European Journal of Pharmacology ∎ (∎∎∎∎) ∎∎∎–∎∎∎

Contents lists available at ScienceDirect

European Journal of Pharmacology

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

Full length article

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

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ARTICLE INFO

Article history:
Received 20 May 2014
Received in revised form
8 October 2014
Accepted 8 October 2014

Chemical compounds studied in this article:
Haloperidol (PubChem CID: 3559)
Mildronate (PubChem CID: 123868)

Keywords:
Stress
Haloperidol
Memory
Protein expression
Mildronate

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.

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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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http://dx.doi.org/10.1016/j.ejphar.2014.10.014
0014-2999/© 2014 Published by Elsevier B.V.

Please cite this article as: Beitnere, U., et al., Carnitine congener mildronate protects against stress- and haloperidol-induced impairment in memory and brain protein expression in rats. Eur J Pharmacol (2014), http://dx.doi.org/10.1016/j.ejphar.2014.10.014

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

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