Behavioural Brain Research 273 (2014) 23–33

Contents lists available at ScienceDirect

Behavioural Brain Research

journal homepage: www.elsevier.com/locate/bbr

Research report

Antidepressant-like effect of the mGluR5 antagonist MTEP in an astroglial degeneration model of depression

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HIGHLIGHTS

- Astroglial degeneration in the rat prefrontal cortex as a model of depression.
- Gliotoxin L-AAA induced depressive-like behavior in the forced swim test.
- Antidepressant-like effect of mGluR5 antagonist MTEP in the gliotoxin model.
- Imipramine antidepressant activity in the gliotoxin model.
- MTEP and imipramine exerted glioprotective effects in L-AAA treated rats.

ARTICLE INFO

Article history:
Received 16 April 2014
Received in revised form 8 July 2014
Accepted 11 July 2014
Available online 18 July 2014

Keywords:
Gliotoxin
L-AAA
Depression
mGluR5 antagonist
MTEP

ABSTRACT

The glutamatergic predominance in the excitatory-inhibitory balance is postulated to be involved in the pathogenesis of depression. Such imbalance may be induced by astrocyte ablation which reduces glutamate uptake and increases glutamate level in the synaptic cleft. In the present study, we tried to ascertain whether astroglial degeneration in the prefrontal cortex could serve as an animal model of depression and whether inhibition of glutamatergic transmission by the mGluR5 antagonist MTEP could have antidepressant potential.

Astrocytic toxins L-or DL-alpha-aminoadipic acid (AAA), 100 μg/2 μl, were microinjected, bilaterally into the rat medial prefrontal cortex (PFC) on the first and second day of experiment. MTEP (10 mg/kg) or imipramine (30 mg/kg) were administered on the fifth day. Following administration of MTEP or imipramine the forced swim test (FST) was performed for assessment of depressive-like behavior. The brains were taken out for analysis on day eight. The astrocytic marker, glial fibrillary acidic protein (GFAP) was quantified in PFC by Western blot method and by stereological counting of immunohistochemically stained sections.

Both L-AAA and DL-AAA induced a significant increase in immobility time in the FST. This effect was reversed by imipramine, which indicates depressive-like effects of these toxins. A significant decrease in GFAP (about 50%) was found after L-AAA. Both the behavioral and GFAP level changes were prevented by MTEP injection.

The obtained results indicate that the degeneration of astrocytes in the PFC by L-AAA may be a useful animal model of depression and suggest antidepressant potential of MTEP.

1. Introduction

A decrease in the density of glial cells in cortical regions, especially in the prefrontal and cingular areas was one of the most consistent findings from postmortem studies of brains of depressed patients [1–5]. Moreover, a decline in the number of astrocytes in the prefrontal cortex was observed in rats subjected to chronic unpredictable stress which is one of the generally accepted animal models of depression [6]. The main functions mediated by astrocytes include trophic support, neuronal differentiation and synaptic efficacy [7]. They regulate the local concentration of ions and neuroactive substances [8–12] which is crucial for keeping the central nervous system (CNS) homeostasis. Astrocytes are an integral part of tripartite synapse where they regulate neuronal excitability [13,14]. In the glutamatergic synapses, the most abundant type of synapses in the CNS, astrocytes control the levels of

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http://dx.doi.org/10.1016/j.bbr.2014.07.019
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extracellular glutamate, the main excitatory neurotransmitter in the mammalian brain, so they maintain the excitatory/inhibitory balance there [15,16].

It has been hypothesized lately that dysregulation of this balance underlies mood disorders and anxiety and that the glutamatergic predominance in this Glu/GABA balance is involved in the pathogenesis of depression [17–20]. Indeed, an increase in glutamate or glutamine level was documented in the brains and cerebrospinal fluid of depressed patients [21,22] as well as in their plasma and platelets [23–25]. Moreover, the inhibition of glutamatergic function had antidepressant effects [26,27]. It was also found that chronic treatment with antidepressants caused the reduction of up-regulated glutamate release [28–30].

As mentioned above, astrocytes have a crucial role in maintaining the proper Glu/GABA balance, as they take up the released glutamate. Hence, the basic consequences of the disappearance or dysfunction of astrocytes include the reduction of glutamate uptake and thus excessive glutamate levels in the synaptic cleft which disturbs the balance and may lead to depression.

Recently, depressive-like behavior has been demonstrated in rats after degeneration of astrocytes by gliotoxins [6]. The authors postulated that astroglial degeneration induced by the astrocytic toxin L-alpha-aminoadipic acid (L-AAA) in the prefrontal cortex was sufficient to trigger depressive-like behavior. Previous studies of other authors performed in cell cultures and mice hypothalamus showed that not only L-AAA but also D-AAA and DL-AAA induced gliotoxic damage [31–34]. Therefore, in the present study we tried to find out whether such gliotoxins, not only L-AAA but also DL-AAA microinjected into the rat medial prefrontal cortex (mPFC), could serve as a new animal model of depression and whether our model of gliotoxin-induced depression may be useful for studying an antidepressant action of compounds. Moreover, we examined if both toxins decreased astrocytes density specifically in the prefrontal cortex. At the beginning, we verified if the classical antidepressant drug, imipramine, antagonized depressive-like effects of the gliotoxins and then we studied the effect of a compound engaged in the regulation of glutamatergic transmission.

As mentioned above, the overactivation of glutamatergic function in the prefrontal and limbic cortices may be essential in the pathophysiology of depression [35]. It was confirmed by the results in which inhibition of glutamatergic activity by NMDA receptor antagonists exerted antidepressant-like effects in laboratory animals [36,37] and had antidepressant properties in depressed patients [38–41]. Ketamine is the first drug of clinical significance which has a mechanism of action associated with the NMDA receptor. This NMDA antagonist exhibits a rapid onset of action and has high therapeutic efficacy. Unfortunately, the high antidepressant activity of ketamine is associated with a risk of serious adverse effects (dissociative and psychotomimetic effects and the potential to abuse) that greatly restrict its application on a larger scale [42]. Since the therapeutic use of direct NMDA receptor antagonism is diminished by their undesirable side-effects, therefore, an indirect inhibition of glutamatergic function via metabotropic glutamate receptors (mGluR) seems to be a much more promising way for treatment of depression than the direct blockade of ionotropic glutamatergic receptors (iGluR). Indeed, mGluR ligands were demonstrated to produce antidepressant-like and anxiolytic effects. Metabotropic GluRs constitute a family of eight subtypes subdivided into three groups on the basis of sequence homology, pharmacological profile and transduction pathways [43]. Group I comprises mGluR1 and mGluR5 are primarily localized postsynaptically and are positively coupled with phosphoinositide hydrolysis, activation of phospholipase C, protein kinase C and calcium release [44]. Many studies showed that stimulation of these receptors potentiated the excitatory effect of glutamate by modulation of ion channels [45], while blockade of these receptors attenuated these excitatory effects. Behavioral studies in the last years have revealed that group I mGluR antagonists produced antidepressant-like activity in several tests and models in rodents. Recently, MTEP (3-[(methyl-1,3-thiazol-4-yl)ethynyl]-pyridine), a highly selective, non-competitive antagonist of metabotropic glutamate receptors subtype 5 (mGluR5), has been described as an antidepressant and anxiolytic compound in animal experiments in the behavioral despair tests and in the bulbectomy-induced model of depression [46–50]. Moreover, in our previous studies we found neuroprotective effect of MTEP which was related to the inhibition of glutamatergic transmission and glutamate release [51].

Therefore, the goal of the present study was to find out if the mGluR5 antagonist MTEP may have potential antidepressant effects in the astrocytic toxin-induced rat model of depression. Such supposition seems to be reasonable as an overactivation of glutamate activity which is postulated to occur in gliotoxic model may be inhibited by MTEP.

2. Materials and methods

2.1. Animals

Male Spraque-Dawley rats (Charles River, Germany) weighing about 250–300 g at the beginning of the experiment, were used in the study. The animals were kept under standard laboratory conditions of constant temperature (19–21 °C), controlled 12:12 light-dark cycle. The rats were age-matched and were housed six to a cage on a 12:12 light-dark cycle, with free access to food and tap water. The rats after cannulae implantation were housed singly. During the experiment, all efforts were made to minimize animal suffering and to reduce the number of animals used, in accordance with the Local Bioethical Commission Guide for the Care and Use of Laboratory Animals.

2.2. Cannulae implantation

The rats anaesthetized with ketamine (75 mg/kg i.m.) and xylazine (10 mg/kg i.m.) were stereotaxically, bilaterally implanted with guide cannulae aimed at the medial prefrontal cortex (mPFC) region using the following coordinates: $AP = +3.2$ mm, $L = +1.0$ mm from the Bregma, $H = -3.5$ mm from the skull, according to the Paxinos and Watson stereotaxic atlas [52]. The guide cannulae (23-gauge stainless steel tubing), secured by dental cement, were anchored to the skull by three stainless steel screws. In order to prevent clogging, stainless steel stylets were placed in the guide cannulae and left until the animals were microinjected. Animals were left for 7 days for recovery.

2.3. Drug treatments

On the 1st and 2nd day of the experiment, the rats were bilaterally microinjected into medial prefrontal cortex (mPFC) with astrocytic toxins L-alpha-aminoadipic acid (L-AAA), or DL-alpha aminoadipic acid (DL-AAA) (Sigma–Aldrich Chemie GmbH, Germany). The gliotoxins were freshly dissolved in 0.1 M phosphate buffer, pH 7.4 and were microinjected at doses of 100 μg/2 μl. Control rats were bilaterally injected with a phosphate buffer. Afterwards depressive-like behavior was assessed by a forced swim test (FST) 24 h, 72 h or 144 h after the second gliotoxins or buffer administration in the time course experiment, and 72 h (on day 5) in all other experiments. The other group of rats was additionally treated with the antidepressant imipramine hydrochloride (Sigma–Aldrich Chemie GmbH, Germany) in a dose of 30 mg/kg, three times: 24, 5 and 1 h before the forced swim test (FST) or with mGluR5 antagonist MTEP (Tocris, USA) in a dose 10 mg/kg, once

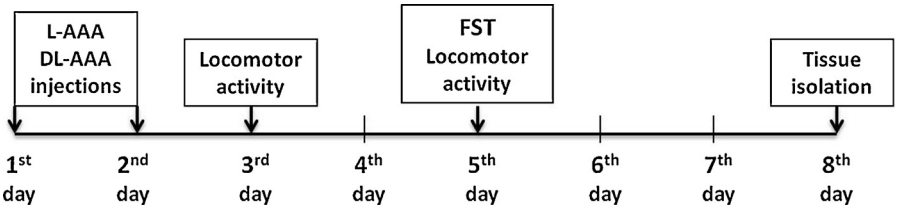

Fig. 1. Experimental design: schedule of treatments and tests in the gliotoxin model. Arrows indicate the time of treatments and tests on the timeline.

20 min before the FST. Imipramine was dissolved in saline, MTEP was suspended in 0.5% methylcellulose and both compounds were administered intraperitoneally (i.p.) in a volume of 2 ml/kg. The schedule of treatment is shown at Fig. 1.

2.4. Behavioral studies

2.4.1. Forced swim test (FST)

The rats were subjected to the FST according to the method described in the paper of Nowak et al. [53]. Briefly, during the pretest the rats were placed individually in a glass cylinders (40 cm high, 18 cm in diameter) containing 15 cm of water, maintained at 25 °C. After 15 min swimming session rats were removed from the cylinders and returned to their home cages. Rats were placed again in the cylinder 24 h later and the total duration of immobility was measured during a 5 min test. Rats were judged to be immobile when it remained floating passively in the water. The effect of L-AAA or DL-AAA alone and in combination with imipramine or MTEP was measured 72 h after the second administration of toxins. To obtain a time course effect of L-AAA administration in the FST, the total duration of immobility in rats was measured in the separated groups 24, 72 and 144 h after the second L-AAA administration.

2.4.2. Locomotor activity

Locomotor activity was recorded individually for each animal in Opto-Varimex cages (Columbus Instruments, USA) linked on-line to a compatible IBM-PC. The behavior of the rats was analyzed using Auto-Track software (Columbus Instruments, Columbus, USA). Each cage (43 cm × 43 × 21 cm) was equipped with a 15 × 15 array of infrared emitters located 3 cm from the floor surface. The number of light beams interrupted by an animal was recorded at 5 min intervals and was presented as the distance traveled in cm. Locomotor activity was recorded 24 or 72 h after the second gliotoxin administration; 1 h after the last dose of imipramine or 20 min after MTEP injection.

2.4.3. Statistical analysis

The obtained data were presented as means ± SEM, and evaluated either by a two-way ANOVA analysis of variance, followed by Bonferroni multiple comparison test (results on Figs. 2 and 4) or Student's *t*-test (results on Fig. 3). The level of significance was determined as $p < 0.05$.

2.5. Western blot analysis

On day eighth (144 h after the second gliotoxin injection) the rats were decapitated, brains were taken out and the glial fibrillary acidic protein (GFAP) level was determined in the prefrontal cortex (PFC), hippocampus, amygdala and posterior cortical areas. Prefrontal cortex was taken by cutting the anterior part of the forebrain at the level of Bregma 2.20 mm according to the Paxinos and Watson stereotaxic atlas [52]. Olfactory bulbs and the anterior striatum were cut off. Therefore, the tissue taken for analysis contained majority of the prefrontal cortex. Additionally NeuN protein level

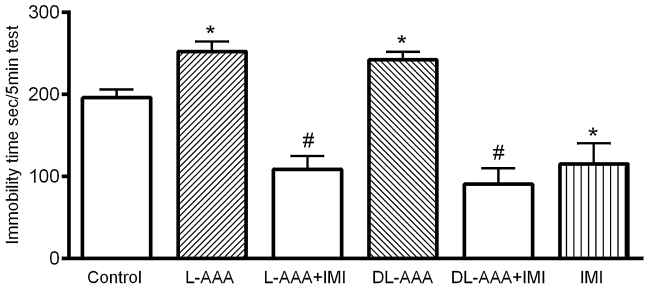

Fig. 2. The effect of combined administration of L-AAA, DL-AAA and imipramine (IMI) on the total duration of immobility in the FST in rats. L-AAA and DL-AAA were administered twice into the rat mPFC, at a dose of 100 μg/2 μl, on the first and the second day of experiment. The FST was performed on day 5 (72 h after the second injection). Imipramine (30 mg/kg) was administered *i.p.*, tree times: 24, 5 and 1 h before the test. The obtained data were presented as the means ± SEM ($n = 10–12$ rats per group) and evaluated by two-way ANOVA, followed by Bonferroni multiple comparison test. $^{*}p < 0.05$ vs control; $^{\#}p < 0.001$ vs L-AAA or DL-AAA treated group.

(biomarker for neurons) was estimated after L-AAA injection in the PFC.

The tissue was homogenized in 2% SDS and centrifuged for 5 min at 10,000 rpm at 4 °C. Protein concentration in the obtained supernatant was determined using a BCA Protein Assay Kit (Pierce). The samples containing 30 μg of protein were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes using an electrophoretic transfer system. Non-specific binding was blocked for 30 min in blocking reagent (Roche). Then, membranes were incubated overnight at 4 °C with mouse monoclonal anti-GFAP antibody (1:5000, Millipore) or anti-NeuN (1:1000, Millipore). The membranes were then washed with TBS-T and incubated for 30 min with a horseradish peroxidase-conjugated anti-mouse IgG secondary antibody (1:1000, Roche). The reaction was visualized by using BM Chemiluminescence Western Blotting Kit, Roche). Chemiluminescence was recorded and evaluated with a luminescent image analyzer (FUJI-LAS-4000). The

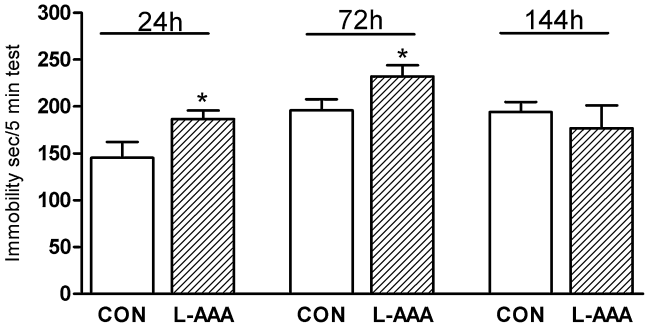

Fig. 3. The effect of L-AAA administration on the total duration of immobility in the FST in rats at different time points. L-AAA was administered twice into the rat mPFC at a dose of 100 μg/2 μl, on the first and the second day of experiment. The FST was performed 24, 72 and 144 h after the second toxin injection. The obtained data were presented as the means ± SEM ($n = 10–12$ rats per group) and evaluated by Student's *t*-test. Significance was assumed at $p < 0.05$.

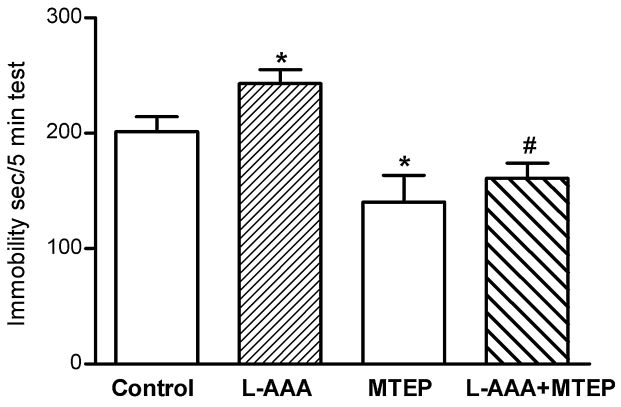

Fig. 4. The effect of combined administration of L-AAA and MTEP on the total duration of immobility in the FST in rats. L-AAA was administered twice into the rat mPFC at a dose of 100 μg/2 μl, on the first and the second day of experiment and MTEP, 10 mg/kg, *i.p.*, was given 20 min before the FST. The obtained data were presented as the means ± SEM (n = 10–12 rats per group) and evaluated by two-way ANOVA, followed by Bonferroni multiple comparison test. $^{*}p < 0.05$ vs control; $^{\#}p < 0.001$ vs L-AAA treated group.

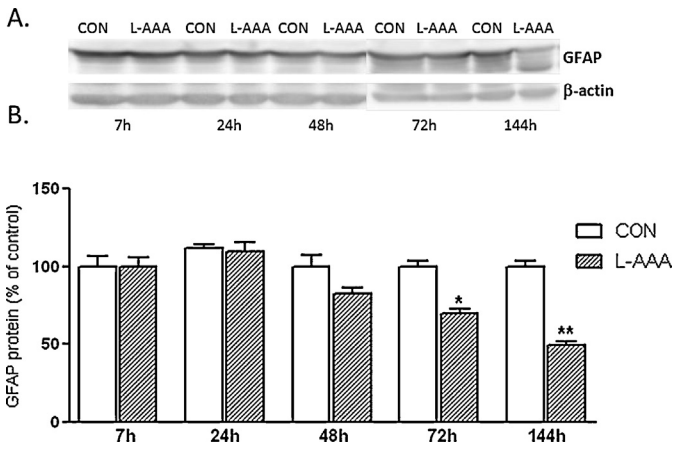

Fig. 5. The effect of L-AAA administration on the GFAP level in the rat PFC, measured at different time points. Western blot analysis. L-AAA was administered twice into the rat mPFC at a dose of 100 μg/2 μl, on the first and the second day of experiment. The GFAP level in the prefrontal cortex (PFC) was determined 7 h, 24 h, 48 h, 72 h and 144 h after the second toxin injection. (A) The immunoblot bands corresponding to GFAP (upper panels) and β-actin (lower panels) are seen. A weaker intensity of the GFAP band is observed in samples taken from rats 72 and 144 h after the L-AAA injection. (B) Analysis of data of the GFAP level. The obtained data were presented as the means ± SEM (n = 6–7 rats per group) and evaluated by a Student's t-test. $^{*}p < 0.05$, $^{**}p < 0.01$ vs control.

relative levels of immunoreactivity were quantified using Image Gauge v 4.0 software. To confirm equal loading of the samples on the gel, the blots were incubated with mouse anti-β-actin antibody (1:5000, Sigma) and then processed as described above. The density of GFAP protein band was normalized to the density of the β-actin.

The Western blot analysis of GFAP was performed in group of rats treated with L-AAA, DL-AAA, L-AAA + imipramine, DL-AAA + imipramine, imipramine, MTEP and L-AAA + MTEP. For the time course effect of L-AAA on GFAP and NeuN protein level in the PFC, rats were decapitated and the brains were collected 24, 48 and 72 h after the second injection of gliotoxin. Western blot analysis was performed as described above.

2.5.1. Statistical analysis

The obtained data were presented as means ± SEM, and evaluated either by a two-way ANOVA analysis of variance, followed by Bonferroni multiple comparison test (results on Figs. 6 and 7) or Student's t-test (results on Fig. 5). The level of significance was determined as $p < 0.05$.

2.6. Histological and immunohistochemical studies

On day eighth (144 h after the second injection of the toxin) the brains were taken for analysis. Rats under deep pentobarbital anesthesia were perfused through the ascending aorta with 0.9% physiological saline, followed by a cold 4% paraformaldehyde (PF) in 0.1 M sodium phosphate buffer, pH 7.4. Next, their brains were removed, postfixed in cold buffered PF for 3 h, and cryoprotected in a buffered 20% sucrose solution for at least 5 days at 4 °C. The brains were then frozen on dry ice, and 40 μm coronal sections were cut at levels containing medial prefrontal cortex (between bregma 4.70 to 1.70 mm, according to the Paxinos and Watson stereotaxic atlas [52]. Free-floating sections were collected and every sixth section were taken for glial fibrillary acidic protein (GFAP) immunohistochemical staining, using anti-GFAP mouse monoclonal antibody (Millipore) diluted at 1:800 in sodium phosphate-buffer (PBS) containing 0.2% Triton X-100 and 1% normal horse serum. Adjacent sections were stained with cresyl violet and used for a histological analysis and verification of the injection sites. The immunostained sections, after reaction with primary antibody were rinsed in PBS and processed by an avidin–biotin peroxidase complex method using an ABC-peroxidase kit (Vector Lab) and diaminobenzidine (DAB) as a chromogen. The stained sections were mounted on slides, dried, dehydrated, cleared in xylene, cover-slipped with Permount and analyzed under a light microscope.

Additionally, some sections from each group were stained by immunofluorescence method using anti-GFAP antibody. After 1 h incubation in a blocking buffer (5% normal donkey serum and 0.2% Triton X-100 in 0.01 M PBS) the sections were incubated (48 h at 4 °C) with the primary anti-GFAP mouse antibody (Millipore) diluted at 1:800 in the blocking buffer. After incubation with the primary antibody, the sections were washed and incubated overnight at 4 °C in the secondary antibody Cy3-conjugated anti-mouse IgG (1:200, Jackson ImmunoResearch, USA). The sections were then washed and mounted with a coverslip overlay. The

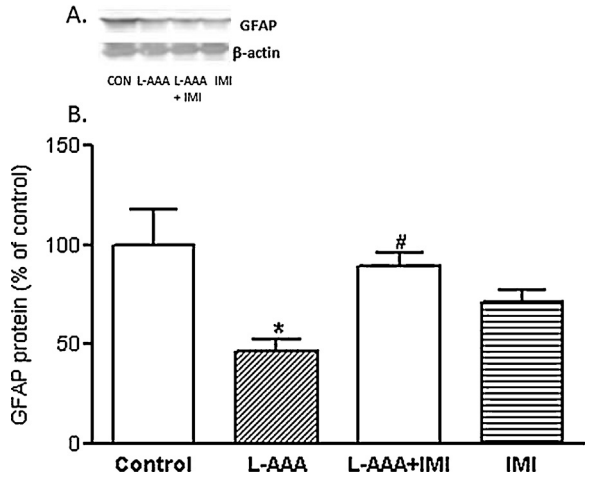

Fig. 6. The effect of L-AAA and imipramine (IMI) administration on the GFAP level in the rat PFC. Western blot analysis. L-AAA was administered twice into the rat mPFC at a dose of 100 μg/2 μl, on the first and the second day of experiment. The GFAP level was determined on day 8 (144 h after the second toxin injection). (A) The immunoblot bands. (B) Analysis of data of the GFAP level. The obtained data were presented as the means ± SEM (n = 6–7 rats per group) and evaluated by two-way ANOVA, followed by Bonferroni multiple comparison test. $^{*}p < 0.05$ vs control; $^{\#}p < 0.05$ vs L-AAA.

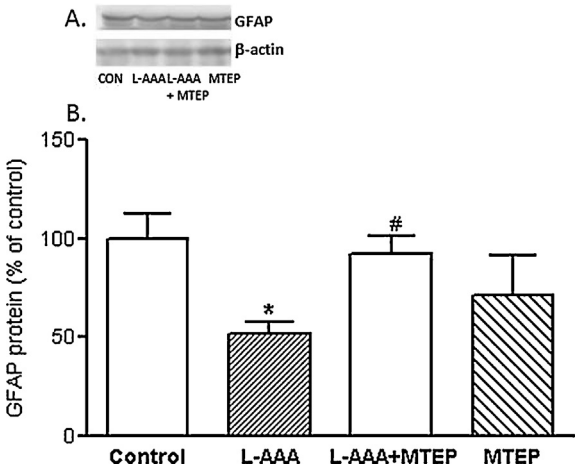

Fig. 7. The effect of L-AAA and MTEP administration on the GFAP level in the rat PFC. Western blot analysis. L-AAA was administered twice into the rat mPFC at a dose of 100 μg/2 μl, on the first and the second day of experiment. The GFAP level was determined 144 h after the second toxin injection. (A) The immunoblot bands. (B) Analysis of data of the GFAP level. The obtained data were presented as the means ± SEM (n = 6–7 rats per group) and evaluated by two-way ANOVA, followed by Bonferroni multiple comparison test. $^{*}p < 0.05$ vs control; $^{\#}p < 0.05$ vs L-AAA.

sections were analyzed with a confocal laser scanning microscope, DMRXA2 TCS SP2 (Leica), with a 63×/1.40–0.70 oil objective (Leica) driven by confocal software (Leica) using the sequential scan settings, as described previously [54]. Working with GreNe laser line emitting at 543 nm was used to excite Cy3-conjugated antibody. The background noise of each confocal image was then reduced by averaging four scans/line and six frames/image. The pinhole value of one airy was used to obtain flat images.

2.7. Stereological counting of GFAP-immunoreactive astrocytes

The number of GFAP-immunoreactive astrocyte cell bodies stained with DAB, in the medial prefrontal cortex (mPFC) was evaluated by stereological counting. The procedures were performed using a microscope (Leica, DMLB; Leica, Denmark) equipped with a projecting camera (Basler Vision Technologies, Germany) and a microscope stage connected to an xyz stepper (PRIOR ProScan) controlled by a computer using Visiopharm New CAST software (Visiopharm, Denmark).

Systemic uniform, random sampling was used to choose the sections for the analysis. For stereological estimates, GFAP-ir astrocytes were counted within the contours of the mPFC [AP = 4.70–1.70 mm] from bregma according to the Paxinos and Watson stereotaxic atlas [52] in at least 8–12 sections at 240 μm intervals. The mPFC was outlined under a lower magnification (5×) and uniformly sampled dissector points were used at random along the whole structure using the meander sampling.

The total number of GFAP-positive cells in the mPFC was unbiasedly estimated using a three dimensional probe under a higher magnification (63×) according to the formula: $N = \Sigma Q \times V(\text{ref})/v(\text{dis}) \times \Sigma P$, where ΣQ is a total count of GFAP-ir astrocytes in the uniformly sampled dissectors, V(ref) – the total volume of the mPFC, v(dis) – the total volume of the dissector [55] and ΣP – the total number of all dissector points. A counting frame of 74.61 μm × 74.61 μm (5567.27 μm^2), a sampling grid of 333.68 μm × 333.68 μm (111,342.3 μm^2) and a dissector height of 20 μm below the surface were employed. Sampling was optimized to produce a coefficient of error well under the observed biological variability [56]. The total volume of the mPFC V(ref) was estimated using Cavalieri's principle [56] according to the formula: $V(\text{ref}) = t \times a(p) \times \Sigma P$, where t is the known distance between the sampled sections, $a(p)$ is the area associated with each point of a grid, ΣP is the total number of counted points over all sections from one rat.

2.7.1. Statistical analysis

The data were presented as means ± SEM, and evaluated by a one-way ANOVA analysis of variance, followed by Tukey's multiple comparison test. The level of significance was determined as $p < 0.05$.

3. Results

3.1. Behavioral studies

3.1.1. Effect of combined administration of L-AAA or DL-AAA and imipramine in the FST

The effect of L-AAA and DL-AAA on the total duration of the immobility time in the FST in rats is shown in Fig. 2. L-AAA and DL-AAA administered twice into the rat mPFC, at a dose of 100 μg/2 μl, induces depressive-like behavior in the FST. The immobility time of rats increased significantly both in L-AAA and DL-AAA treated group as compared to control group. Administration of imipramine significantly decreased the immobility time of rats and antagonized the effects elicited by L-AAA and DL-AAA administration. Two way ANOVA demonstrated significant effect of gliotoxin [$F(2,47) = 3.32$, $p = 0.0448$]; significant effect of imipramine [$F(1,47) = 83.87$, $p = 0.0001$] and significant interaction [$F(2,47) = 4.03$, $p = 0.0242$].

3.1.2. Effect of L-AAA in the FST at different time points

The time course effect of L-AAA on the total duration of the immobility time in the FST in rats is shown in Fig. 3. Depressive-like behavior, observed as increased immobility time in the FST was found 24 h ($p = 0.0445$) and 72 h ($p = 0.0491$) but not 144 h ($p = 0.5103$) after the second gliotoxin injection.

3.1.3. Effect of combined administration of L-AAA and MTEP in the FST

The effect of combined administration of L-AAA and MTEP on the total duration of the immobility time in FST in rats is shown in Fig. 4. L-AAA administered alone significantly increased the immobility time in FST. MTEP given alone significantly decreased the immobility time in rats and antagonized the effect induced by L-AAA. Two way ANOVA demonstrated the significant effect of MTEP [$F(1,39) = 13,49$, $p = 0.0007$], significant effect of L-AAA [$F(1,39) = 5.44$, $p = 0.0249$] and significant interaction [$F(1,39) = 4.21$, $p = 0.047$].

3.1.4. Effect of L-AAA and DL-AAA alone or in combined administration with imipramine and MTEP on the locomotor activity in rats

The effect of L-AAA and DL-AAA administration alone or in combined treatment with imipramine and MTEP on the locomotor activity in rats is shown in Table 1.

L-AAA and DL-AAA administered alone did not influence the locomotor activity in rats (Table 1A). However, the imipramine alone or in combined administration with L-AAA and DL-AAA significantly decreased the locomotor activity in rats, measured 72 h after the second injection of toxin (Table 1B). No significant effect on the locomotor activity was found after MTEP and combined L-AAA and MTEP injections. Only a tendency for decrease was observed (Table 1C). Two way ANOVA demonstrated no effect of gliotoxins $F(2,25) = 1.01$, $p = 0.3802$, significant effect of

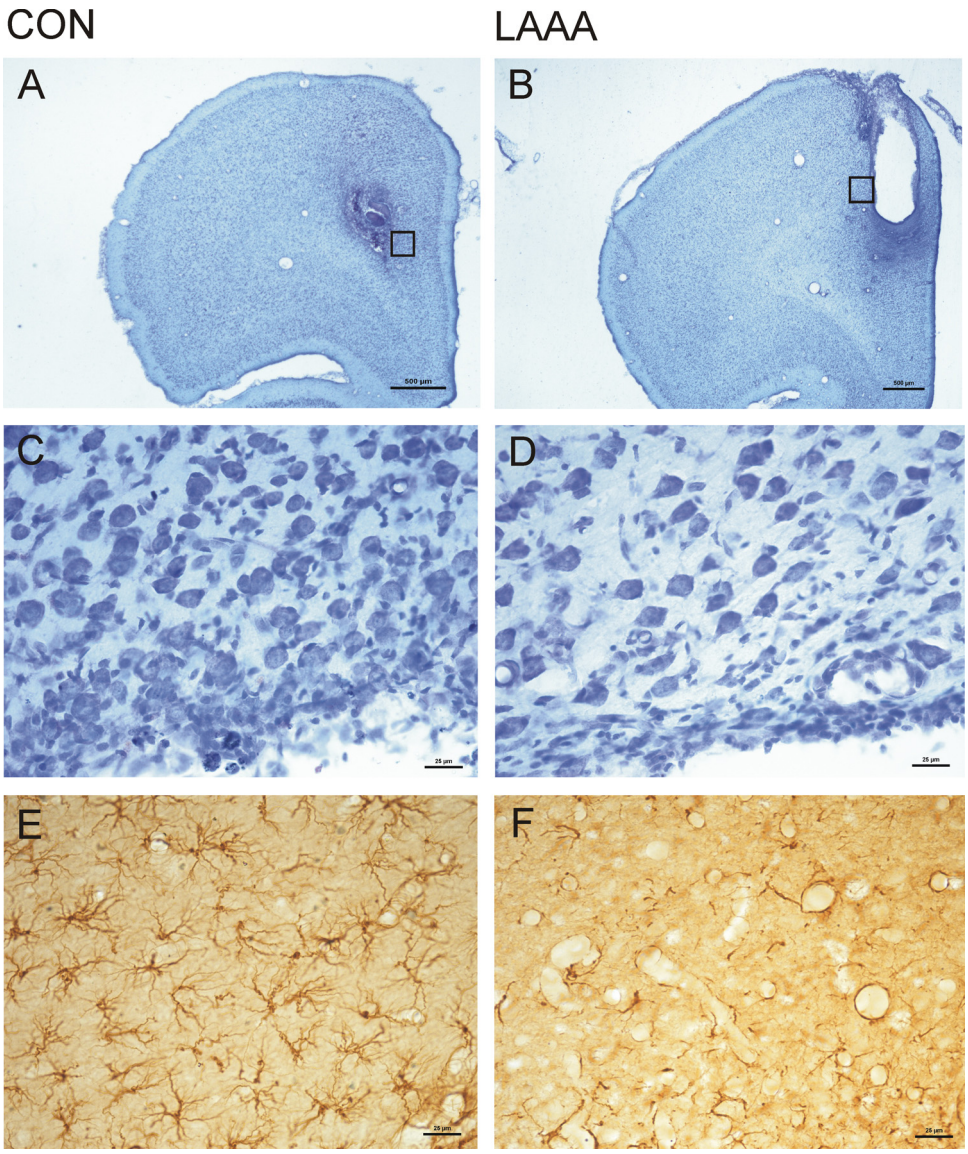

Fig. 8. Microphotographs of coronal sections of the rat brain mPFC. (A, B, C and D) Cresyl violet staining. General view of the sections of control (A) and L-AAA (B) treated rats under lower magnification. Glial scar (in A) and tissue destruction (in B) is seen in the injection sites. Black squares point the regions presented under higher magnification on C and D respectively. Stained nerve cell bodies are seen near the injection site both in a control rat (C) and in an L-AAA injected rat (D). Glial scar is visible at the bottom of the photographs. (E and F) Sections immunostained with GFAP antibody. Numerous GFAP positive astrocytes are seen in the section from a control rat (E), in contrast to nearly no astrocytes in the section from an L-AAA treated rat (F). Calibration bars for A and B 500 μm, for (C), (D), (E), (F) 25 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

imipramine $F(1,25) = 56.50$, $p < 0.0001$ and not significant interaction $F(2,25) = 0.15$, $p = 0.8642$.

3.2. Western blot analysis

3.2.1. Effect of L-AAA administration on the GFAP and NeuN protein level in the rat PFC at different time points and in different structures

The time course effect of L-AAA on the GFAP protein level in rat PFC is shown in Fig. 5. Significant decrease in the GFAP protein level was observed 72 h ($p = 0.0298$) and 144 h ($p = 0.0195$) after second L-AAA administration. No changes in the GFAP protein level were observed 7, 24 and 48 h after L-AAA treatment. L-AAA injection did not influence the NeuN level in PFC at any time points (data not shown). No changes in the GFAP level were found in the posterior cortical areas, hippocampus and amygdala (data not shown) after the toxin administration.

3.2.2. Effect of L-AAA, DL-AAA and imipramine administration on the GFAP protein level in the rat PFC

The effects of L-AAA alone and combined administration of L-AAA with imipramine on the GFAP protein level in PFC (144 h after the toxin injection) are shown in Fig. 6.

L-AAA administration induced a significant reduction in the GFAP protein level in the rat PFC (to 46% of control level). Imipramine alone had no effect on GFAP protein level however antagonized the effect induced by L-AAA. Two way ANOVA demonstrated no effect of L-AAA [$F(1,21) = 2.18$, $p = 0.1544$]; no effect of imipramine [$F(1,21) = 0.33$, $p = 0.569$] and significant interaction [$F(1.21) = 8.85$, $p = 0.007$]. No significant changes were found

Table 1
The effect of L-AAA and DL-AAA alone or in combined administration of L-AAA and MTEP on the locomotor activity in rats.

Treatment	Activity counts (5 min test)
A	
Control	662.08 ± 66.1
L-AAA	670.37 ± 32.5
DL-AAA	693.79 ± 49.3
B	
Control	583.7 ± 10.08
Imipramine	213.9 ± 43.03[a]
L-AAA + imipramine	311.5 ± 81.43[a]
DL-AAA + imipramine	212.9 ± 28.25[a]
C	
Control	622.5 ± 64.99
MTEP (10 mg/kg)	466.54 ± 37.19
L-AAA + MTEP (10 mg/kg)	487.5 ± 45.55

Locomotor activity was examined; (A) 24 h after the second L-AAA or DL-AAA administration; (B) 72 h after the second administration of L-AAA and 1 h after the last dose of imipramine (triplicate injections of imipramine 24, 5, 1 h before the test); (C) 72 h after the second administration of L-AAA and 20 min after the MTEP treatment. The values represent mean ±SEM (n = 6–8 rats per group).

[a] $p < 0.05$ vs control.

after DL-AAA alone and in combination with imipramine (data not shown).

3.2.3. *Effect of combined administration of L-AAA and MTEP on the GFAP protein level in the rat PFC*

The effects of L-AAA alone and the effect of combined administration of L-AAA with MTEP on the GFAP protein level in PFC (144 h after the toxin injection) are shown in Fig. 7.

L-AAA administration induced significant reduction in the GFAP protein level in the rat PFC (to 52,8% of control). MTEP antagonized the effect induced by L-AAA but did not change the GFAP protein level when given alone. Two way ANOVA demonstrated no effect of L-AAA [$F(1,30) = 0.75$, $p = 0.3523$]; no effect of MTEP [$F(1,30) = 0.44$, $p = 0.5102$] and significant interaction [$F(1.30) = 7.54$, $p = 0.0101$].

3.3. *Histological and immunohistochemical analysis*

3.3.1. *Effect of L-AAA and DL-AAA alone or in combined administration of L-AAA and MTEP on the number of GFAP-positive cells*

Histological analysis of sections stained with cresyl violet showed the proper localization of the injection sites (in mPFC), and CV stained neurons were seen in the whole mPFC (Fig. 8A and B), also very near a scar (Fig. 8C and D) GFAP immunohistochemistry showed a decrease in a density of astrocytes in the mPFC of rats treated with L-AAA (Fig. 8E and F). Stereological counting showed a strong, significant decrease in the number of GFAP-immunoreactive (ir) astrocytes in mPFC after L-AAA in comparison to control rats (to 54,18% of control) (Fig. 9A). The effect of DL-AAA on the number of GFAP-ir astrocytes was weak and insignificant. The decreasing effect of L-AAA on the number of GFAP-ir cells was significantly inhibited by MTEP, reaching 81,2% of control (Fig. 9B). The glioprotective effect of MTEP found in stereological counting was confirmed by the morphological observation of astrocytes in the mPFC immunostained with an anti-GFAP antibody. A density of GFAP immunofluorescent astrocytes decreased by prior L-AAA administration was nearly normalized after MTEP treatment (Fig. 10).

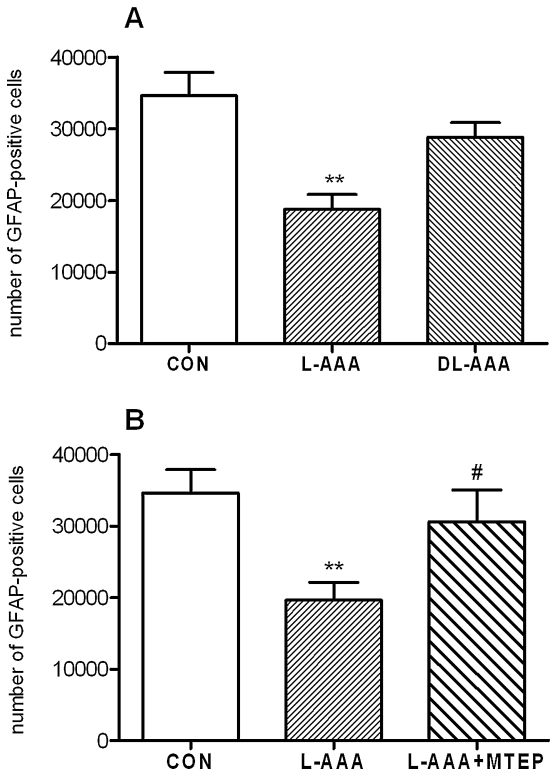

Fig. 9. The effect of L-AAA and DL-AAA administration (A) and the effect of combined administration of L-AAA and MTEP (B) on the number of GFAP positive cells in the rat mPFC counted by stereological method. L-AAA was administered twice into the rat mPFC at a dose of 100 μg/2 μl, on the first and the second day of experiment and MTEP 10 mg/kg, *i.p.*, was injected on day 5 (20 min before FST). Brains were taken for analysis on day 8 (144 h after the second toxin injection). The data are presented as the means ± SEM, (n = 5–8 rats per group) and evaluated by one-way ANOVA, followed by Tukey's multiple comparison test. $p < 0.05$ was considered significant. $^{**}p < 0.01$ vs control; $^{\#}p < 0.05$ vs L-AAA.

4. Discussion

Our results indicate that gliotoxin induced degeneration of astrocytes in the rat medial prefrontal cortex may be used as a good experimental model of depression. The results are in line with the idea arising from numerous studies indicating that astrocyte dysfunction plays a crucial role in pathogenesis of depression. Actually, anhedonia was observed in rats after the blockade of astrocytic glutamate uptake in the prefrontal cortex [57]. Intracerebroventricular infusion of dihydrokainate, an astrocytic glutamate uptake blocker, also induced some signs of anhedonia observed in the intracranial stimulation and place conditioning tests [58]. The blockade of astrocytic glutamate uptake in the amygdala, a limbic structure engaged in the regulation of mood and anxiety has been reported to induce some behavioral effects characteristic of depressive-like symptoms [59]. Reduced glutamate transporter activity and expression was reported to occur in the hippocampus in the mouse stress model of depression and in the cortex in the learned helpless model in rats [60]. Also in human post mortem studies, downregulation of genes coding for astrocytic glutamate transporters was observed in the cingulate and prefrontal cortex of depressed subjects [61]. Dysfunction of astrocyte gap junction communication was also found in the rat prefrontal cortex after chronic unpredictable stress and it was reversed by treatment with antidepressants, moreover, the pharmacological gap junction blockade in the PFC induced depressive-like behavior in rats [62]. All these findings indicate that damage or impairment of proper function of astrocytes in the prefrontal cortex may be a good model of depression.

In our studies, we damaged astrocytes in the rat mPFC by the gliotoxin, alpha-aminoadipic acid (AAA). It was demonstrated to be a specific toxin for astrocytes already in 1971 by Olney and coworkers [63], and its specificity was confirmed in later years both in vivo

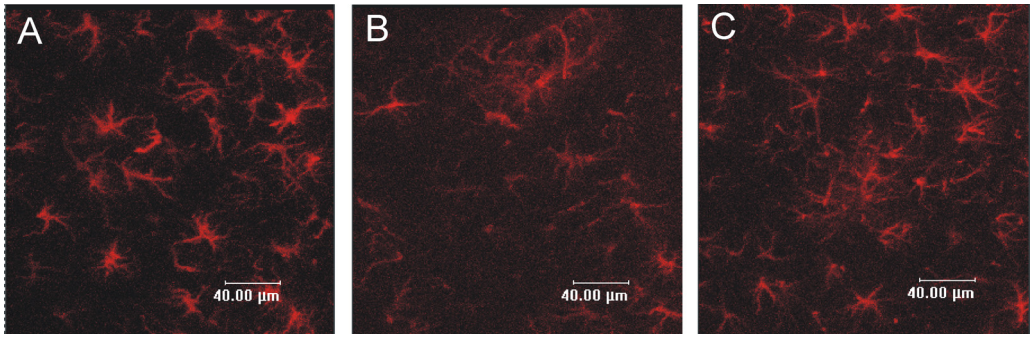

Fig. 10. Microphotographs of coronal section of the rat brain mPFC, showing GFAP positive astrocytes stained by immunofluorescence method. (A) Numerous astrocytes are seen in the section from a control rat; (B) the decrease in astrocyte density and diminution of their processes are found after L-AAA injection; (C) MTEP partially prevents the L-AAA effect: numerous astrocytes are visible like in the control section.

and in vitro [32,33,64]. It is taken up specifically by astrocytes and accumulating in these cells exerts its gliotoxic effect by interference with protein synthesis [31,34,65].

Studies of other authors concerning the effects of D- and L-isomers or racemic form of AAA gave conflicting, heterogeneous results. When administered subcutaneously into infant mice, it exerted a toxic effects in the arcuate hypothalamic nucleus, whereas D-AAA and DL-AAA induced, respectively, a mild to extreme gliotoxic but not neurotoxic damage and L-AAA induced not only gliotoxic but also some neurotoxic changes, moreover D-isomer had even anti-neurotoxic properties [32,64,66]. Similar results were obtained in astrocytes or mixed neuronal/astrocyte cultures [33] but additionally D-isomer appeared to be toxic only for mitotic cells [31]. The effect of AAA on astrocytes was also studied in adult rats after intracerebral injection into different brain structures. A single L-AAA injection into the amygdala induced loss of astrocytes and their markers (GFAP, S-100) in a wide area around the injection site, while neurons remained undamaged [67]. Astroglial degeneration was also observed after L-AAA injection into the striatum [68], substantia nigra and locus coeruleus [69]. On the other hand Saffran and Crutcher [70] failed to observe gliotoxic properties of L-AAA or DL-AAA in the hippocampus and striatum after injections into those structures. All the above mentioned studies, were focused only on the cellular effects of the toxins, and the authors did not examine their behavioral effects.

In our studies we used L-AAA and DL-AAA as a model of depression based on astrocyte ablation in the medial prefrontal cortex (mPFC). Such model was developed by Banasr and Duman [6]. The authors demonstrated that a double microinjection of gliotoxin L-alpha-aminoadipic acid (L-AAA) into the rat medial prefrontal cortex induced astroglial degeneration in the prefrontal cortex and triggered a depressive-like behavior similar to that observed after chronic unpredictable stress, a generally accepted model of depression. Doses of toxin and general schedule of our experiment were similar but we used not only L-AAA but also DL-AAA, as this racemic form has been suggested by some authors to be more specific for astrocytes (as already mentioned above).

Our present results showed that both gliotoxins L-AAA and DL-AAA microinjected into the medial prefrontal cortex of the rat induced depressive-like behavioral effects, because an increase in immobility time in the forced swim test (FST) without changing locomotor activity was observed after these toxins. The effect was observed 24 and 72 h after the second gliotoxin injection but not after 144 h. The results are in agreement with the findings of Banasr and Duman [6] who demonstrated L-AAA-induced depressive-like behavioral changes in rats in the sucrose preference test and forced swim test, in the novelty suppressed feeding model and active avoidance test at similar time points, but they did not study the behavioral effect after 144 h. We found that not only L-AAA but also DL-AAA revealed similar depressive-like action and, moreover, the effect was prevented by the classical antidepressant drug, imipramine, used in doses and schedule typical for such studies [71]. These results indicate that the intra-mPFC gliotoxin injection may be a good model for studying antidepressant effects of drugs.

We verified also the effects of the gliotoxins on astrocytes by studying the appearance and the level of GFAP, a protein characteristic of astrocytes [72], using immunohistochemical and Western blot analysis. Both methods showed a decrease in GFAP-ir in the PFC after L-AAA microinjection, which indicated a damage of astrocytes. The effect is in line with the results of Banasr and Duman [6] who found a decrease in density of GFAP positive cells 144 h after L-AAA in chosen fields of the prefrontal cortex. DL-AAA displayed a tendency toward decreasing of the above parameters but the effect was statistically non-significant probably because of high variability of the results. For that reason, we have chosen L-AAA for further studies.

The Western blot analysis of the time-course of L-AAA-induced changes showed a progressive decrease in GFAP level displaying a non-significant lowering 48 h and a strong significant decrease 72 and 144 h after the second toxin injection. No such time-course studies were performed earlier. It is difficult to explain the discrepancy between low GFAP level 144 h after the toxin and the lack of depressive-like behavioral effect in the FST at the same time point. We may hypothesize that some compensatory changes might take place after the longer time period, even in other brain structures, which influenced animal behavior. This hypothesis seems to be supported by our preliminary findings which showed that GAD (glutamic acid decarboxylase) protein level, a marker of GABA neurons, increased in the hippocampus 144 h after the L-AAA injection (our unpublished observations). As depression is postulated to be a result of GABA/Glu imbalance with glutamatergic overactivity (see introduction), the activation of GABA function may have positive antidepressant effects on behavior.

We verified whether our gliotoxin model may be a useful model of depression. For this purpose we administered imipramine, a standard antidepressant drug, to L- and DL-AAA injected rats. Imipramine was given in triple doses which is routine treatment for screening antidepressive effects in FST (according to [73,74,75]). We evidenced that imipramine reversed the behavioral effects of both L-AAA and DL-AAA. The time of immobility decreased even below the control level. The imipramine effect seems to be specifically connected with an antidepressant action, because the drug decreased not increased, the locomotor activity.

We also studied whether imipramine prevented the gliotoxin induced astrocytic degeneration in the PFC using Western blot method. While imipramine reversed decreasing effect of L-AAA on GFAP level, the effect of DL-AAA was only weakly and

non-significantly influenced (data not shown). Summing up, L-AAA toxin seems to be more promising as a model of depression.

There are no studies yet on the effect of imipramine or other antidepressants in the gliotoxic model both on behavior and on astrocyte density and GFAP protein level. However, in another model, chronic unpredictable stress in rats, both astrocyte pathology and depression-like behavior were reversed by antidepressants clomipramine, fluoxetine and magnolol [76–79]. The effect of antidepressant therapy on astrocytes was demonstrated both in experimental studies and in depressed patients [80].

We also examined whether the toxins used in our experiments, L-AAA and DL-AAA, killed neurons besides astrocytes. We did not observe neuronal loss in histological and Western blot studies. No diminution of nerve cells density was seen in brain sections from the mPFC, stained with cresyl violet. Nerve cell bodies were observed even near the cannulae track and glial scar (Fig. 8A and B). Western Blot analysis of NeuN protein level was focused on the effect of L-AAA only, because, as mentioned above, L isomer of AAA was postulated by some authors to have some neurotoxic effects [32,33]. No changes in NeuN level were found during 7 h to 144 h after the toxin injection. This result is in agreement with the observations of Banasr and Duman [6] who found no noticeable neuronal loss in the prefrontal cortex after L-AAA in sections stained immunohistochemically with NeuN antibody.

As mentioned in the introduction, the glutamatergic overactivity has been postulated to be essential in pathogenesis of depression, and the model of astrocyte ablation is based on the dysregulation of the Glu/GABA balance. Therefore, in the present study, we used potential antidepressant compound, MTEP, to examine whether it can prevent the depressive-like effects of L-AAA toxin, as measured by behavioral test and astrocyte degeneration markers. MTEP is a highly selective negative allosteric modulator of mGlu5 receptors [81–83].

Antidepressant-like effect of MTEP was observed in previous studies in rat and mouse models. Pałucha et al. [47] showed that a single intraperitoneal injection of MTEP produced a significant, dose dependent decrease in the immobility time of mice in the tail-suspention test (TST) and chronic treatment with this compound inhibited bulbectomy induced hyperactivity in rats. Antidepressant-like effects of MTEP and other mGluR1 and mGluR5 antagonists were also demonstrated in the forced swim test in rats and the TST in mice [84–86]. Similarly, the other negative allosteric modulator of mGluR5, GRN-529, given orally, 1 h before the test, in a single dose into mice induced antidepressant-like and anxiolytic effects [83].

Our results indicate that MTEP possesses antidepressant-like activity also in the gliotoxin induced model of depression. This compound injected once 20 min before the forced swim test (FST), prevented the L-AAA induced increase in immobility time of rats. It also reversed the decreasing effect of L-AAA on GFAP level in PFC, measured 144 h after the toxin using Western blot method, and on the number of GFAP-ir cells determined by stereological counting.

It is especially interesting that the antidepressant-like effect of MTEP was found after a single injection. Till now, most of the studies concerning antidepressant drugs described that they were effective after chronic treatment only, both in human and animal experimental studies [87,88], but those studies were focused mainly on the drugs acting through the monoaminergic mechanisms and they needed weeks or months for developing their therapeutic effects. Moreover, about 30–50% of patients remains resistant to treatment [89]. On the other hand, the compounds acting by inhibition of glutamatergic activity, like NMDA receptor blockers (i.e. dizocilpin), revealed their antidepressant activity quickly after a single dose [90,91]. Metabotropic Glu5 receptors are anatomically and functionally coupled with NMDA receptors [92–94] and their activation potentiates NMDA receptor activity, so the mGluR5 antagonists indirectly inhibit them [95].

MTEP counteracted not only the depressive-like behavior after L-AAA but also prevented the astrocytic degenerations in the mPFC induced by the toxin. The mechanism of such protective effects is not clear but it may be connected with mGluR5 inhibition and in consequence with a diminution of NMDA receptor function. NMDA receptors are present on astrocytes and other glial cells [96]. Metabotropic Glu5 receptors are very abundant in astrocytes [97–101] and are found to be connected with NMDA receptor currents [102–104]. Inhibition of mGluR5 and NMDA receptors has neuroprotective and glioprotective effects [51,105,106]. It is worthwhile noting that L-AAA-induced astrocytic degeneration developed gradually (see results, Fig. 5), and at 72 h after the toxin, when MTEP was injected, the level of GFAP was still maintained at about 50%, so the drug could affect these remaining astrocytes. Many studies have shown that antidepressants stimulate proliferation of astrocytes and production of trophic factors by these cells, such as BDNF, GDNF, FGF and others modulators which may restitute proper neuroglia network and their function [79,107–111]. Moreover, such effects may appear even after a short treatment and a single dose. The first molecular responses in ERK/MAPK signaling pathway in astrocytes may appear already after acute administration of desipramine or fluoxetine [110]. Primary cultures of rat astrocytes treated with fluoxetine revealed induction of MAPK, BDNF, GDNF and their receptor genes already 2 h after the beginning of the treatment [112]. Especially fast effects were found after the compounds acting through the glutamatergic receptors, but most of those studies investigated only behavioral changes, or receptor responses [113,114]. In clinical studies, rapid (during 2 h) antidepressant effects were produced by the NMDA receptor antagonist ketamine or the low-trapping NMDA channel blocker AZD6765 [26,115]. The behavioral antidepressant-like effects were also revealed soon after a single mGluR5 antagonist treatment (as mentioned above).

Therefore we can hypothesize that antidepressant-like and antigliotoxic effects of MTEP demonstrated in the present gliotoxic model of depression, may result from its inhibitory action on mGluR5 and indirectly on NMDA receptors both in neurons and astrocytes. The astrocytes surviving after the toxin application may be stimulated for proliferation and production of growth factors and gliotransmitters which can improve structure and glia-neuronal connections in the prefrontal cortex and restore proper behavior. However, this hypothesis needs further studies.

Acknowledgments

We wish to thank Assoc. Prof. Marzena Mackowiak for her valuable comments and assistance in using confocal microscopy.

This study was supported by the grant “Depression-mechanisms-therapy” POIG.01.01.02-12-004/09.

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