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Carnitine congener mildronate protects against stress- and haloperidol-induced impairment in memory and brain protein expression in rats

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ABSTRACT

The present study investigates the efficacy of mildronate, a carnitine congener, to protect stress and haloperidol-induced impairment of memory in rats and the expression of brain protein biomarkers involved in synaptic plasticity, such as brain-derived neurotrophic factor (BDNF), acetylcholine esterase and glutamate decarboxylase 67 (GAD67). Two amnesia models were used: 2 h immobilization stress and 3-week haloperidol treatment. Stress caused memory impairment in the passive avoidance test and induced a significant 2-fold BDNF elevation in hippocampal and striatal tissues that was completely inhibited by mildronate. Mildronate decreased the level of GAD67 (but not acetylcholine esterase) expression by stress. Haloperidol decrease by a third hippocampal BDNF and acetylcholine esterase (but not GAD67) expression, which was normalized by mildronate; it also reversed the haloperidol-induced memory impairment in Barnes test. The results suggest the usefulness of mildronate as protector against neuronal disturbances caused by stress or haloperidol.

1. Introduction

Learning and memory can be impaired by different causes. 60–70% of dementia in Alzheimer's disease and Parkinson's disease are attributed to brain neurodegenerative processes (Levy and Chelune, 2007). A decline in cognitive functions is also the most debilitating symptom of stroke (Leys et al., 2005), schizophrenia (O'Carroll, 2000) and severe stress (Sandi and Pinelo-Nava, 2007). Recently, attention has been paid to memory disturbances induced by long-term use of psychotropic drugs (Breggin and 1990, 2011), particularly antidepressants (Geerlings et al., 2012; Maxmen, 2010) and neuroleptics (Breggin, 1990, 2011). Thus, haloperidol, the first generation anti-schizophrenia drug, impairs learning and memory performance in humans (Harrison and Therrien, 2007; Legangneux et al., 2000; Lustig and Meck, 2005), as well as in experimental animal models (Hutchings et al., 2013). Deficit in cognition was demonstrated also by chronic treatment of atypical antipsychotics (Rosengarten and Quartermain, 2002; Terry, 2003; Terry and Mahadik, 2007).

During the last 2–3 decades, the role of proteopathies (e.g. deposition of misfolded proteins) is traditionally regarded as crucial factors leading to cell death and dementia (Rodrigue et al., 2009); however growing evidence indicates that protein pathologies are strongly associated with synaptic dysfunction (Selkoe, 2002) that involves trophic deficits (Butterfield et al., 2001). In this light, neurotrophins are considered as critical molecules, which support the plasticity of brain function throughout life (Chen et al., 2011; Thoenen, 1995). Among these, BDNF is particularly important for synaptic plasticity by inducing longlasting structural changes at dendritic spines (Verpelli et al., 2010), and hence, plays a major role in learning and memory (Minichiello, 2009). In addition, BDNF expression is regulated by different neurotransmitter systems, particularly cholinergic and glutamatergic (Angelucci et al., 2005; da Penha Berzaghi et al., 1993), impairment of which is related to memory loss in schizophrenia, depression, Alzheimer's disease (Li et al., 2000), stress (Bowers et al., 1998), and chronic treatment with antipsychotics (De Souza et al., 1999).

In the present study we investigated whether mildronate, a carnitine congener, may regulate changes in cognitive functions and

Abbreviations: BDNF, brain-derived neurotrophic factor; GAD67, glutamate decarboxylase 67

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the expression of protein biomarkers related to synaptic plasticity, such as neurotrophin BDNF, and enzymes of cholinergic (acetylcholine esterase) and glutamatergic (glutamate decarboxylase 67, GAD67) systems in the hippocampus and the striatum, in two separate amnesia models, one caused by stress, one caused by haloperidol.

Although mildronate is traditionally used as a cardioprotective drug (Simkhovich et al., 1988), the rationale behind the design of the present study was our previous data demonstrating memory-enhancing effect of mildronate. This was achieved via mechanisms involving proliferation of neural progenitor cells, enhancement of synaptic metabolism and activation of transcription factors, as well as activation of glutamatergic and cholinergic processes in trained rats (Klusa et al., 2013b). Moreover, mildronate in different neurotoxicity models normalized protein expression, impaired by neurodegeneration, neuroinflammation, apoptosis (Klusa et al., 2010; Pupure et al., 2010; Isajevs et al., 2011; Beitnere et al., 2014), mitochondrial dysfunction (Pupure et al., 2008), and hypoxia (Rumaks et al., 2012).

2. Materials and methods

2.1. Animals

Male Wistar rats were obtained from the Laboratory of Experimental Animals, Riga Stradins University, Riga, Latvia. Animals weighed 200 ± 10 g at the beginning of each experiment. All experimental procedures were performed in accordance with the EU Directive 2010/63/EU on the protection of animals used for scientific purposes and were approved by the Animal Ethics Committee of the Food and Veterinary Service (Riga, Latvia). All possible effort was made to minimize animal suffering and to reduce the number of animals used.

2.2. Drugs, antibodies and chemicals

Mildronate [3-(2,2,2-trimethylhydrazinium) propionate dihydrate] (Grindeks, Riga, Latvia), was dissolved in physiological saline and prepared as a 2% stock solution. Haloperidol (Gedeon Richter, Hungary) was obtained as 0.5% solution for injection; ketamine 10% and xylazine 2% solution (Alfasan, The Netherlands). For Western blotting, the following primary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were used: rabbit polyclonal against BDNF (sc-20981), rabbit polyclonal acetylcholine esterase antibody (sc-11409), mouse monoclonal GAD67 (sc-28376), and β-actin (ab8224; AbCam, UK). Secondary antibodies: goat anti-mouse IgG-HRP (sc-2005; Santa Cruz Biotechnology) or goat anti-rabbit IgG-HRP (A9169; Sigma). Chemiluminiscence reagents were ECL Prime Western Blotting Detection Reagent RPN2232V2 (GE Healthcare – Amersham UK).

For immunohistochemical studies the following antibodies from AbCam, UK were employed: rabbit polyclonal antibody against BDNF (ab101747), mouse monoclonal GAD65 antibody (ab26113), mouse monoclonal GAD67 antibody (ab26116); rabbit polyclonal antibody against acetylcholine esterase (sc 11409, Santa Cruz Biotechnology, Santa Cruz Biotechnology, CA, USA). The EnVision detection kit, peroxidase-conjugated polyclonal goat anti-rabbit IgG and 3,3- diaminobenzidine (DAB) were from DAKO (Glostrup, Denmark).

2.3. Stress model

2.3.1. Immobilization stress and passive avoidance response test (PAR)

Experimental rats (n=8–10 per group) were treated daily intraperitoneally for 2 weeks with mildronate (50 mg/kg) or physiological saline (control). 1 h after the last administration, animals were subjected to the PAR training (foot-shock 0.2 mA, 2 s). The step-through latencies, i.e. time spent in the light compartment before entering the dark chamber, were measured. On the next day, rats were exposed to immobilization stress according to a method described elsewhere (Nooshinfar et al., 2011) with slight modifications. Rats were placed in a perspex restrainer device with an adjustable door that allowed adjusting the box length for each rat, and held for 2 h completely immobilized with no space to move for the duration of immobilization. 30 min after immobilization, i.e. 24 h after the first PAR procedure, the retention test (without footshock) was carried out. Differences between retention and training day data were calculated. The step-through latency maximum testing limit was 300 s for both training and retention days. Non-stressed animals (kept in standard rat cages) received saline or mildronate served for the control.

2.4. Haloperidol model

2.4.1. Haloperidol treatment

Rats for this experiment were randomly divided into four groups (n=8–10 per group), and were administered intraperitoneally daily for 21 days: physiological saline (control), haloperidol (1 mg/kg), mildronate (50 mg/kg), and haloperidol (1 mg/kg)+ mildronate (50 mg/kg). After a two-day washout period, animals were checked for absence of catalepsy before the Barnes maze test.

2.4.2. Barnes maze test

Each rat was placed individually on the Barnes maze (1 m diameter table with 18 holes in the periphery at equal distances, of which only one was open and led to a target or escape box). On the first day, animals were acclimated to the maze; a bright light and fan were turned on as they were guided from the center of the maze to the target hole. After entering, the bright light and fan were turned off, and rats were left undisturbed for 30 s. Animals underwent 4 days of training consisting of three maximum 180 s trials separated by 15 min intervals in the home cage. On the fifth day of testing, animals were given a 90 s probe trial in which the escape box was blocked off. The number of errors was scored during all trials. The error was defined as an attempt to enter the maze hole with no escape box under it.

2.5. Biomarker assessment

After completion of the behavioral tests, rats were anesthetized by intraperitoneal injection with ketamine (75 mg/kg) and xylazine (10 mg/kg) and perfused through the ascending aorta with ice-cold saline. The hippocampus was removed, stored at −80 °C and processed for Western blot analysis and immunohistochemical assessment.

2.5.1. Western blot analysis

A whole hippocampus of the left cerebral hemisphere dissected by a routine method was used to quantify the expression levels of BDNF, acetylcholine esterase, and GAD67 proteins by Western blot. Briefly, the hippocampal tissue samples were homogenized and lysed in RIPA buffer (Sigma) supplemented with 1% protease inhibitor cocktail. The protein concentration was determined by a bicinchoninic acid (BCA) assay method. 20 μg of total protein was resolved by 15% SDS-PAGE for about 2 h at 20 mA per gel and transferred onto a nitrocellulose membrane. After blocking the membrane with 5% (wt/vol) non-fat dry milk in Tris buffered saline (TBS) containing 0.01% Tween-20 for 30 min, the proteins were immunoblotted overnight with rabbit anti-BDNF(1:200), rabbit anti-acetylcholine esterase (1:200) and mouse anti-GAD67 (1:100). After washing steps, the membrane was incubated with goat anti-mouse IgG-HRP (1:2000) or goat anti-rabbit IgG-HRP

(1:2000) at room temperature for 1 h. The excess secondary antibody was washed off, and the blots were developed by using chemiluminescence reagents. The same blot was restained with mouse anti-β-actin (1:5000) as a loading control. Density of bands was measured in ImageJ and normalized to β-actin.

2.5.2. Immunohistochemical assessment

The striatal tissue was cut into 10 μm-thick sections at −20 °C using a cryostat (Leica CM1850, Leica Microsystems, Germany). Twenty-four sections through the striatum were obtained. The sections were transferred onto polylysine-coated slides (three sections per slide). The slides were air-dried for 15 min and then immersed in ice-cold acetone for 15 min, followed by air drying for 2 h. The slides were wrapped in aluminum foil and stored at −20 °C until use. Tissue sections were immunostained to visualize cells that were positive for BDNF, GAD67, and acetylcholine esterase. Endogenous peroxidase activity was blocked with 3.0% H_2O_2 for 10 min. Nonspecific primary antibody binding was blocked with normal horse serum prior to antibody incubation. The slides were incubated overnight at 4 °C with mouse monoclonal anti-GAD67 antibody (1:500), rabbit polyclonal anti-acetylcholine esterase antibody (1:1000) and rabbit polyclonal anti-BDNF (1:300). Bound antibodies were detected using 1 h incubation with EnVision reagent. The immunoperoxidase reaction color was developed by incubating the slides with diaminobenzidine for 5 min. A negative control that omitted the primary antibody was included for each experiment.

For each experimental group, BDNF, GAD67 and acetylcholine esterase-immunopositive cells were counted from six independent sections. The total number of immunopositive cells was quantified using a magnification of 400 times. Regions of interest were captured using a Motic digital camera (Motic, China) mounted on a microscope (Motic BA400) using the Motic Image Advanced 3.2 software. The results were expressed as the number of immunopositive cells per mm^2.

2.6. Statistics

GraphPad Prism 5 software was used for the statistical analysis. The behavioral data (PAR and Barnes maze tests) were expressed as the mean ± S.E.M and analyzed using one-way ANOVA with Bonferroni's comparison test for selected pairs of columns as post-hoc analysis. For Western blot, differences between the groups' relative intensities were analyzed using oneway ANOVA followed by Bonferroni's post-hoc test, and immunohistochemical data were analyzed by two-way ANOVA followed by Bonferroni's post-hoc test. These results were expressed as mean ± S.D. In all tests, $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effects in the stress model

The results showed that subjecting rats to 2 h immobilization stress resulted in impairment of their PAR acquisition task performance. The difference in latency between retention and training days in stressed animals was about a third of that in non-stressed control animals ($F3,31 = 4.105$, $P = 0.0146$) (Fig. 1).

Mildronate pre-treatment improved the stress-induced memory impairment, and the latency values were comparable to that in the control group. Mildronate in non-stressed rats did not influence behavioral responses in this test (Fig. 1).

Western blot analysis (Fig. 2) showed that in the hippocampus, stress induced approximately 2-fold overexpression of BDNF, whereas mildronate significantly reduced the stress-induced

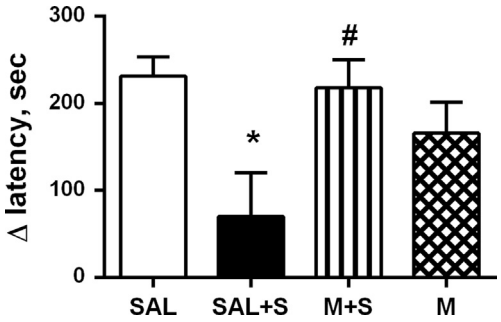

Fig. 1. Passive avoidance responses in rats after 2 h immobilization stress (*S*). Rats ($n = 8$–10 per group) were pre-treated intraperitoneally for 2 weeks with mildronate 50 mg/kg (*M*) or saline (SAL) for control. Differences of step-through latencies between retention and training days were calculated. Data are expressed as mean ± S.E.M. $^{*}P < 0.05$ vs. SAL; $^{\#}P < 0.05$ vs. SAL+*S*; one-way ANOVA with Bonferroni's comparison test for selected pairs of columns as post-hoc analysis.

increase. Mildronate did not alter the BDNF expression in non-stressed animals.

In the striatum (Fig. 3), stress increased the number of BDNF-positive cells (assessed immunohistochemically) 2.5-fold in comparison to control (Fig. 3, 48 ± 12 vs. 20 ± 5 cells/mm^2, $P = 0.03$). Mildronate pre-treatment normalized BDNF expression to close to the control values 18 ± 4 vs. 48 ± 12 cells/mm^2, $P = 0.03$ (Fig. 3).

Stress caused decreased hippocampal acetylcholine esterase- and increased GAD67 expression levels (Fig. 2, Western blot data); mildronate reversed the GAD67 expression in stressed animals to control values, while it did not change expression of acetylcholine esterase, (Fig. 2).

In the striatum (immunohistochemical data), stress increased the number of acetylcholine esterase-positive nerve fibers in comparison to control (15 ± 5 vs. 4 ± 3, $P = 0.01$, Fig. 4A). Mildronate did not influence this increase. The number of the striatal GAD67-immunopositive cells was not changed by stress or by mildronate (Fig. 4B).

3.2. Effects in haloperidol model

In Barnes maze test, haloperidol treatment for 3 weeks impaired spatial learning and memory, that was shown as an increased number of errors to reach the escape box on the 4th training day compared with saline treatment group data ($F3,29 = 3.126$, $P = 0.0409$; Fig. 5), whereas mildronate attenuated this spatial learning impairment. Similarly, during the probe trial test (5th day), in which the escape box was blocked off, the haloperidol treatment group showed more errors vs. control, while mildronate reduced the number of visits to incorrect holes to control level ($F3,32 = 3.797$, $P = 0.0195$, Fig. 5). Mildronate alone showed similar effect to control. It had no influence on the rat's locomotor activity in this test.

Western blot analysis showed that haloperidol decreased the BDNF and acetylcholine esterase expressions in the hippocampus, and mildronate normalized them to control levels (Fig. 6). GAD67 expression was not influenced in the haloperidol test (Fig. 6). Mildronate alone had no effect.

4. Discussion

In this study, we wanted to test whether mildronate, a carnitine congener molecule, protects against stress- and haloperidol-induced impairment in memory and how that correlates with the expression of brain proteins BDNF, acetylcholine esterase and GAD67, which are essentially involved in synaptic plasticity. The link between these models is the use of antipsychotic drugs in

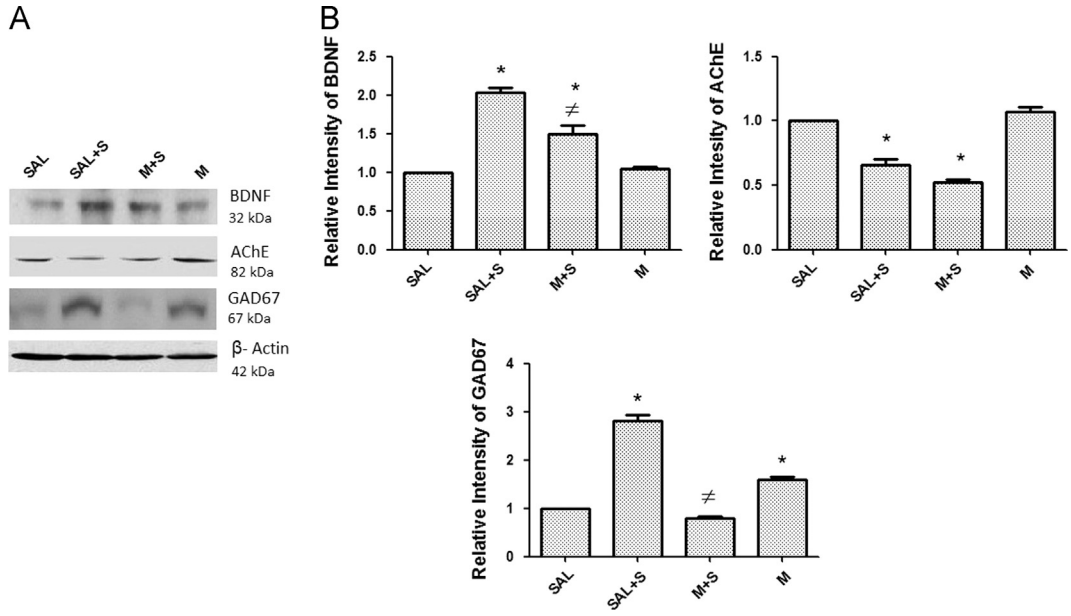

Fig. 2. (A) Representative Western blots of hippocampal tissue stained for BDNF, acetylcholine esterase (AChE), GAD67 and β-Actin. Rats were treated intraperitoneally by saline (SAL) or mildronate 50 mg/kg (*M*) for 2 weeks, followed by 2 h immobilization stress (*S*). (B) Relative intensity graphs between groups ($n=3$), $^{*}P<0.05$ vs. SAL; $^{\neq}P<0.05$ vs. SAL+*S*; one-way ANOVA followed by Bonferroni's post-test.

some forms of stress, for instance post-traumatic stress (Adetunji et al., 2005). The changes in protein expression were assessed in the hippocampus and the striatum, the brain structures more susceptible to different stress conditions (Giachero et al., 2013; Nooshinfar et al., 2011; Robinson et al., 2013) and chronic use of antipsychotics (Lipska et al., 2001). The dose 50 mg/kg of mildronate was chosen as the most effective in our previous experiments (Isajevs et al., 2011; Pupure et al., 2010).

Comparison of the structures of mildronate and carnitine clearly demonstrates their similarity (Klusa et al., 2013a). Carnitine is known to play a vital role in the transport of fatty acids to mitochondria for energy production, including brain cells (Nalecz et al., 2004). Moreover mildronate is transported into cells with slightly higher efficiency than that of carnitine by the carnitine transporter, the organic cation transporter type 2 (OCTN2) (Grigat et al., 2009). Therefore, mildronate can be used as replacement therapy in energy deficiency caused by different aversive situations. A striking evidence for mildronate effectivity in brain was demonstrated by our previous studies in animal models. Mildronate normalized nerve cell functioning, particularly by restoring brain protein expression impaired by neurodegeneration, neuroinflammation and apoptosis in an azidothymidine neurotoxicity mouse model (Pupure et al., 2010), in a Parkinson's disease (6-OHDA) rat model (Klusa et al., 2010; Isajevs et al., 2011) and in Alzheimer's disease transgenic mice (Beitnere et al., 2014). Therefore, although the traditional use of mildronate is as a cardioprotective agent due to inhibition of carnitine biosynthesis (Simkhovich et al., 1988; Dambrova et al., 2002), we focused on its carnitine-like action necessary for the regulation of nerve cell processes (Klusa et al., 2013a).

The data obtained in the present studies showed that mildronate is capable to regulate protein expression in both amnesia models. Thus after subjecting the rats to 2 h immobilization stress we observed the impairment of their PAR-task performance. This coincided with an increase in expression of BDNF in both hippocampus and the striatum, and these results are in good agreement with the data demonstrated by other authors (Marais et al., 2009; Nooshinfar et al., 2011). Mildronate reversed either memory deficiency (retention impairment in PAR test) in stressed animals, or normalized BDNF expression completely in the striatum and partially in the hippocampus.

The precise neurobiological explanation of stress still remains unclear; however chronic stress as the factor capable to modify neuronal plasticity have been put forward already since the 1980s, when elevated glucocorticoid concentration was shown to promote neuronal death, particularly in the hippocampus (McEven and Sapolsky, 1995). Recent data indicate that stress induced memory decline and significantly decreased the expression of cell adhesion molecule nectin-3 (Wang et al., 2013) and synaptophysin (Jin et al., 2013); however most information is related to BDNF expression (Licinio and Wong, 2002; Smith et al., 1995). BDNF is considered very important in cellular processes, including transcription, intracellular trafficking, secretion, proteolytic processing and receptor signaling, that makes BDNF a key protein for synapse-specific modulation in activity-dependent synaptic plasticity underlying cognitive functions such as learning and memory (Lu and Woo, 2009). Depending on the stress stimulus intensity, this influence is shown as opposite: chronic stress reduces it (Smith et al., 1995; Ueyama et al., 1997), whereas a single immobilization stress challenge induces rapid induction of BDNF expression in the hippocampus (Marmigère et al., 2003). Interestingly that similarly to stress, BDNF mRNA up-regulation in the hippocampus was observed also at learning/training, and short- or long-term memory formation (Huang et al., 2006; Mizuno et al., 2003), while deprivation of endogenous BDNF results in impairment of special learning and memory in adult rats (Mu et al., 1999).

In our stress experiment, mildronate ameliorated (to the control level) also the expression of hippocampal GAD67, which was considerably increased in stressed animals. Our data agree with other author's reports that demonstrated the increased GAD67 mRNA expression in several stress relevant brain regions, including the hippocampus (Bowers et al., 1998). In the striatum, however, GAD67 was not altered in stressed animals and not influenced by mildronate. Although literature data showed that stress causes the increase in the expression of the acetylcholine esterase gene and increased brain acetylcholine esterase activity (Kaufer et al., 1998), we observed distinct effects: increased acetylcholine esterase expression in the striatum but decreased in the hippocampus. Acetylcholine esterase levels altered in our stress experiment was not influenced by mildronate, indicating that cholinergic mechanism was not affected by mildronate in the stress model.

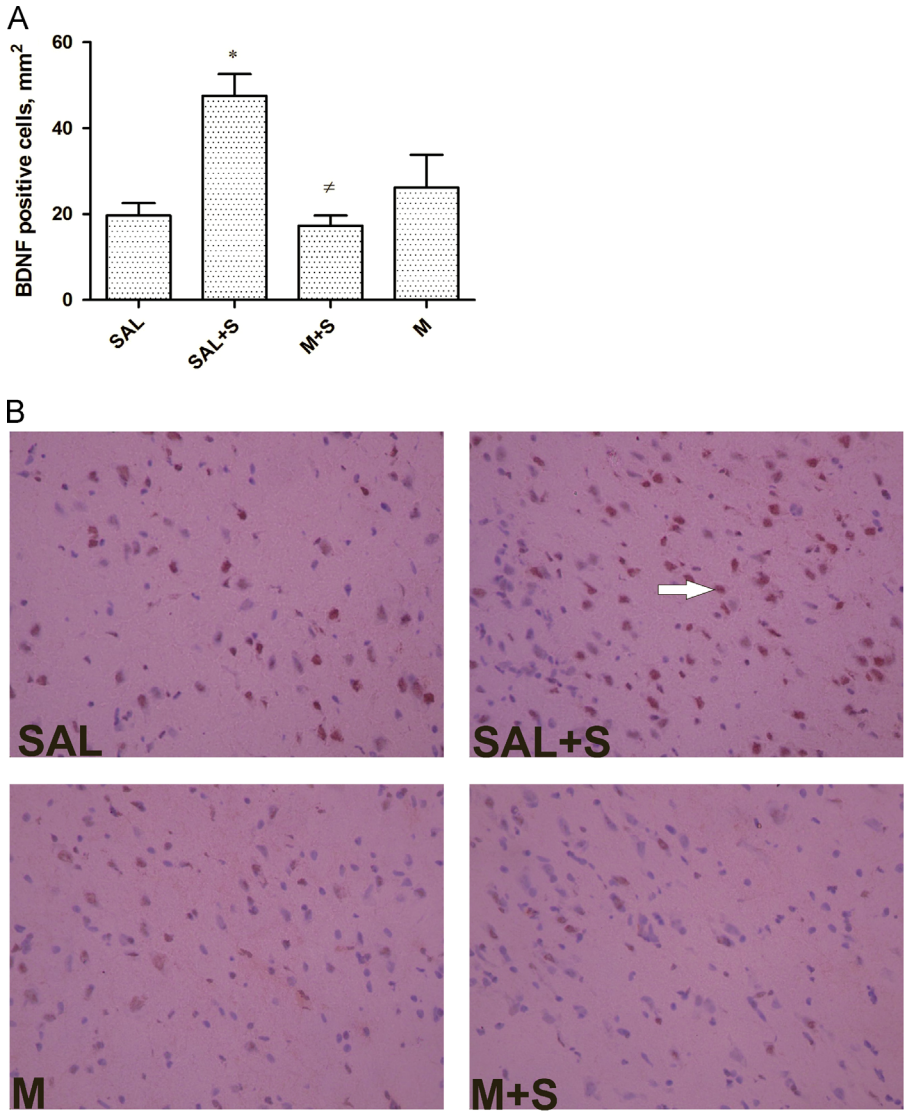

Fig. 3. (A) Immunohistochemical assessment of the number of BDNF-positive cells per mm^2 in rat striatal tissue and (B) a representative microphotograph. Rats were treated intraperitoneally for 2 weeks by mildronate 50 mg/kg (*M*) and saline (SAL) for control, followed by 2 h immobilization stress (*S*). Arrow indicates BDNF-positive cells. Magnification 400 ×. The results were expressed as mean ± S.D. (n=8–10). *$P<0.05$ vs. SAL; $^{\neq}P<0.05$ vs. SAL+*S*; two-way ANOVA followed by Bonferroni's post-test.

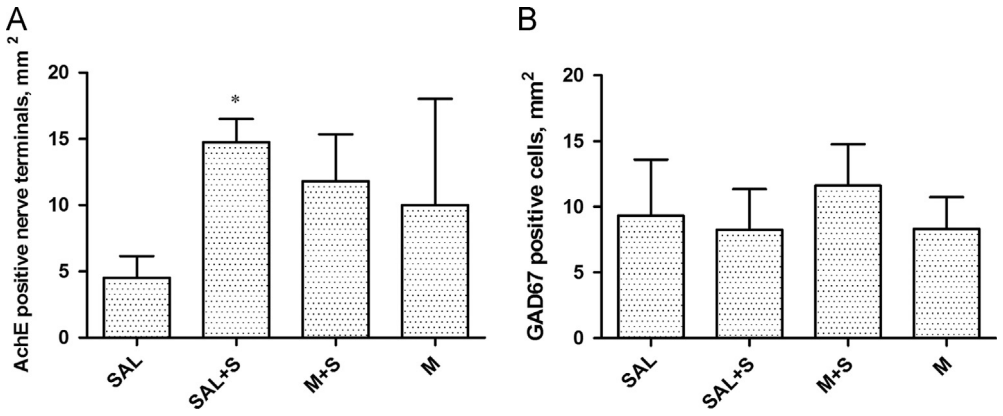

Fig. 4. (A) Immunohistochemical assessment of the number of acetylcholine esterase (AChE) positive fibers and (B) GAD67-positive cells per mm^2 in rat striatal tissue. Rats were treated intraperitoneally for 2 weeks by mildronate 50 mg/kg (*M*) and saline (SAL) for control, followed by 2 h immobilization stress (*S*). The results were expressed as mean ± S.D. (n=8–10). *$P<0.05$ vs. SAL; two-way ANOVA followed by Bonferroni's post-test.

In the three-week haloperidol treatment, a significant decrease in the expression of hippocampal BDNF and acetylcholine esterase (GAD67 level was not changed) was observed. Mildronate counteracted the decrease of these protein expressions by normalizing them to control levels, which coincided with the reversal of the impaired spatial memory in Barnes test. Concerning lowering

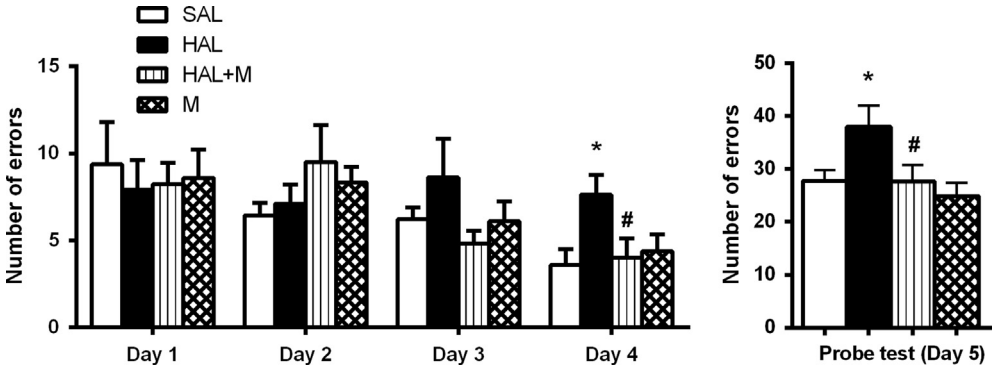

Fig. 5. Number of errors in training days and in probe test in Barnes maze. Rats were pre-treated intraperitoneally for 3 weeks with haloperidol 1 mg/kg (HAL), or mildronate 50 mg/kg (*M*), or saline (SAL) for control, and HAL+*M*. Number of errors (visits to false holes) per day (average of 3 trials) was calculated. Number of animals per group ($n=9$–10). Data are expressed as mean ± S.E.M. $^{*}P<0.05$ vs. SAL; $^{\#}P<0.05$ vs. HAL, corresponding day; one-way ANOVA with Bonferroni's comparison test for selected pairs of columns as post-hoc analysis.

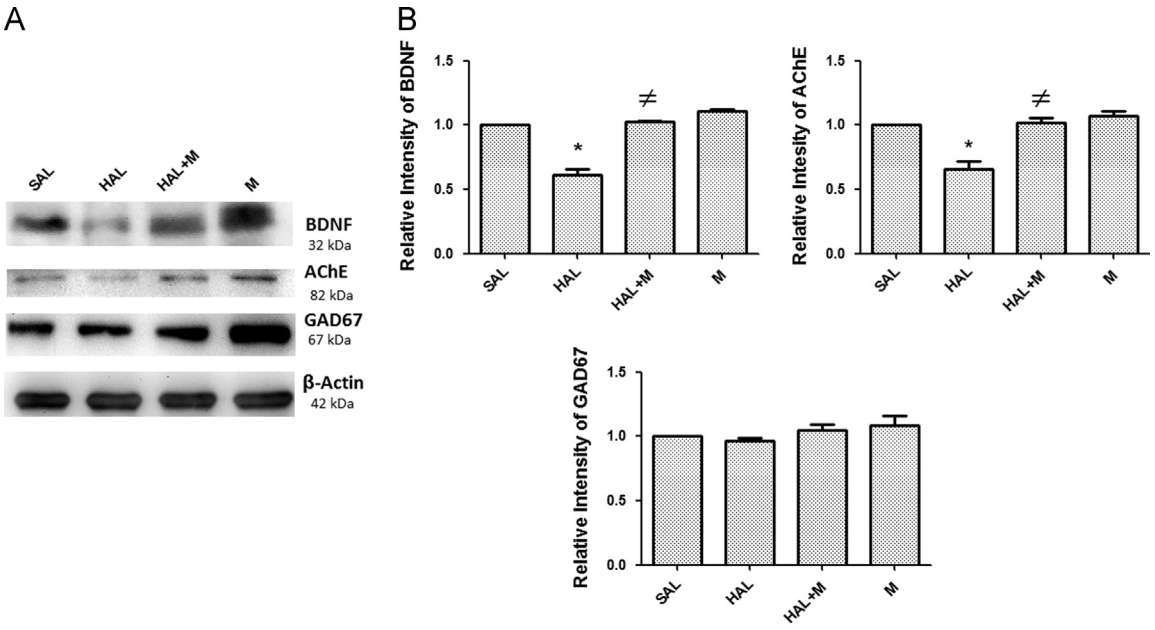

Fig. 6. (A) Representative Western blots of hippocampal tissue stained for BDNF, acetylcholine esterase (AChE), GAD67 and β-Actin. Rats were treated intraperitoneally by saline (SAL), haloperidol 1 mg/kg (HAL), mildronate 50 mg/kg (*M*), and HAL+*M* for 3 weeks. (B) Relative intensity graphs between groups ($n=3$). $^{*}P<0.05$ vs. SAL, $^{\neq}P<0.05$ vs. HAL; one-way ANOVA followed by Bonferroni's post-test.

of BDNF, our data are in good agreement with those reported elsewhere (Lipska et al., 2001; Nandra and Agius, 2012), while some authors did not notice any influence on BDNF by haloperidol (Valvassori et al.,2008). At the same, alterations in cholinergic activity were shown to be strongly associated with haloperidol-induced impairment of cognitive functions (Mahadik et al., 1988; Terry and Mahadik, 2007).

Our results obtained in haloperidol test suggest that the haloperidol-induced decrease in acetylcholine esterase level can be explained by the specific antagonism of dopamine receptors that halts dopaminergic signaling into the cell. Furthermore, the reduced dopaminergic activity becomes insufficient to provide its negative influence on the cholinergic system, hence leading to the hyperactivity of the latter. The over-stimulated cholinergic activity depends to a large extent on the reduced expression of acetylcholine degrading enzyme acetylcholine esterase. In human beings, such an imbalance between the dopamine and acetylcholine systems manifests as parkinsonism and cognition impairment after the long-term use of dopamine antagonistic anti-schizophrenia drugs. Mildronate regulates the expression of acetylcholine esterase and can be considered a beneficial effect leading to balance between dopaminergic and cholinergic systems that is very important to protect both the decline in memory and the manifestation of extrapyramidal symptoms in patients – dopamine receptor antagonist users – suffering from parkinsonism.

In summary, the present data demonstrated mildronate's ability to protect stress- and haloperidol-induced memory impairment that correlated with regulation of the expression of proteins, which are essentially involved in synaptic plasticity, hence in memory formation. Although stress and haloperidol caused opposite influence on BDNF expression, mildronate effectively normalized its expression in both situations, indicating BDNF as an important target, particularly because neurons require balanced BDNF expression. BDNF overexpression by itself may lead to memory impairment (Nooshinfar et al., 2011), or promotion of epileptogenesis via activation of its cognate TrkB receptor (He et al., 2010). However, not only the regulation of hippocampal BDNF but also GAD67 in the stress experiment, and acetylcholine esterase in the haloperidol experiment, seems to play an important role in mildronate's action to normalize cell functioning and hence, to improve memory impaired by these aversive situations.

Since mildronate has been shown to regulate a wide spectrum of brain proteins (for review, Klusa et al., 2013a), mildronate is suggested to stabilize protein expression due to its carnitine-like

action. The essential role of carnitine to act as protein stabilizer was identified previously (Calvani, 2002). In specific experimental situations mildronate normalizes the misbalance caused by specific drugs, neurotoxins, stressful stimuli.

In conclusion, the results suggest that mildronate might be used as neuroregulatory agent to stabilize expression of proteins essentially involved in cell functioning and which are necessary to perform cognitive tasks. Therefore one may expect mildronate to be clinically important in preventing or delaying the memory impairment processes triggered by stress situations and during chronic use of haloperidol (and/or other dopamine antagonists).

Acknowledgments

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