Published in final edited form as:

Pharmacol Res. 2016 November ; 113(Pt B): 781–787. doi:10.1016/j.phrs.2016.06.020.

Memory-enhancing and brain protein expression-stimulating effects of novel calcium antagonist in Alzheimer's disease transgenic female mice

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Abstract

The prevalence of Alzheimer's disease (AD) is higher in females than in males, and causes more severe cognitive, memory and behavioral impairments. Previously, in male transgenic (Tg) APPSweDI mice, we reported that the novel lipophilic 1,4-dihydropyridine (DHP) derivative AP-12 crossed the blood-brain barrier, blocked neuronal and vascular calcium channels, changed brain protein expression and improved behavior. In this study, we used female Tg APPSweDI mice to assess the effects of AP-12 on behavior, and brain protein expression, with a particular focus on those of the GABAergic system. The results showed that in female Tg mice, similar to male Tg mice, AP-12 improved spatial learning/memory performance in the water maze test and demonstrated anxiolytic effect in the elevated zero maze (after single administration of AP-12) and elevated plus maze (after chronic injections of AP-12). In addition, we demonstrated upregulated expression of glutamate decarboxylase 67 (GAD67) and vesicular GABA transporter (VGAT) in the cingulate cortex and hippocampus, pointing to the role of the GABAergic system as one of the neural networks dysregulated in AD. In both female and male mice, AP-12 did not change the expression of hippocampal Homer-1, a protein which is involved in synaptic plasticity. However, in cingulate cortex, the staining density of Homer-1 was significantly increased in female mice. Further, female mice (similar to male mice) did not show changes in brain AChE expression and in the amyloid beta load in the hippocampus and cingulate cortex. In conclusion, the memory enhancing, anxiolytic and protein expression effects of AP-12 did not show sex specificity in APPSweDI mice. Considering the ability of AP-12 to block brain calcium channels and improve memory by enhancing the GABAergic and synaptic plasticity processes, AP-12 is a promising

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Conflict of interest statement

None declared.

compound which merits further pre-clinical studies to investigate its usefulness in the treatment of AD.

GRAPHICAL ABSTRACT

Keywords

1,4-dihydropyridine derivative; memory; anxiolytic effect; protein expression; Alzheimer's disease; mice

1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder that leads to dementia [1]. The treatment options currently available are limited. Acetylcholinesterase inhibitors and N-methyl-D-aspartic acid receptor antagonist (memantine) only slow the progression of the disease and do not provide a cure. The pathogenesis of AD is still not clearly understood, but likely, there are multiple mechanisms, such as the extracellular deposition of β -amyloid ($A\beta$), intraneuronal tau pathology, synaptic loss, impaired neural transmission, $A\beta$ angiopathy and inflammatory processes, together impairing neurons in the hippocampus and cerebral cortex [1]. In addition, there is evidence that altered neuronal Ca^{2+} homeostasis can also facilitate neurodegeneration in AD [2–6], therefore attention has been turned to the calcium channel blocking drugs of the 1,4-dihydropyridine (DHPs) series as neuroprotectors in both Parkinson's disease [7, 8] and AD [9].

Recently we have described the novel lipophilic 1,4-dihydropyridine (DHP) derivative AP-12 and its memory-improving, anxiolytic effects in transgenic AD model (Tg APPSweDI) male mice. This compound was also shown to cross the blood-brain barrier [10] and exert Ca^{2+} antagonistic properties in neuroblastoma and in the vascular smooth muscle cell line [11].

The present study examined how this compound affected behavior and protein expression in female transgenic (Tg APPSweDI) mice. In this context, it is noteworthy to emphasize that females are more affected by AD than males, in terms of prevalence and severity [12, 13].

Thus, in female transgenic (Tg APPSweDI) mice we tested the effects of AP-12 treatment on spatial learning/memory (water maze), anxiolytic (in the elevated zero maze after single and elevated plus maze after repeated administration) behavior, as well as the expression of cortical and hippocampal proteins related to GABA (glutamate decarboxylase 67, GAD67; vesicular GABA transporter, VGAT) and cholinergic (acetylcholine esterase, AChE) systems, synaptic plasticity (Homer-1) and amyloid-beta deposition.

2. Materials and methods

2.1. Animals and housing

Alzheimer's disease APPSweDI [14] female mice were obtained from the breeding colony at the University of Alabama at Birmingham, USA. The animals were housed 5 per cage in a controlled environment at the University of Alabama at Birmingham Animal Care Facility (temperature 21–22°C, humidity 50–60%, and with a 12-h light/dark cycle) and received food and water *ad libitum*. For this study Tg APPSweDI mice were used at 3 months of age. All efforts were made to minimize animal suffering and to reduce the number of animals used. All procedures for this study were carried out in accordance with recommendations made in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, and the animal protocol was approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC), and approved protocol was strictly adhered to.

2.2. Drugs and experimental design

The 1,4-dihydropyridine derivative AP-12 (3',5'-bisethoxycarbonyl-2',6'-dimethyl-1-dodecyl-1',4-dihydro[3,4']bipyridinyl-1-ium bromide), MW 579.61, was synthesized at the Latvian Institute of Organic Synthesis, Riga, Latvia and it has a purity of 98% according to high performance liquid chromatography [15]. For intraperitoneally (ip) injections, AP-12 was dissolved in saline (with 0.1% Tween and using ultrasonication). The dose of 1 mg/kg of AP-12 was chosen as that demonstrating anxiolytic and memory-enhancing effects obtained in our previous study using Tg APPSweDI male mice [11].

Female Tg APPSweDI mice were randomly divided into 2 groups (n=8–10 per group): 1) control (saline) and 2) AP-12, 1mg/kg. Saline or AP-12 was administered once daily ip for 21 days. Three hours after the first injection mice were tested in the elevated zero-maze test. Thirteen days after the start of the treatment the animals were tested in elevated plus maze. From day 17 to day 21 of AP-12 treatment, water maze test was performed for 5 days. All behavioral tests were done 3 h after AP-12 or saline injection and conducted between 11:00 and 14:00. At the end of the behavioral testing, i.e., on day 22 after the start of the treatment, mice were sacrificed; they were deeply anesthetized with ketamine/xylazine and perfused transcardially with cold saline for immunohistochemical analysis. Brains were removed immediately after perfusion and immersed in 4% buffered paraformaldehyde for 24 h, cryoprotected in 30% sucrose in 0.1M phosphate buffer and stored in antifreeze solution at –20°C until the time of the sectioning.

2.3. Elevated zero maze test

Elevated zero maze (EZM) is an anxiety test similar to the elevated plus maze. One advantage to the zero-maze is that there is no central point, so the animal is always in closed or open parts. The EZM apparatus consists of a circular maze of 70 cm diameter that is raised 40 cm above the table divided into two opposites, open parts (open areas) and two opposite, closed parts (closed areas). Closed parts have 15-cm-high sides of nontransparent material whereas the open parts have only a 0.5-cm-high side wall for guiding the animal and preventing from falling. The room was illuminated with two ceiling-mounted LED lights. The mouse was allowed to explore the maze for 4 min and then returned to its home cage. The time spent in the open parts, total number of entries and distance moved in the EZM were recorded. An increased time spend on the open areas indicate anxiolytic action of the tested drug.

2.4. Elevated plus maze test

The elevated plus maze (EPM) consists of two open arms (31 cm×5 cm) and two closed arms (31 cm×5 cm×15 cm) extending from a central platform (5 cm×5 cm), and was raised 40 cm above the table. On day 13 after the beginning of AP-12 treatment, the experiment was started by placing the mouse onto the central platform of the EPM facing one of the open arms. The mouse was allowed to explore the maze for 4 min and then returned to its home cage. The time spent in the open arms, the total number of arm entries and distance moved in the EPM were recorded. An increase in time on the open arms indicates anxiolytic action of the drug.

2.5. Water maze test

The water maze consisted of a blue circular tank (diameter 120 cm, height 40 cm) filled with clear water ($23 \pm 1^\circ\text{C}$). In the water maze test, mice were trained for four 60 s trials per day for 5 days to swim from one of the four starting points of the pool to find a fixed, hidden platform submerged below the water (0.5 cm) in one of the four quadrants of the pool. If the animal did not locate the platform during that time, it was placed upon platform by the experimenter and left there for 10 s. Inter-trial time was 1 min. The latency to find the hidden platform (escape latency) and the swimming speed was recorded for each trial. In the probe trial, which was performed after the last trial on day 5, the platform was removed from the pool and animals were allowed to swim for 60 s. The time spent in each quadrant was determined; similarly, the swimming speed was recorded.

2.6. Immunohistochemistry

At the end of the experiment mice were anesthetized with ketamine/xylazine (100/10 mg/kg) and perfused transcardially with cold saline. The brains were removed and cut in half sagittally, and the right hemisphere of the brain was placed in 4% paraformaldehyde overnight. Following fixation, the right half of the brain was put for 24h in 30% sucrose for cryoprotection; and 30- μm thick coronal sections were cut on a freezing-sliding microtome. Free-floating sections were incubated with the following primary antibodies: W0-2 antibody (mouse anti-human $\text{A}\beta_{4-10}$; 1:5,000, MABN10), Homer-1 (rabbit anti Homer1, 1:500, ABN37), GAD67 (mouse anti-GAD67, 1:1000; MAB5406), all from Millipore, Temecula,

CA, USA, and VGAT (rabbit anti VGAT, 1:1000; cat#131 002 Synaptic Systems, Gottingen, Germany). For A β staining, free-floating sections were pretreated for 30 minutes in 85°C sodium citrate solution (pH=6.5). The other series of sections were transferred to a solution containing the appropriate primary antibody; this solution consists of TBS pH 7.4 with 0.5% Triton X-100 added (TBS-T). Following incubation in this solution for 18 h on a shaker table at room temperature (20°C), the sections were rinsed three times in TBS-T and transferred to the solution containing the appropriate secondary antibody (goat anti-mouse* biotin; Sigma or goat anti-rabbit*biotin; Millipore, Temecula, CA, USA or donkey anti rabbit Cy3, Jackson ImmunoResearch, West Grove, PA, USA. After 2 h, the sections were rinsed three times with TBS-T and transferred to a solution containing mouse ExtrAvidin (1:1000; Sigma, Saint Louis, MO, U.S.A.) for 2 h. Following rinsing, the sections were incubated for 3 min with Ni-enhanced DAB. All stained sections were mounted on gelatin-coated slides and coverslipped. It should be noted that to obtain similarly stained material, sections from both animal groups were always stained simultaneously in the same staining tray. One series of sections was stained for AChE according to a modified method of Koelle and Friedenwald [16, 17], mounted and coverslipped.

2.7. Quantification

The appropriate areas [18] of the brains were digitized using an Olympus DP70 digital camera. To avoid changes in lighting 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, i.e., in the same staining tray. The percentage of area covered by the reaction product to A β was measured [19] in hippocampus (stratum oriens of CA1, CA3 and the dentate gyrus); the density of Homer-1, VGAT and GAD67 staining was measured by the optical density of the staining in the appropriate hippocampal and Cg (cingulate) cortex areas using the ScionImage (NIH) program [20]. The density of AChE staining was measured by the optical density in the appropriate hippocampal area (stratum pyramidale, CA3 and radiatum of area CA1). All density measurements were done in duplicate. These measurements were done by an observer blinded to the treatment of the animal.

2.8. Data analysis and statistics

To track the activity and movements of the animals in the elevated zero maze, plus maze and water maze a camera driven tracker system (Ethovision 8.5, Noldus, The Netherlands) was used. Behavioral data are expressed as mean $\pm$ SEM, and statistics were performed with GraphPad Prism 5 software (GraphPadSoftware Inc., CA, USA). For the statistical analysis of the water maze data, ANOVA followed by Bonferroni Multiple Comparison post-test was used. Immunohistochemical quantification data, elevated zero-maze and plus maze test data were analyzed with unpaired t test. In all cases, a p value < 0.05 was taken as the criterion of statistically significant difference.

3. Results

3.1 Acute injection of AP-12 induced anxiolytic effect in Tg APPSweDI female mice

The time spent in open area in zero-maze test was significantly increased ($p=0.009$) by acute injection of AP-12 (1 mg/kg) compared to saline control group mice (Fig. 1A), whereas no significant difference between groups ($p=0.86$) in number of total area entries (Fig. 1B) and in the mean distance ($p=0.92$; Fig. 1C) travelled in the elevated zero-maze test were observed.

3.2. Chronic administration of AP-12 demonstrated anxiolytic and learning/memory-improving activity in Tg APPSweDI female mice

The obtained data showed that administration of AP-12 (1 mg/kg; ip once daily) for 13 days in Tg APPSweDI mice significantly increased the time spent in open arms of the elevated plus maze compared to saline injected control group mice ($p=0.024$; Fig. 2A) whereas no significant difference between groups ($p=0.668$) in number of total arms entries (Fig. 1B) and mean distance moved (saline: 1473.0 ± 122.3 cm; AP-12: 1484.4 ± 100.7 cm; $p=0.942$; Fig. 2C) in arms were observed.

On day 17th of treatment, the water maze test was started. AP-12 treatment improved the spatial learning performance in water maze test by shortening the latency to find the hidden platform vs. saline control group on days 3–5 ($p=0.002$; $p=0.001$ and $p=0.029$, respectively) (Fig. 3A). In the probe trial, no difference in the preference for the target quadrant was found between the groups (Fig. 3B). No significant differences were observed in the swimming speed of AP-12 treated mice vs. saline control group data on day 4 (saline: 18.71 ± 1.43 cm/sec; AP-12: 17.74 ± 0.85 cm/sec; $p=0.553$) neither on any other days.

3.3. Chronic administration of AP-12 upregulates VGAT, GAD67 and Homer-1 expression in brain of Tg APPSweDI female mice

Immunohistochemical analysis demonstrated that AP-12 (1 mg/kg) treatment altered VGAT expression in the hippocampus and cingulate (Cg) cortex. The staining density for VGAT was significantly increased in the CA3 area of hippocampus (Fig. 4AB, top row) in AP-12 treatment group compared with the saline group (saline: 22.5 ± 3.56 vs. AP-12: 37.8 ± 2.61 ; $p<0.0001$), VGAT expression was also increased in the anterior Cg cortex (Fig. 4AB, bottom row) of AP-12 treated mice compared with saline control group (saline: 21.7 ± 3.68 vs. AP-12: 36.6 ± 3.80 ; $p<0.0001$).

Similarly, to VGAT, GAD67 staining density was significantly increased in the anterior Cg cortex of AP-12 treated mice (Fig. 5AB, top row) compared with saline treated mice (saline: 55.9 ± 1.85 vs. AP-12: 69.1 ± 1.66 ; $p<0.001$). GAD67 staining density was also significantly increased in the hippocampus CA1 pyramidal cell layer and stratum radiatum (Fig. 5AB, middle row) in AP-12 treatment group compared to the saline control group (saline: 56.2 ± 4.76 vs. AP-12: 65.8 ± 1.78 ; $p=0.01$). Further, chronic administration of AP-12 (saline: 149.0 ± 15.84 vs. AP-12: 166.2 ± 14.79 ; $p=0.02$) significantly increased the Homer-1 immunoreactivity density in the Cg cortex to compare with saline treated animals (Fig. 5AB, bottom), but not in the hippocampus (saline: 123.1 ± 24.6 vs. AP-12: 130.4 ± 8.5 ; $p=0.13$;

data not shown in figure). Fig.4B and 5B represents bar graphs showing the quantification of the density measurements for the analyzed regions. There were no changes in the amyloid beta load and AChE staining density in hippocampus and Cg cortex of the treated mice compared with saline treated-animals (data not shown in figures).

4. Discussion

Nowadays, the neurodegenerative processes in AD are considered as multifactorial involving formation of misfolded proteins, dysregulation of proteasomal degradation, mitochondrial damage, unbalanced neurotransmitter signaling, disturbances in synaptic plasticity etc. Therefore, each of these events may serve as the drug target to protect against synaptic loss and cell death. For instance, development of AD therapy targeting mitochondria [21], oxidative stress [22] and many other approaches have been proposed for prevention and management of AD. Recently, a special emphasis was focused on impaired intracellular redox state which mediates a shift in Ca^{2+} regulation and alteration in synaptic plasticity [23]. Moreover, emerging concept of oxidant/antioxidant redox homeostasis based on activation of vitagen system (the complex of several genes modulating redox-dependent mechanisms) are considered as potential target in cytoprotection, particularly in early stages of AD [24, 25].

In light of the above mentioned mechanisms, calcium antagonists of the 1,4-DHP series are promising compounds targeting oxidative stress [26] and mitochondrial processes [27].

The aim of the present study was to evaluate the effects of AP-12, the amphiphilic 1,4-DHP, on memory and anxiolytic behavior, and brain protein expression in AD Tg female mice (APPSweDI). Previously [10] in male Tg mice, we have found that this compound at 1 mg/kg accelerated spatial learning (water maze test) after 21-day administration and exerted anxiolytic action (elevated plus maze) after 13-day intraperitoneal injections, as well as increased protein expression in brain structures (hippocampus and Cg cortex) which are responsible for memory formation. For this study in Tg APPSweDI female mice the same dose 1 mg/kg was chosen.

Our interest was to know whether the effects of AP-12 in female mice are similar or distinct from those of males because gender-related differences were found in AD patients [12, 13]. It is thought that this distinction is based on hormonal differences between sexes, such as the decline in estrogen [28] and progesterone [29] levels in aged women. Females also show an exacerbated effect of ApoE genotype [30–32], the factor which putatively can impair memory. Moreover, females are more likely than males to have accelerated age-related myelin breakdown, white matter loss and hence, damage of neural network connectivity [33, 34]. Gender-specific AD therapy [35] is not common, only some studies demonstrated beneficial effects of anticholinesterase drug rivastigmine in the female subpopulation compared to men [36, 37].

The data obtained in the present study in female Tg mice showed that the spatial memory-enhancing (water maze) and anxiolytic effects (elevated plus maze) of AP-12 were similar to those found in male Tg mice. AP-12 showed significant anxiolytic effect also after single

intraperitoneal injection in female mice in the zero maze. The influence on GABAergic processes was found to predominate in both sexes because of upregulated GAD67 expression in the Cg cortex and hippocampus, indicating that AP-12 may promote GABA synthesis. Moreover, AP-12 also upregulated the expression of the vesicular GABA transporter VGAT, which is highly expressed in GABA-ergic nerve terminals [38] and can be considered as the best marker for inhibitory nerve terminals. GABA is known to be a very important neurotransmitter in contributing to learning/memory stabilization and anxiolytic action, which are impaired in AD due to reduced GABA transmission [39]. Tg AD model mice have been shown to display hippocampal hyperactivity as a result of loss of inhibitory interneuron function [40]. Similarly, it is shown that alterations in the function of GABAergic interneurons leads to increased neural activity in the CA3 region of the aged hippocampus [41, 42]. Another recent study [43] has also highlighted the importance of the therapeutic strategy to enhance inhibitory neurotransmission in the AD suggesting GABAergic therapy for the compensatory effect on the unbalanced neurotransmitter signaling.

Interestingly, a key molecule at the postsynaptic membrane, protein Homer-1 was found to be significantly increased by AP-12 treatment only in the Cg cortex. However, the expression of Homer-1 was not influenced in hippocampal tissue of both female and male mice. Homer-1 is related to the protein family that takes part in synaptic plasticity, suggesting that it may play a role in the network of complex regulating cell connectivity, thus influencing both learning and memory.

The influence of AP-12 on brain AChE expression was found neither in male nor female mice hippocampus and Cg cortex. No effect was found also on amyloid beta load in these structures after 3 week of AP-12 administration, indicating that the cholinergic system and amyloid load do not play a crucial role in AP-12 stimulatory effects on learning/memory. At the same time, it is worthwhile to stress the calcium channel blocking properties of AP-12, because it is known that dysregulated Ca^{2+} homeostasis in AD patients may affect signaling processes and related pathways [3, 6]. Taking together, the memory enhancing and anxiolytic activities and the influence on brain protein expression obtained in the present study on Tg female mice and did not show a considerable sex specificity if compare with the previously found results in Tg male mice [11].

5. Conclusion

The novel 1,4-DHP compound AP-12 in Tg female mice demonstrated spatial learning/memory-enhancing and anxiolytic effects with the predominant mechanisms based on regulation of GABAergic and synaptic plasticity processes in brain. One may suggest AP-12 as promising compound for further pre-clinical studies to clarify its eventual usefulness in the treatment of AD patients.

Acknowledgments

This study is supported by grants: NIH P30 NS47466; Norwegian Financial Mechanism co-financed project No. NFI/R/2014/023.

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Figure 1. Acute anxiolytic effects of AP-12 (1 mg/kg, ip) in Tg APPSweDI (n=8–10 mice/group) female mice in the elevated zero-maze

Three hours after the first injection mice were tested in the elevated zero-maze. (A) Data represent time in seconds spent in the open arms $\pm$ SEM (A); (B) total number of entries and (C) the mean distance travelled in the elevated zero-maze. Significance was measured by unpaired t-test. ** $p < 0.01$ vs. saline control group.

Figure 2. Anxiolytic effect of chronic AP-12 (1 mg/kg) ip administration in Tg APPSweDI (n=8–10 mice/group) female mice in the elevated plus maze
Elevated plus maze was started 3h after AP-12 or saline injections on day 13. (A) Data represent time in seconds spent in the open arms $\pm$ SEM; (B) total number of entries and (C) the mean distance travelled in the elevated zero-maze test. Significance was measured by unpaired t-test (A; C) and Mann-Whitney test (B). *p<0.05 vs. saline control group.

Figure 3. Influence of AP-12 (1mg/kg) on spatial memory in Tg APPSweDI mice (n=8 mice/group) in water maze test

Training was started 3h after the injection of AP-12 on day 17. (A) The two curves (control and AP-12-treated) showed the learning. Daily mean latency (mean $\pm$ SEM) were calculated from four trials. Unpaired t-test was used. * $p < 0.05$ vs. saline control group, ** $p < 0.01$ vs. saline control group. (B) In probe trial test the time spent in the correct quadrant was registered.

Figure 4. Photomicrographs of the hippocampus and cingulate cortex stained for VGAT of Tg APPSweDI mice after treatment with saline (control) or AP-12 (1 mg/kg)
 Cg – cingulate cortex (A) Top row: two photomicrographs of the hippocampus stained for VGAT. Bottom row: two photomicrographs of the anterior Cg cortex stained for VGAT. (B) Bar graphs showing the quantification of the density measurements. Significance was measured by unpaired t-test. *** $p < 0.001$ vs. saline control group.

Figure 5. Photomicrographs of the hippocampus and cingulate cortex stained for GAD67 and Homer-1 of Tg APPSweDI mice after treatment with saline (control) or AP-12 (1 mg/kg) Cg – cingulate cortex, sp – stratum pyramidale, sr – stratum radiatum. (A) Top row: the anterior Cg cortex stained for GAD67. Middle row: the hippocampus stained for GAD67. Bottom row: two photomicrographs of the anterior Cg cortex stained for Homer-1. Significance was calculated using unpaired t-test. (B) Bar graphs showing the quantification of the density measurements. *** $p < 0.001$ vs. saline control group; * $p < 0.05$ vs. saline control group.