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Mildronate Improves Cognition and Reduces Amyloid- β Pathology in Transgenic Alzheimer's Disease Mice

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Abstract

Mildronate, a carnitine congener drug, previously has been shown to provide neuroprotection in an azidothymidine-induced mouse model of neurotoxicity and in a Parkinson's disease rat model. The aim of this study was to investigate the effects of mildronate treatment on cognition and pathology in Alzheimer's disease (AD) model mice (APP_{Swe}DT). Mildronate was administered i.p. daily at 50 or 100 mg/kg for 28 days. At the end of treatment, the animals were behaviorally and cognitively tested, and brains were assessed for AD-related pathology, inflammation, synaptic markers, and acetylcholinesterase (AChE). The data show that mildronate treatment significantly improved animal performance in water maze and social recognition tests, lowered amyloid- β deposition in the hippocampus, increased expression of the microglia marker Iba-1, and decreased AChE staining, although it did not alter expression of proteins involved in synaptic plasticity (GAP-43, synaptophysin, and GAD67). Taken together, these findings indicate mildronate's ability to improve cognition and reduce amyloid- β pathology in a mouse model of AD and its possible therapeutic utility as a disease-modifying drug in AD patients.

Keywords

mildronate; Alzheimer's disease; transgenic mice; cognition; hippocampus

Alzheimer's disease (AD) is associated with cognitive decline, leading to functional impairment and loss of independence. It has been widely recognized that AD represents a challenge for society and for health-care providers (Wimo et al., 2011). Therefore, the need to develop effective therapies to treat and/or delay the onset of this disease remains urgent.

The two pathological hallmarks of AD brains are the extracellular accumulation of amyloid- β peptide (A β) in neuritic plaques and intracellular neurofibrillary tangles composed of hyperphosphorylated tau protein (Karran et al., 2011). Furthermore, a reduction in the number of neurons, particularly in the hippocampus and entorhinal cortex (Lippa et al., 1992; West et al., 1994) and synapses (Terry et al., 1991) is seen, with neurochemical

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changes affecting cholinergic pathways (Craig et al., 2011). In addition, inflammatory processes and mitochondrial dysfunction may play an important role in disease progression (Ballard et al., 2011).

Currently existing Alzheimer's drugs provide some symptomatic benefits for up to 12 months, but still no disease-modifying treatments are available (Corbett et al., 2012). Recently, failures of numerous novel disease-modifying therapies in clinical trials indicate that a complementary strategy based on repositioning drugs that are approved for other indications could be more promising. Drug repositioning is the application of established drug compounds to new therapeutic indications and has been the basis of successful therapies (Hubsher et al., 2012), particularly with their established safety record, which is an essential advantage over novel compounds (Ashburn and Thor, 2004).

Recently, we have found that mildronate, developed as a cardioprotective drug (Dambrova et al., 2002), also has learning- and memory-enhancing properties that were shown to correlate with the proliferation of neural progenitor cells and activation of transcription factors in trained rats (Klusa et al., 2013). Traditionally, the main molecular mechanism of action of mildronate is based on carnitine biosynthesis inhibition aimed to prevent accumulation of cytotoxic intermediate products of fatty acid β -oxidation in ischemic tissues. Mildronate has been shown to block this highly oxygen-consuming process. Alternatively the drug acts via stimulation of nitric oxide (NO) production in the vascular endothelium (Sjakste et al., 2005). However, because of the structural similarity of carnitine and mildronate, the latter can be regarded as a carnitine analogue. Moreover, we have recently found that mildronate may have effects similar to those produced by carnitine, such as mitochondria-regulating, anti-apoptotic, anti-inflammatory effects. In previous studies, mildronate has been demonstrated to have neuroprotective properties in the mouse azidothymidine neurotoxicity model by suppressing inflammation and apoptotic processes in the brain, i.e., reduced lymphocyte infiltration in the brain and decreased activated caspase 3 levels (Pupure et al., 2010). In the Parkinson's disease 6-hydroxydopamine rat model (Klusa et al., 2010; Isajevs et al., 2011), it reduced proteins related to inflammation, such as glial; fibrillary acidic protein (GFAP), inducible NO synthase (iNOS), and ionized calcium-binding adapter molecule 1 (Iba-1). Furthermore, mildronate treatment effectively normalized neurological disturbances and reduced the infarct size in the ischemic stroke endothelin-1 model in rats (Rumaks et al., 2012). In addition, mildronate previously has been shown to act as a mitochondria-protecting agent, particularly at the level of complex I (Pupure et al., 2008). Together, these data indicated that mildronate might have beneficial effects in AD animal models.

The present study investigated for the first time the effect of mildronate in AD transgenic APP^{SweDI} mice. These mice express human amyloid precursor protein (APP) with Swedish, Dutch, and Iowa mutations on a C57BL/6 background. This mouse model develops early-onset and robust accumulation of A β in the brain with a high association with cerebral microvessels, and it also develops early cognitive deficits (Davis et al., 2004). We tested the mice in different behavioral (open field, zero maze) and cognitive (social recognition and water maze) tasks. Furthermore, AD pathology was evaluated in the hippocampus immunohistochemically and by Western blot. AD pathology was evaluated for A β , GFAP,

Iba-1, glutamate decarboxylase 67 (GAD67), growth associated protein 43 (GAP-43), synaptophysin, and acetylcholinesterase (AChE). The 28-day treatment with mildronate significantly improved cognitive performance in the water maze and social recognition tasks and lowered A β pathology and the expression of AChE.

MATERIALS AND METHODS

Animals

The original Tg APP_{SweDI} mice were obtained from Dr. Van Nostrand (SUNY Stony Brook, Stony Brook, NY) and are locally maintained in a colony. For this study, we used three groups of female mice 4 months of age ($n = 36$). The animals were housed five per cage in a controlled environment at the University of Alabama at Birmingham Animal Care Facility (temperature 22°C, humidity 50–60%, with a 12-hr light/dark cycle); food and water were available ad libitum. All efforts were made to minimize animal suffering and to reduce the number of animals used. The experiments were conducted in accordance with the local Institutional Animal Care and Use Committee (IACUC) guidelines.

Chemicals

Mildronate [3-(2,2,2-trimethylhydrazinium) propionate dihydrate] was obtained from the Joint Stock Company “Grindex” (Riga, Latvia), dissolved in saline, and prepared as a 1% stock solution.

Experimental Design

Mice were randomized to one of three weight-matched experimental groups ($n = 12/\text{group}$); average weight was 21 ± 2 g; and mildronate at doses of 50 mg/kg (M50 group) or 100 mg/kg (M100 group) or control (saline) was administered intraperitoneally (i.p.) every day for 28 days. Two weeks after the start of treatment, the animals were tested in two behavioral tasks, i.e., open field test and zero maze. During the following week they were tested in the social recognition and water maze tests. At the end of the behavioral testing, i.e., 4 weeks after the start of the treatment, the animals were sacrificed for histopathological and biochemical analysis.

Mice were deeply anesthetized and sacrificed by a transcardial perfusion with cold saline. Brains were removed immediately after decapitation and cut in half in the sagittal plane. The left hemisphere was dissected into four pieces (rostral cortex, caudal cortex, hippocampus, and midbrain/brainstem) and stored at -80°C for Western blotting (WB) analysis. The right hemisphere was immersion fixed in 4% paraformaldehyde for 24 hr, cryoprotected in 30% sucrose in 0.1 M phosphate buffer for an additional 24 hr, and stored in antifreeze solution at -20°C until the time of sectioning.

Behavioral and Cognitive Assessment

Briefly, open field and zero maze test were used to assess locomotion, exploratory activity, and anxiety. The social recognition and water maze test were used to measure the cognitive abilities of the animals. Animals were put in the arena and observed with a camera-driven tracker system, i.e., Ethovision (Noldus, The Netherlands). The open field maze is an arena

of 42 by 42 cm square with clear plexiglass sides (20 cm high). The arena is subdivided into two areas, the open center area, and the side by the wall. This test measures activity and anxiety, i.e., time spent in the “open” center vs. the “safe” side. The zero maze consists of a circular maze (70 cm diameter) that is raised 40 cm above the table and divided into four equal parts. Two parts have 15-cm-high sides of nontransparent material, and two parts have only a 0.5-cm-high wall, i.e., two open areas and two closed areas. The test measures anxiety in the animal; i.e., how much time does the animal spent in open vs. the closed, “safe” areas?

Social recognition was tested in a rectangular, three-chambered box from clear polycarbonate. Quantification of entries and duration in the chambers was automatically recorded for 10 min on the first day and for 4 min on the second day. Animals used as test subjects were male C57BL6 mice that were placed under a wire cup in each side chamber. On the first day, two “subject” mice were present under the wire cups in the arena; on the next day, one of the “old” mice and a “new” test mouse were present under the wire cups. Measurements taken included time spent in each chamber, time spent sniffing each test mouse, and number of entries into the test chambers.

Our version of the water maze consisted of a blue circular tank of clear water ($23^{\circ}\text{C} \pm 1^{\circ}\text{C}$). In the water maze test, mice were trained for three 60-sec trials per day to swim from one of the three starting points of the pool to find a fixed, hidden platform, submerged below the water (0.5 cm) in one of the quadrants of the pool. If the animal did not locate the platform during that time, it was placed upon it by the experimenter and left there for 10 sec. Once the mouse has learned the task (day 5, trial 15), a probe trial was given on day 8. In the probe trial (i.e., trial 16), the platform was removed from the pool and animals were allowed to swim for 60 sec. Mice with intact memory function typically remain searching for a much longer time in the “correct” quadrant, whereas impaired mice would explore more evenly among the four quadrants. All behavioral tests were performed by an individual who was blinded to the treatment groups of animals. Data were analyzed by one-way ANOVA and the Bonferroni’s multiple-comparisons posttest were carried out to determine the source of the significant main effect or interaction.

Immunohistochemistry

The right cerebral hemispheres were coronally sectioned (six series) at 30 μm on a freezing, sliding microtome. Free-floating sections of half of series one and two were then stained with primary antibodies: W0-2 antibody (mouse anti-human $\text{A}\beta_{4-10}$; 1:5,000; Millipore, Temecula, CA), GFAP (mouse anti-GFAP; 1:1,000; clone G-A-5; Sigma, St. Louis, MO), Iba-1 (rabbit anti-Iba1; 1:5,000; ab5076; Wako, Richmond, VA), and GAD67 (mouse anti-GAD67; 1:1,000; MAB5406; Millipore). The other series were stored in antifreeze for later use. The half-series of sections was transferred to a solution containing the primary antibody; this solution consists of TBS with 0.5% Triton X-100 added (TBS-T). After incubation in this solution for 18 hr on a shaker table at room temperature (20°C), the sections were rinsed three times in TBS-T, pH 7.4, and transferred to the solution containing the appropriate secondary antibody (goat anti-mouse biotin [Sigma] or goat anti-rabbit biotin [Millipore]). After 2 hr, the sections were rinsed three times with TBS-T and transferred to a

solution containing mouse ExtrAvidin (1:1,000; Sigma) for 2 hr. After being rinsed, the sections were incubated for 3 min with Ni-enhanced diaminobenzidine (DAB). All stained sections were mounted on gelatin-coated slides and coverslipped. It should be noted that, to obtain similarly stained material, sections from all three animal groups were always stained simultaneously in the same staining tray. One half of the third series of sections was stained for AChE according to a modified method of Koelle and Friedenwald (1949), mounted, and coverslipped.

Quantification

The appropriate areas (dorsal hippocampus) of the brains were digitized using an Olympus DP70 digital camera. To avoid changes in lighting, which might affect measurements, all images were acquired in one session. Furthermore, to avoid differences in staining density between sections, the measurements were performed on sections that were stained simultaneously in the same staining tray. The percentage of area covered by the reaction product to A β was measured (van Groen et al., 2006) in hippocampus (stratum oriens of CA1 and the dentate gyrus) in Scion Image (NIH; Kadish and Van Groen, 2002). The density of Iba-1 and GFAP staining was measured by the optical density of the staining in the appropriate hippocampal area (stratum oriens of CA1 and the dentate gyrus) in Scion Image. The density of AChE staining was measured by the optical density in the appropriate hippocampal area (stratum pyramidale and radiatum of CA1). All density measurements were performed in duplicate. These measurements were made by an observer blinded to the treatment of the animal. Data were analyzed by ANOVA (GraphPad Prism; between groups), and Bonferroni post tests were carried out to determine the source of a significant main effect or interaction. Statistical significance was set at $P < 0.05$.

WB Analysis

The left hemisphere's hippocampus was used to quantify the expression levels of GAP43, synaptophysin, and GAD67 proteins. Briefly, the hippocampal tissue samples were homogenized and lysed in RIPA buffer supplemented with 1% protease inhibitor cocktail. The protein concentration was determined by BCA assay method. Twenty micrograms of total protein was resolved by 15% SDS-PAGE for about 1 hr at 100 V and transferred onto a nitrocellulose membrane. After blocking the membrane with 5% (wt/vol) nonfat dry milk in TBS containing 0.01% Tween-20 for 30 min, the proteins were immunoblotted overnight with one of the following primary antibodies: GAD67 (mouse anti-GAD67; 1:5,000; MAB5406; Millipore), GAP43 (rabbit anti-GAP43; 1:800; sc-10786; Santa Cruz Biotechnology, Santa Cruz, CA), and synaptophysin (mouse anti-synaptophysin; 1:1,000; S5768; Sigma). After washing steps, the membrane was incubated with goat anti-mouse IgG-HRP (1:2,000; sc-2005; Santa Cruz Biotechnology) or goat anti-rabbit IgG-HRP (1:2,000; sc-2004; Santa Cruz Biotechnology) at room temperature for 1 hr. Excess secondary antibody was washed off, and the blots were developed by using chemiluminescence reagents. The same blot was restained with mouse anti- α -tubulin (1:5,000; T5168; Sigma) as a loading control. Density of bands was measured in ImageJ and normalized to α -tubulin. Statistical evaluation was performed by using one-way ANOVA followed by Bonferroni's post hoc test. Difference was considered significant at $P < 0.05$.

RESULTS

Animals

At the end of the study, M50 and M100 group animals had gained body weight similarly to the saline group (final weights: saline 24.1 ± 0.8 g, M50 23.7 ± 0.8 g, and M100 23.4 ± 0.8 g, respectively). Two animals were removed from the study because of “floating” in the water maze task, one in the M50 group and one in the M100 group.

Behavioral Outcome

After 2 weeks of mildronate treatment, the three groups of mice (saline, M50, and M100) were tested in the open field and zero maze to investigate their motor activity and anxiety levels. There were no significant differences in time spent in the center of the open field between the groups (saline 5.3 ± 1.5 , M50 4.5 ± 1.4 , M100 3.7 ± 0.9), nor were differences present in the zero maze (time in open area: saline 82.8 ± 10.6 , M50 68.5 ± 5.9 , M100 77.9 ± 8.0). Thus, all animals had similar levels of motor activity (not illustrated) and similar levels of anxiety.

To test cognition, the social recognition and the water maze tests were performed. In the social recognition test, both mildronate groups had significant improvements in the recognition of the new mouse (Fig. 1), spending on average more time (%) with the new mice (M50 60.4 ± 5.8 vs. saline 43.2 ± 5.3 , $P < 0.05$; M100 60.0 ± 7.2 vs. saline, $P < 0.05$).

In the water maze test, all groups improved their performance during the week of training. The average time for finding the platform on day 5 was saline 25.5 ± 7.2 , M50 16.9 ± 2.5 , M100 21.7 ± 3.4 sec. Although there is a tendency for learning faster for both mildronate groups, there was a significant difference ($P < 0.05$) only between the M50 and the saline groups in the water maze test in the time to find the hidden platform on day 5 (Fig. 2A). Similarly, only the M50 group showed a preference for the correct quadrant in the probe trial compared with the saline group (Fig. 2B). There was no significant difference in the swimming speed of the mice (saline 18.8 ± 0.8 cm/sec, M50 19.0 ± 0.8 cm/sec, M100 19.1 ± 0.6 cm/sec).

Immunohistochemical Data

AD pathology was assessed by measuring the A β load in selected areas of the hippocampus. In the A β -stained sections, there was a significant decrease of A β deposition in the M50 group (M50 vs. saline, $P < 0.05$) and a tendency to decrease A β load in stratum oriens of CA1 in the M100 group (Fig. 3. AA,B). Similarly, there was a significant decrease of A β deposition in the dentate gyrus in the M50 group (M50 vs. saline; $P < 0.05$) and a tendency to decrease A β load in the M100 group (percentage load: saline 1.34 ± 0.24 , M50 0.59 ± 0.23 , M100 0.79 ± 0.23 ; not illustrated). Staining with the microglia marker Iba-1 demonstrated an increased staining density in both treatment groups compared with the saline group (Fig. 3. AA,B). The average density in the groups was saline 174.4 ± 2.3 , M50 183.2 ± 1.5 , M100 186.6 ± 1.9 , with a significant difference between the M50 and the saline groups ($P < 0.05$; M100 vs. saline, $P < 0.05$).

Staining for the astroglial marker GFAP (Fig. 3. AA,B) did not show any significant differences among groups in average density of GFAP expression (saline 179.3 ± 2.4 , M50 173.5 ± 1.6 , M100 176.9 ± 1.6). There was also no significant difference between AChE average staining density, measured in stratum radiatum (saline 174.5 ± 2.0 , M50 165.7 ± 3.9 , M100 169.5 ± 3.4) in the hippocampus. However, the average staining density for AChE in stratum pyramidale was significantly ($P < 0.05$) decreased in the M50 group compared with the saline group (saline 113.6 ± 2.5 , M50 106.5 ± 2.7 , M100 110.5 ± 2.6 ; Fig. 3. AA,B).

WB Analysis

To understand the molecular basis that led to the cognitive improvement, WB analysis was undertaken for GAD67, synaptophysin, and GAP43. WB analysis revealed no significant changes between the saline and the treatment groups in any of these proteins (Fig. 4).

DISCUSSION

The main goal of this study was to assess whether mildronate would be able to improve cognition in transgenic APP_{SweDI} mice and alleviate brain pathology. The rationale for undertaking such a study was that mildronate had previously been demonstrated to have memory-enhancing properties; similarly, it has been shown to promote proliferation of neuronal progenitor cells and enhance synaptic protein expression (Klusa et al., 2013). Mildronate treatment decreased protein expression related to inflammation and apoptosis, but increased expression of endogenous chaperone Hsp70, in a Parkinson's disease rat model (Isajevs et al., 2011) and in an azidothymidine neurotoxicity model in mice (Pupure et al., 2010).

In the present study, the APP_{SweDI} mice were treated i.p. for 28 days with mildronate at doses of 50 and 100 mg/kg. These doses have previously been shown to be the most effective in other experimental disease models (Klusa et al., 2006; Pupure et al., 2010; Isajevs et al., 2011). Mildronate treatment did not change basic motor activity and anxiety levels in either the open field or the zero maze. Mildronate significantly improved social recognition in the treated AD-model mice compared with the saline-treated group. This phenomenon can be taken as the critical ability to identify individuals in social groups. Social recognition is associated with the hippocampal processes in rodents (Maaswinkel et al., 1996; Feinberg et al., 2012). Impaired sociability and dysregulated social behavior have been implicated as hallmarks not only in AD pathology but also in many psychiatric disorders, such as autism and schizophrenia. Moreover, marked deficits in social cognition and social behavior disrupt social relationships and result in an increased risk of depression (Abdi and Sharma, 2004; Sasson et al., 2011). Therefore, drugs that might improve social cognition can be considered beneficial therapeutic agents. In the water maze test, only the M50 group significantly improved their cognitive performance; however, it is not unlikely that the nonsignificant outcome for the M100 group was affected by the relatively unimpaired performance of the saline-group mice, which also improved their performance significantly during the week of training.

Another important finding was that mildronate at a dose of 50 mg/kg significantly lowered A β plaque load in hippocampus, whereas the higher dose showed a trend toward decreased

A β load. Our AD-model mice develop both parenchymal A β deposition and cerebral amyloid angiopathy (CAA), so we evaluated whether mildronate treatment reduced either A β pathology. We measured A β load in stratum oriens of CA1, where there is both CAA and parenchymal amyloid deposition, and in dentate gyrus, where only parenchymal A β depositions are present. Mildronate treatment at 50 mg/kg significantly decreased the A β load in both hippocampal regions, whereas the higher dose did not significantly reduce A β load. Unexpectedly, mildronate at both doses slightly, but significantly, increased microglial expression. This finding is in contrast with previously reported mildronate treatment effects on microglia, in which mildronate reduced activated microglia levels in several experimental neuro-degenerative disease animal models. The altered results for microglial activation in our study might be explained by different animal models of neurodegeneration used in previous studies; previous studies used chemically induced neurodegeneration, but we employed Tg AD mice. In animals with chemically induced neurodegeneration, brain immune cells might initiate slightly different inflammatory pathways to overcome acute injury compared with immune cells in our transgenic animals, in which certain proteins have been expressed for much longer times and primed the microglia and astrocytes to different conditions (Block et al., 2007). We suggest that microglial activation might be one of the reasons for the ability of mildronate to decrease A β load in the hippocampus. Drugs that improve memory deficits by inhibiting A β toxicity and increasing microglial activation in APPswe/PS1dE9 transgenic mice have been shown before (Xue et al., 2012). For instance, the anti-inflammatory drug NCX-2216 shared both the activation of microglia and a dramatic reduction of A β load (Jantzen et al., 2002). The relationship between the clearance of A β and microglia remains controversial; some studies have shown that anti-inflammatory action by nonsteroidal anti-inflammatory drugs (NSAIDs) such as ibuprofen significantly reduced microglial activation (i.e., CD11b and CD45 expression) in APP-PS1 transgenic mice and A β load (Lim et al., 2000). Similarly, the treatment with a higher dose of HCT-1026 (an NO-donating analog of flurbiprofen) decreased activation of microglia (lower levels of MAC1 expression) in the immediate vicinity of plaques near fibrillar A β deposits (van Groen and Kadish, 2005; van Groen et al., 2011). One of the hypotheses for the cause of A β deposits is that hyperactive microglia are the proximal event of AD-associated neurotoxicity and contribute directly to the neuropathology characteristic of the disease (McGeer and McGeer, 1999, 2004). However, it should be taken into account that the neuroinflammation in AD has two faces (Eikelenboom and van Gool, 2004); microglial activation and inflammation can also contribute to phagocytosis and clearance of A β deposits (Matsuoka et al., 2001; D'Andrea et al., 2004).

Similarly, in transgenic mice overexpressing the APP transgene only, a reduced number of plaques and reduced levels of inflammation markers IL-1 β and GFAP were found after ibuprofen treatment (Lim et al., 2000). In our study, mildronate did not affect GFAP expression. It is, however, possible that the relatively large variation in the staining density for GFAP between animals prevented us from seeing any significant differences between the groups.

Mildronate did not change the levels of any of the synaptic plasticity markers that we measured (i.e., GAP-43, synaptophysin, and GAD67). Most likely, no significant changes were present, because the Western blots were performed on the whole hippocampus, and the

changes that were measured in the immunohistochemistry were present at only specific parts of areas of the hippocampus, e.g., stratum oriens of CA1 or stratum pyramidale of CA1.

Dysregulated cholinergic signaling is an early hallmark of AD and is caused by loss of cholinergic neurons, likely because of A β accumulation in the hippocampus (Craig et al., 2011). The enzyme AChE is responsible for the breakdown of acetylcholine at the neuronal synapse and plays an important role in synaptic transmission. Blocking this enzyme's activity was the first approach to alleviate the memory deficits in AD (Martorana et al., 2010). In our study, mildronate affected AChE levels only in area CA1 in the mice that had been treated with M50 but not in the M100 group. This, however, correlates with the improved cognition in this group compared with the M100 group. The decrease in AChE expression in area CA1 likely causes higher levels of acetylcholine and thereby better functioning of area CA1 in spatial learning and memory, as indicated by the significantly improved performance in the water maze in the M50 group. Similarly, a decreased number of AChE-positive nerve fibers was found previously in mildronate-treated rats that improved their performance in the conditioned avoidance response (CAR) test (Klusa et al., 2013).

Alternatively, it is conceivable that mildronate acts through other pathways to improve cognitive abilities in AD model mice. One of the possibilities is that mildronate augments cerebral blood flow and by doing so improves soluble A β outflow from the brain. In support of this concept are data from Sjakste et al. (2005), who suggest that CNS effects of mildronate could be mediated by stimulation of the NO production in the vascular endothelium by modification of γ -butyrobetaine and its esters pools.

Another possibility is that mildronate affects the vitagene network. Recently, L-carnitine and its metabolite acetyl-L-carnitine have been demonstrated to be neuroprotective through the activation of hormetic pathways, including vitagenes (Calabrese et al., 2012). We point out the structural similarity of carnitine and mildronate, leading us to consider the latter as a carnitine analogue. In addition, it is worthwhile to note the ability of mildronate to stimulate HSP70 expression in 6-OHDA-lesioned striatum and substantia nigra (Isajevs et al., 2011). Vita-genes encode heat shock proteins, and neuroprotective action can be explained at least in part through the activation of stress response and hormetic pathways. However, we do not have data on whether (or how) mildronate regulates the HSP system in AD model animals, but one may expect a similar effect evoked by acetyl-L-carnitine, which upregulates HSP and protects cortical neurons against A β 42-mediated oxidative stress and neurotoxicity (Abdul et al., 2006).

Given the results from current study, we suggest that the 50 mg/kg dose is more effective in improving cognition and in lowering AD-related pathology in our AD-model mice. In most data sets of the current study it appears that M50 has a more positive outcome than the M100 group, indicating that the M100 dose might be too high and have undesired side effects. These effects could be species-related; it has been shown that doses of up to 200 mg/kg in rats do have protective effects in rat stroke models (Svalbe et al., 2011).

The data show that mildronate has several positive effects in AD-model mice, including improvement of social recognition and spatial learning in the water maze and a reduction of

A β load. Furthermore, microglia are activated in the mildronate-treated mice, and AChE expression is decreased in area CA1. Together these findings indicate that mildronate could have therapeutic utility as a disease-modifying drug in AD patients. Our AD-model mice at this age do not have tau pathology, so in subsequent studies it will be important to evaluate whether the mildronate treatment in AD mouse models with tau pathology would have similar effects. In addition, future studies in preclinical AD models are needed to understand better the molecular mechanisms through which mildronate acts to improve cognition and decrease the pathology.

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The authors have no conflicts of interest to declare.

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Fig. 1.

Bar graphs showing time spent (%) with the new mouse in the social recognition test. Both M50 (50 mg/kg mildronate) and M100 (100 mg/kg mildronate) groups spent significantly more time with the new mouse compared with the saline group. * $P < 0.05$.

Fig. 2.

Three line graphs showing the learning curves of the three groups of mice. **A:** Daily mean performance in the water maze; on day 5, the M50 (50 mg/kg mildronate) group found the escape platform significantly ($*P < 0.05$) faster than the saline group. **B:** Probe trial performance of the three groups of mice. Note that only the M50 group has a preference for the correct quadrant compared with the saline group. $*P < 0.05$.

Fig. 3.

A. Four sets of photomicrographs of the dorsal hippocampus showing three groups, saline, M50 (50 mg/kg mildronate), and M100 (100 mg/kg mildronate), stained for Aβ₄₋₁₀ (W0-2; top row), micro-glia (Iba-1; second row), astrocytes (GFAP; third row), and AChE (bottom row). Aβ deposition was significantly ($P < 0.05$) decreased in the M50 group compared with saline group; Iba-1 staining density was increased in both treatment groups ($P < 0.05$) compared with saline. There were no significant changes in GFAP staining density. The M50 dose of mildronate significantly ($P < 0.05$) decreased AChE staining density in CA1 pyramidal layer of hippocampus of treated animals compared with the saline group. so, Stratum oriens; sp, stratum pyramidale; sr, stratum radiatum; cc, corpus callosum. **B:** Bar graphs showing quantification of the density measurements in the immunohistochemical stainings. * $P < 0.05$.

Fig. 4. Representative Western blots of hippocampal homogenates from saline (sal)-, M50 (50 mg/kg mildronate)-, and M100 (100 mg/kg mildronate)-treated mice stained for GAD67, GAP43, syn-aptophysin, and α -tubulin. There were no significant differences in any of these markers between saline and treatment groups.