.31)} = 31.91$, $P < 0.0001$) (Fig. 9).

4. Discussion

In the present studies, we show that L-arginine derivatives (ADMA/SDMA/NMMA) may interfere with NO˙ production in pharmacologically developed models of cognitive dysfunction related to Alzheimer's disease or schizophrenia. Concomitant impairments in DDAH1 or PRMT5 expressions were observed. Chronic administration of mGlu receptors

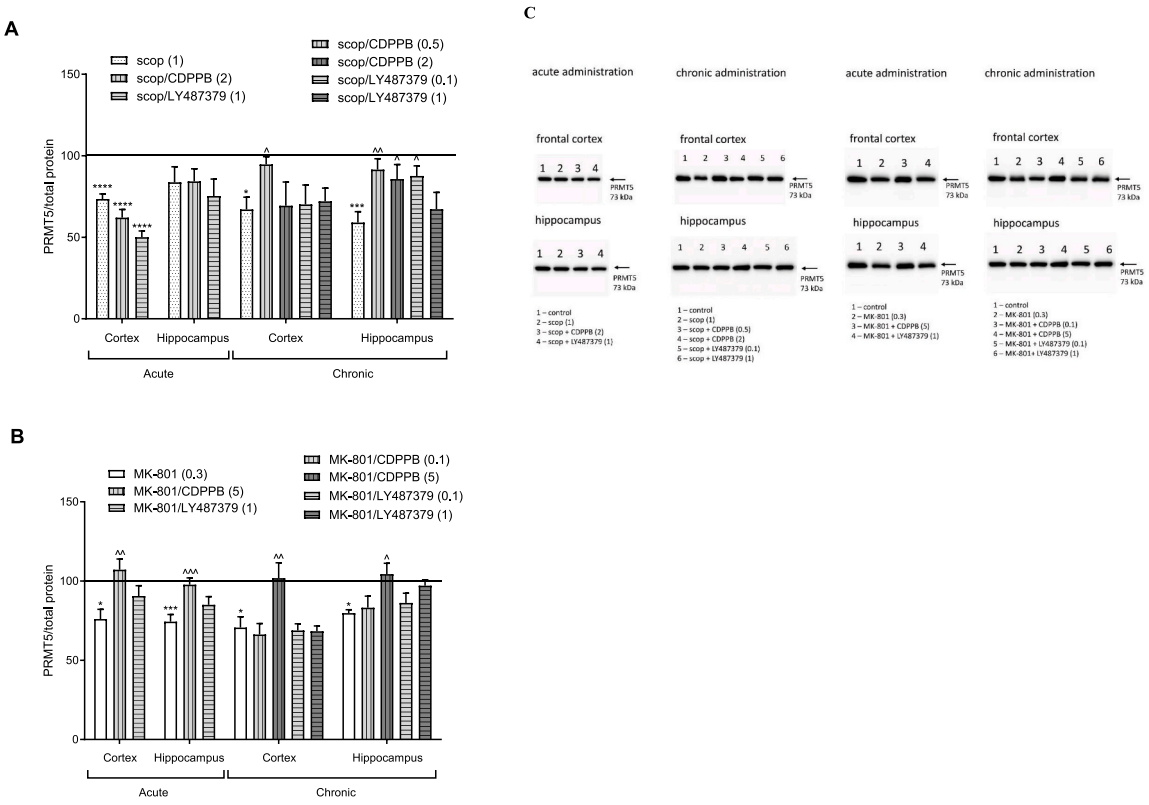

Fig. 8. The impact of CDPPB or LY487379 administration on PRMT5 expression in cortex and hippocampus of scopolamine- (A) or MK-801-treated (B) animals, after acute or chronic administration. The doses of the compounds used are indicated in parentheses and expressed in mg/kg b.w. The data are presented as a percentage of control ± SEM. The differences are significant according to one-way ANOVA followed by Tukey's post-hoc test. At least * $P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$ compared to control groups, and at least ˆ $P < 0.02$, ˆ $P < 0.004$, and ˆ $P < 0.0005$ comparing to scopolamine- or MK-801-treated groups accordingly. Representative blots (C).

activators, CDPPB or LY487379, normalized L-arginine derivatives in animal model of schizophrenia which was positively related with antipsychotic activity of the compounds. In contrast, in scopolamine-induced AD model only lower dose of CDPPB normalized L-arginine derivatives, and no effect of LY487379 was observed.

L-arginine is an essential substrate for NO˙ production, and L-arginine derivatives are endogenous compounds that interfere with NO˙ production which results in cascade of consequences as presented on Fig. 10.

ADMA is most abundant in plasma, and its increased level constitutes a risk factor for vascular dysfunction and hypertension [28], while SDMA or NMMA are more dominant in the brain, where they together constitute ~70 % of methylamines [29]. The imbalanced proportions of L-arginine and its derivatives may result in impaired NO˙ signaling and development of brain disorders, in which memory impairments are frequently observed [19,30,31]. In particular, decreased cGMP synthesis may affect LTP, as the molecule is crucial part of glutamate–NO–cGMP axis that regulates the process, and may contribute to decrease in some learning abilities [32]. In addition, the formation of free radicals is generated instead as shown on Fig. 10, which is the fundametal factor in the pathophysiology of he majority of brain disorders, including schizophrenia, autism or AD [33–39]. Thus, all treatments that normalize L-arginine bioavalibility may contribute to the improvement of cognitive functions. Clinical studies reveal higher levels of all arginine derivatives in AD and schizophrenic patients, calculated in comparison to L-arginine level [40–44]. Due to obvious ethical standards, the measurements were performed only in plasma. In the present studies similar dysfunctions were observed in scopolamine- or MK-801-treated mice regarded as pharmacological models of AD or schizophrenia, indicating that face validity of the models is adequate in this context. Accordingly, normalizing the changes within NO-related pathways could fit with clinical needs and contribute to safer and satisfactory action of the drugs. Up to date, no relevant research, except those from our group, has been published. Some reports indicate that ADMA exacerbated Aβ-induced toxicity and oxidative stress in human cells and *Caenorhabditis elegans* models of AD [45].

For years, mGlu receptor ligands have been investigated as alternatives for presently used pharmacotherapy, but their impact on NO-related pathways has not been well recognized. Recently, we showed that mGlu5 and mGlu2 activators may promote eNOS uncoupling through the induction of dimer dissociation, exaggerating the effect of scopolamine or MK-801 [26]. This risk places a shadow on the safety of the treatment using mGlu ligands, particularly for a longer time and at top doses. The current results rehabilitate to some extent mGlu2 and mGlu5 activators, as both CDPPB and LY487379 restored the L-arginine bioavailability in the brain; in plasma, the overall impact of LY487379 on MK-801-induced dysfunctions seems better than CDPPB.

To elucidate, at least in part, the mechanisms by which the compounds impact the level of L-arginine derivatives, the expression of DDAH1 was investigated. The enzyme plays pivotal role in the regulation of nitric oxide levels by degrading the main endogenous NOS inhibitors, in particular ADMA and NMMA. Dysregulation of DDAH1 was found in schizophrenia patients [46] and its role in the treatment of AD was also proposed [47]. Both tool compounds decreased DDAH1 amount in the brain, which stays in line with observed increased level of ADMA and NMMA after chronic treatment. As only CDPPB at one dose reversed this effect, it is questionable to what extend the counteraction of this effect is critical in the antipsychotic or antialzheimers potency of drugs, especially that in contrast to ADMA and NMMA, SDMA is not

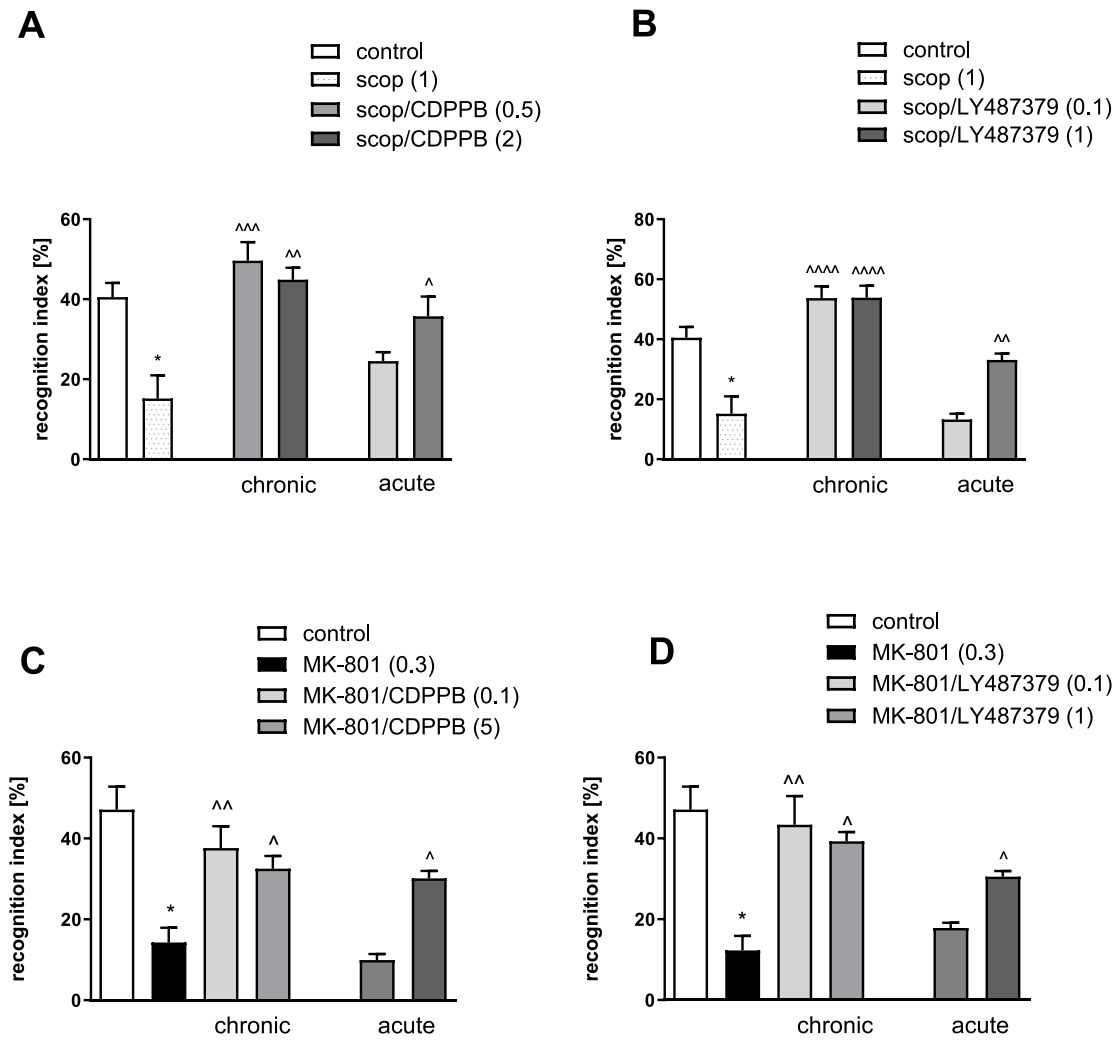

Fig. 9. The impact of CDPPB or LY487379 administration on recognition index (RI) in the novel object recognition test in scopolamine (A, B) and MK-801 (C, D) treated animals. The doses of the compounds used are indicated in parentheses and expressed in mg/kg b.w. The data are presented as means ± SEM. The differences are significant according to one-way ANOVA followed by Tukey's post-hoc test. At least * $P < 0.01$ compared to the control group, and at least $^{\wedge} P < 0.02$, $^{\wedge\wedge} P < 0.001$, $^{\wedge\wedge\wedge} P < 0.0001$ and $^{\wedge\wedge\wedge\wedge} P < 0.0005$ compared to the scopolamine- or MK-801-treated group.

metabolized by DDAH1 [19,46–48]. The possibility that DDAH1 undergoes rapid degradation or that the compounds change the activity of the enzyme through the regulatory role for e.g the Zn(II)-binding site [49] cannot be excluded.

NOS or DDAH1 are among the enzymes that also catalyze the other L-arginine metabolic pathways (conversion to L-citrulline, L-ornithine, L-glutamate or L-glutamine) [46,50,51]. These amino acids are important in maintaining the integrity of the blood-brain barrier (BBB) and brain homeostasis. Citrulline restores nitric oxide levels and cellular uptake at the brain capillary endothelial cell line (TR-BBB cells) with glutamate cytotoxicity [50], L-ornithine may improve the level of arginine [51] and the glutamate-glutamine cycle is a cyclic metabolic pathway which maintains a balance of the glutamate/GABA in the central nervous system. The administration of CDPPB or LY487379 normalized the level of all those amino acids that were dysregulated in the MK-801 model. This aspect of mGlu activators' activity may be an important factor in their action.

Up to date, no convincing data on L-arginine derivatives or DDAH1 in response to presently used pharmacological intervention in schizophrenic patients are available. The abnormal concentrations of ADMA, SDMA, citrulline, and ornithine in bipolar disorder patients were described [44,46]. Our results suggest that mGlu activators could serve not only as antipsychotic drugs [16,17,52], but potentially may be considered as the therapeutic solution for developing dementia. However, more studies regarding this are required as available data indicate that mGlu2 or mGlu5 antagonists may also be considered as anti-alzheimer's compounds [53–56]. Therefore mGlu2 or mGlu5 receptors could serve as double-edged swords in AD treatment [56].

Another important component in many pathologies including cancer, inflammation and immune responses is aberrant arginine methylation mediated by arginine methyltransferases and demethylases. The arginine residues mono- and di-methylation in histone or non-histone substrates is catalyzed by PRMTs, and as the result SDMA or ADMA are formed. Histone arginine methylation is crucial for controlling DNA damage repair, transcriptional activation or repression, and chromatin dynamics while non-histone proteins can act as substrates for PRMTs to control e.g receptor trafficking, protein stability, transcription, and DNA repair. Among eleven PRMT family members, PRMT5 is expressed in all types of brain cells, including neurons, astrocytes, microglia, and oligodendrocytes and is linked to crucial physiological processes such as

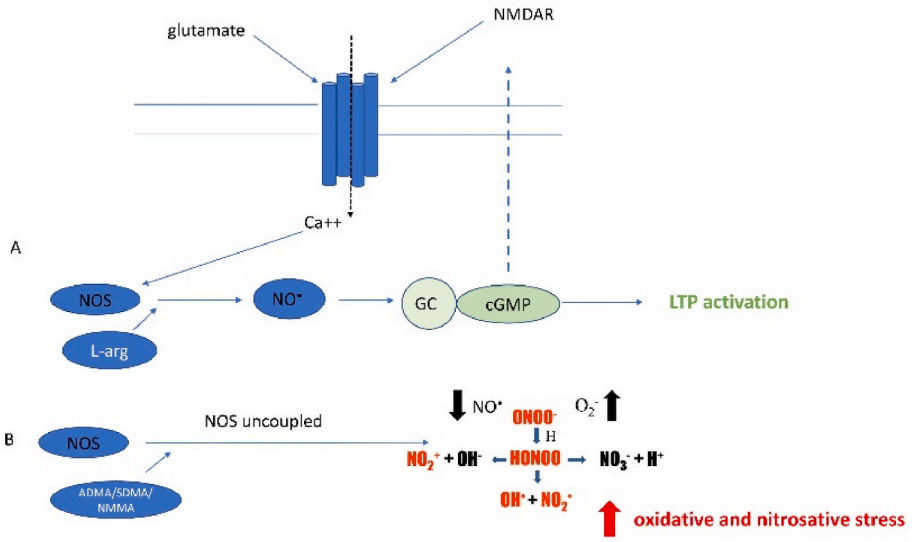

Fig. 10. The glutamate-nitric oxide-cGMP pathway (A). Activation of NMDA receptors leads to increased calcium in the post-synaptic neuron. Calcium activates nitric oxide synthase (NOS), increasing the formation of nitric oxide (NO), which, in turn, activates soluble guanylate cyclase (GC) and increases the formation of cGMP, activating long-term potentiation (LTP). Part of the cGMP formed is released to the extracellular fluid. (B) Under increased levels of L-arginine derivatives (ADMA/SDMA/NMMA) uncoupled NOS isoforms generate simultaneously high amounts of both $NO^{\bullet}$ and $O_2^{\bullet-}$ favoring production of $ONOO^-$. The uncoupling of NOS isoforms can be additionally potentiated by $O_2^{\bullet-}$ overproduction from other enzymatic sources in the cells. Oxidative and nitrosative stress develops [18].

differentiation, cell proliferation, gene regulation and for maintaining cerebrovascular homeostasis [57,58]. Available data indicate that overexpression of PRMT5 has been associated with brain cancers such as glioblastoma or medulloblastoma [22], but its deficiency has been linked with Huntington's disease pathogenesis [59] or Aβ-induced toxicity [60]. Up to date, no data on the expression of the PRMT5 gene or protein in schizophrenia or AD patients, eventually in animal models, are available. Our studies are the first on this subject and indicate on dysfunction in PRMT5 protein in MK-801 or scopolamine model. Investigated compounds, to some extent, prevented PRMT5 dysfunction, and in both models, the effect of CDPPB was more evident. Thus it seems that impaired PRMT5 function contributes to brain disorders while its overexpression promotes cancer development. Although the research on arginine methylation is still on early stages, there are indications that cells depleted of PRMT1, PRMT5 or PRMT7 are hypersensitive to DNA damage [57,58].

The studies were performed in scopolamine or MK-801-treated animals only. The data concerning the activity of the compounds alone were not made. The decision was made consciously and in relation to ethical standards concerning the use of laboratory animals in research. Majority of our previous studies revealed the lack of efficacy of investigated compounds when given alone. Moreover, drugs are rarely administered to healthy individuals, especially psychotropic drugs, therefore their effect alone is less significant comparing to their impact on already dysregulated system.

The present behavioural studies indicate that the efficacy of the low doses of compounds increases after prolonged administration along with MK-801 or scopolamine, and the efficacy of the top doses remains at the same level comparing to acute administration. It confirms that the tolerance towards pharmacologically active doses does not develop, and the sensitization for the pharmacological effect of lower doses increases. In conclusion, prolonged treatment with low doses of the compounds can be less burdened with the induction of adverse effects and have satisfactory effectivity. Based on recent investigations, the co-administration of NO donors may enhance the activity of mGlu ligands, especially at low doses [26]. Also, the simultaneous administration of CDPPB or LY487379 with the ligands of other receptors (muscarinic, GABAB, or 5-HT1A) can be considered [25,61]. In a variety of previous articles, the administration of ineffective doses of that combination exerted a synergistic effect. The other components of NO-dependent pathways, including cGMP production, s-nitrosilation and free radicals formation after mGlu activators administration are in progres and will be published soon.

To sum up, glutamate-induced dysfunction seems to be more vulnerable to mGlu-based treatment compared to cholinergic deprivation with scopolamine. Our results confirm that the antipsychotic indication of mGlu2 and mGlu5 activators is still one of the most promising targets in neuropsychopharmacology, but requires refinement when it comes to doses and treatment schemes. Still, more evidence proves the mGlu2 receptor is a better candidate. Considering the activity of the ligands in a broader scope of anti-dementia and neuroprotective actions requires further studies, and the observed detrimental effect on eNOS expression [26] must be considered. Low doses administration as adjuvant therapy seems to be optimal. The present results confirm that biochemical changes and preventive effects of the compounds are more stable and convincing after chronic and not single administration.

CRediT authorship contribution statement

Agata Płoska: Methodology, Investigation, Formal analysis. **Adrianna Radulska:** Methodology, Investigation, Formal analysis. **Anna Siekierzycka:** Methodology, Investigation, Formal analysis. **Paulina Cieślik:** Methodology, Investigation, Formal analysis. **Michał Santocki:** Methodology, Investigation. **Iwona T. Dobrucki:** Methodology, Investigation, Validation. **Leszek Kalinowski:** Supervision, Funding acquisition, Conceptualization, writint. **Joanna Wieronska:** Writing – original draft, writing-review, Supervision, Funding acquisition, Data curation, Conceptualization.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.niox.2024.11.010.

Data availability

Data will be made available on request.

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