Original Article

Sildenafil Decreases BACE1 and Cathepsin B Levels and Reduces APP Amyloidogenic Processing in the SAMP8 Mouse

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Abstract

The senescence-accelerated mouse-prone 8 (SAMP8), used as a model of aging, displays many established pathological features of Alzheimer’s disease. Cognitive impairments and increased levels of hyperphosphorylated tau are found in the hippocampus of SAMP8 mice along with an increased β-secretase activity and amyloid-β (Aβ) depositions that increase in number and extent with age. Based on a previous study from our laboratory showing an amelioration of cognitive impairments and tau pathology by sildenafil, in this study we tested whether this drug could also modulate the amyloid precursor protein amyloidogenic processing in this mouse model. Our results show that the protein levels of the β-secretases β-site amyloid precursor protein cleaving enzyme 1 and cathepsin B are higher in the hippocampus of 9-month-old SAMP8 mice than those of age-matched senescence-resistant-1. Sildenafil (7.5 mg/kg for 4 weeks) attenuated learning and memory impairments shown by SAMP8 mice in the passive avoidance test. The increased expression of β-site amyloid precursor protein cleaving enzyme 1 was also reduced by sildenafil, an effect paralleled to decreases in the activities of two β-site amyloid precursor protein cleaving enzyme 1 modulators, calpain and cyclin-dependent kinase 5 protein. Interestingly, sildenafil enhanced both Akt and glycogen synthase kinase-3β (ser9) phosphorylation, which could be mediating the reduction in cathepsin B levels found in the hippocampus of sildenafil-treated SAMP8 mice. Sildenafil-induced reduction in β-site amyloid precursor protein cleaving enzyme 1 and cathepsin B expression in SAMP8 mice was associated with a decrease in hippocampal Aβ42 levels which, in turn, could mediate the parallel decline in glial fibrillary acidic protein expression observed in these animals. These findings highlight the therapeutic potential of sildenafil in Alzheimer’s disease pathogenesis.

Key Words: Amyloid-β—BACE1—Cathepsin B—SAMP8—Sildenafil.

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Alzheimer's disease (AD) is a progressive neurodegenerative disorder and the leading cause of dementia in the elderly population. The deposition of amyloid-β (Aβ) peptides and the activation of glial cells surrounding senile plaques in brain areas involved in cognitive functions are assumed to participate in the initiation of a pathological cascade resulting in synaptic dysfunction, synaptic loss, and neuronal death (1,2). Aβ is produced by sequential proteolytic cleavage of amyloid precursor protein (APP) by β- and γ-secretases (3–5), being β-secretase the rate-limiting enzyme in Aβ genesis. β-Secretases include, among others, the β-site APP cleaving enzyme 1 (BACE1) and cathepsin B (6), both considered potential new drug targets for the treatment of AD (7–9).

The senescence-accelerated mouse (SAM) was developed as a model of spontaneously accelerated senescence after a normal process of development. This model consists of nine senescence-accelerated mouse-prone (SAMP) substrains and three senescence-accelerated mouse-resistant (SAMR) substrains. Among the former substrains, SAMP8 is characterized by accelerated brain aging and age-related learning and memory deficits compared to SAMR1, which shows a normal aging (10,11). Furthermore, SAMP8 mice also show age-related key neuropathological features seen in AD patients, such as Aβ deposition, increased levels of hyperphosphorylated tau, oxidative stress, and gliosis (12–17). For these reasons, the SAMP8 mouse has been proposed as an excellent model to investigate fundamental mechanisms underlying sporadic AD (18).

Mounting evidence strongly suggests that abnormal expression of Aβ contributes to the cognitive decline in aged SAMP8 mice. In this sense, approaches to reduce brain Aβ levels, using antibodies or antisense oligonucleotides, were shown to diminish oxidative stress and to improve learning and memory deficits in these animals (19–22). Interestingly, it has been recently demonstrated an age-related increase in β-secretase activity in the hippocampus of SAMP8 mice versus age-matched SAMR1 (23). The phosphodiesterase-5 (PDE-5) inhibitor sildenafil has been shown to improve cognition and to decrease tau hyperphosphorylation in SAMP8 mice (24), but its effects on APP processing are unknown. This study was designed to investigate this issue, including the effects of sildenafil on BACE1, cathepsin B and their regulatory pathways.

Materials and Methods

Drugs and Chemicals

1-[4-Ethoxy-3-(6,7-dihydro-1-methyl-7-oxo-3-propyl-1H-pyrazolo[4,3-d]pyrimidin-5-yl) phenylsulfonyl]-4-methylpiperazine citrate was from Pfizer (sildenafil citrate, Viagra); all other chemicals were from Merck (Darmstadt, Germany).

Animals, Treatments, and Experimental Design

Experiments were carried out in male SAMP8 (28–30 g) and SAMR1 mice (32–35 g) obtained from Harlan (Harlan Iberica, Barcelona, Spain). Animals were housed (5 per cage) in constant conditions of humidity and temperature (22 ± 1°C) with a 12-hour/12-hour light-dark cycle (lights on at 7:00 hours). Food and water were available ad libitum. All the procedures followed in this work were in compliance with the European Community Council Directive of November 24, 1986 (86/609/EEC) and were approved by the ethical committee of the University of Navarra.

To study the effect of sildenafil on cognitive impairment, 8-month-old male SAMP8 and SAMR1 mice were treated intraperitoneally once daily with sildenafil (7.5 mg/kg) or saline for 4 consecutive weeks. The dose of sildenafil chosen is the same that has been shown to reverse memory impairments in 6- and 9-month-old SAMP8 mice (24).

Solutions of sildenafil were prepared by grinding Viagra tablets into powder and dissolved in saline. The drug solutions were filtered (22-µm pore size) before administration.

Passive Avoidance Test

During the fourth week of sildenafil treatment, learning and memory were assessed in a passive avoidance test. Mice were trained in a passive avoidance apparatus (Shutavoid-01, Panlab S.L. Technology for Biosearch, Cornellá, Barcelona, Spain), which consisted of two compartments, one light (25 × 25 × 20 cm) and one dark (13.5 × 7.5 × 7.5 cm) connected via a guillotine door. The first day, all animals were placed in the light compartment for 3 minutes, with the door closed for an acclimation period. On the next day, in the acquisition session, mice were placed in the bright compartment and, after 30 seconds, the door was opened. When the mouse stepped into the dark compartment (with all four limbs), the door was closed and a punishing shock of 0.3 mA for 2 seconds was delivered to the animal via the floor. Afterwards, mice were picked from the box and put in the home cage. A milder paradigm in terms of the severity of the aversive stimuli applied compared with that used in previous studies (25–28) avoid an overtraining that may prevent the detection of a possible memory enhancement effect. Twenty-four hours later, retention trial was carried out. For this, mice were placed in the bright compartment again and time to re-enter the dark compartment (latency) was measured for up to 300 seconds. No shock was administered during this testing phase. The equipment for experiments was cleaned after each mouse was tested by wiping with water and 70% ethanol. Sildenafil (7.5 mg/kg intraperitoneally) or saline were injected 30 minutes prior to the acquisition phase.

Preparation of Protein Extracts

Western blot analysis was carried out in hippocampal tissues collected from mice killed 3 hours after the last administration of sildenafil. Tissue homogenates were obtained as described elsewhere (29).

Immunoblotting

Proteins (20 µg) were separated by electrophoresis on a sodium dodecyl sulfate–polyacrylamide gel (10%) under reducing conditions. Membranes were probed overnight at 4°C with the following primary antibodies: rabbit monoclonal anti-p35/p25 and rabbit monoclonal anti-cyclin-dependent kinase 5 (Cdk5); mouse anti-phospho Akt (p-Akt and Ser473); rabbit monoclonal anti-Akt; rabbit polyclonal anti-phospho-glycogen synthase kinase-3β (p-GSK-3β) and ser9); mouse monoclonal anti-glial fibrillary acidic protein (GFAP; 1:1,000, Cell Signaling Technology, Beverly, MA); rabbit polyclonal anti-GSK-3β (1:5,000, Santa Cruz Biotechnology, Santa Cruz, CA); rabbit polyclonal anti-BACE1 (1:1,000, AB 2077, Abcam, Cambridge, UK); mouse monoclonal anti-α-fodrin (AB16156 and MAB1622, respectively; Chemicon, Millipore, Billerica, MA); cathepsin B (1:500, Santa Cruz Biotechnology); and mouse monoclonal anti-β-actin (1:15,000, Sigma-Aldrich, St Louis, MO). Proteins were visualized using an enhanced chemiluminescence Western blotting detection reagent (Amersham, Buckinghamshire, England), and band intensity was estimated densitometrically on a GS-800 calibrated densitometer (Biorad One, Madrid, Spain).
RNA Extraction and Real-time Reverse Transcriptase–PCR
RNA was extracted following the manufacturer’s instructions using the RNAeasy lipid tissue mini kit acquired from Qiagen and then was reverse transcribed using High-Capacity cDNA Reverse Transcription kit (Applied Biosystems). Real-time reverse transcriptase–PCR amplification assays for gene targets were performed with a total volume of 20 μL in each well containing 10 μL of PCR Master Mix (Applied Biosystems), 2 μL of complementary DNA corresponding to 10 ng of RNA, and 1 μL of each TaqMan Gene Expression Assays (Applied Biosystems). Glyceraldehyde 3-phosphate dehydrogenase messenger RNA (mRNA) was used as endogenous controls. The relative quantification of all targets was carried out using the comparative cycle threshold method, \( 2^{-\Delta\Delta Ct} \), where \( \Delta\Delta Ct = (Ct_{\text{target gene}} - Ct_{\text{endogenous control}})_\text{treated} - (Ct_{\text{target gene}} - Ct_{\text{endogenous control}})_\text{untreated} \) (30). Each sample was measured in triplicate. Relative transcription levels \( (2^{-\Delta\Delta Ct}) \) were expressed as a mean ± standard error of the mean.

Quantification of Aβ by Enzyme-Linked Immunosorbent Assay
For analysis of total Aβ42 burden, the hippocampus was homogenized in 70% formic acid (15 mg of tissue in 100 μL of 70% formic acid). Homogenates were then centrifuged at 100,000g for 60 minutes. The supernatant was neutralized with a 20-fold dilution in 1 M Tris-base, and Aβ42 levels were measured using a high-sensitive enzyme-linked immunosorbent assay kit from Wako (cat#292–64501, Wako Chemicals, Richmond, VA) following the manufacturer’s instructions. This kit is constructed as a sandwich enzyme-linked immunosorbent assay format with two kinds of antibodies: the monoclonal antibody BNT77, which epitope is Aβ (11–28), and the monoclonal antibody, BC05, which specifically detects the C-terminal portion of Aβ42.

Immunohistochemistry
Mice were deeply anesthetized with pentobarbital and perfused with 0.9% saline followed by ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer (pH = 7.4). Brains were removed, post-fixed (2 h), cryoprotected in a 30% sucrose solution (in phosphate buffer), and stored at 4°C until they sank. Serial coronal brain slices (thickness: 30 μm) were cut with a freezing microtome through the hippocampus (Bregma –1.60 to –3.60) and were stored in cryoprotectant solution.

Floating tissue sections comprising the hippocampus were processed for GFAP immunohistochemistry. Free-floating slices were washed 3 times in Tris-buffer (Tris-saline, pH 7.4, 0.05% Triton X-100) and incubated in blocking solution (phosphate-buffered saline containing 0.5% Triton X-100, 0.1% bovine serum albumin, and 2% normal goat serum) for 2 h at room temperature. Sections were then incubated at 4°C for 48 hours with GFAP primary antibody diluted in Tris-buffer containing 2% normal goat serum (1:500 mouse monoclonal Cell Signaling Technology). Next, slices were washed with phosphate-buffered saline and incubated with secondary antibody (Alexa 546 donkey anti-mouse IgG, 1:200, Molecular Probes, Eugene, OR) for 2 hours at room temperature, protected from light. Sections were mounted on gelatin-coated slides, air-dried, and coverslipped. To ensure comparable immunostaining, sections were processed under identical conditions. For the assessment of nonspecific immunostaining, some sections from each experimental group were processed without primary or secondary antibody; no immunostaining was observed. Fluorescence images were collected with a confocal microscope LSM 510 Meta (Carl Zeiss) using a Plan-Neofluar 403 objective, Z-series in 0.4-mm steps were acquired and analyzed using LSM 510 Meta software.

Statistical Analysis
Results were expressed as mean ± standard error of the mean. Data were analyzed by two-way analysis of variance (strain*treatment) followed by Tukey post hoc test. In Results section, the F values concerning the neurochemical data represent the F of interaction followed by the p-value of the corresponding post hoc test. In those cases where the F of interaction was not statistically significant, the F value shown represents the main effect observed of strain or treatment. Treatment differences were considered statistically significant at p < .05. Data analyses were performed using the Statistical Program for the Social Sciences (SPSS for Windows, 15.0; SPSS, Chicago, IL).

Results
Effects of Sildenafil on Learning and Memory
Deficits of SAMP8 Mice in the Passive Avoidance Test
Figure 1 shows the latency time to enter the dark compartment on the first day (acquisition trial) and on the second day (retention trial) of the test. On the first day, all the groups showed similar step-through latencies, indicating no differences in locomotor activity or anxiety-like behavior among the groups. Twenty four hours later, during the passive avoidance retention trial (Day 2), latencies to re-enter the dark compartment were shorter in SAMP8 compared to SAMR1 animals, revealing the existence of learning deficits in SAMP8 mice (F = 15.225, p < .01; main effect of strain). Sildenafil treatment resulted in a marked improvement in the behavioral performance of both SAMP8 and SAMR1 mice (F = 10.992, p < .05; main effect of treatment; Figure 1B).

Effects of Sildenafil on BACE1 Levels and on Cdk5/p25–p35 Pathway
Since it has been reported that BACE1 mRNA expression is increased in the hippocampus of old SAMP8 mice (23), we first analyzed BACE1 protein levels in the hippocampus of 9-month-old SAMP8 and SAMR1 mice treated with saline or sildenafil. As shown in Figure 2A, a statistically significant interaction was found between strain and treatment (F = 6.510, p < .05). Further post hoc analysis revealed that BACE1 levels were significantly higher in SAMP8 mice than those found in age-matched SAMR1 mice (p < .05). Of notice, sildenafil treatment significantly decreased BACE1 protein expression in the hippocampus of SAMP8 compared to saline-treated animals (p < .05).

We next analyzed BACE1 mRNA levels in order to check whether sildenafil was modulating BACE1 synthesis. As shown in Figure 2B, sildenafil treatment reduced the increased levels of BACE1 mRNA observed in SAMP8 animals (F = 13.230, p < .05). It has been recently demonstrated the key role of calpain and Cdk5/p25 in the regulation of BACE1 expression (31, 32). Accordingly, we next analyzed the effect of sildenafil on the expression and activity of these proteins. To analyze calpain activity, we measured the cleavage of fodrin, a calpain substrate. As shown in Figure 3A, we found a selective and higher accumulation of the 145/150-kDa fodrin breakdown products in saline-treated SAMP8 animals compared to
SAMR1 saline-treated mice ($F = 11.944, p < .05$), indicating that calpain activity was significantly higher in SAMP8 compared to SAMR1 mice. Noteworthy, intensity of the doublet was reduced to SAMR1 levels in SAMP8 mice treated with sildenafil ($p < .01$).

We next evaluated the changes in p25, the proteolytic fragment resulting from calpain cleavage of p35, which is responsible for Cdk5 hyperactivation. We found a slight p25/p35 ratio increase in saline-treated SAMP8 compared to SAMR1 mice, which did not reach statistical significance perhaps due to the high variability observed in the SAMP8 group (Figure 3B). Sildenafil treatment, however, produced a significant decrease of p25 levels in both SAMP8 and SAMR1 animals ($F = 15.427, p < .01$; main effect of treatment).

When levels of total Cdk5 were analyzed, results revealed that Cdk5 expression was significantly higher in the hippocampus of saline-treated SAMP8 mice compared to SAMR1 ($F = 10.998, p < .05$), an effect reversed by sildenafil ($p < .05$; Figure 3C).

**Effects of Sildenafil on Cathepsin B Levels and on Akt/GSK-3β Pathway**

Several recent studies have demonstrated the importance of another β-secretase, cathepsin B, for Aβ production ($9,33,34$). Therefore, we next analyzed the expression of this protease.
Figure 3. Effect of sildenafil on Cdk5/p25–p35 pathway. Sildenafil (7.5 mg/kg intraperitoneally) was administered every 24 hours for 4 weeks to 8-month-old senescence-accelerated mouse-prone 8 (SAMP8) and senescence-accelerated mouse-resistant 1 (SAMR1) mice. Mice were killed 3 hours after the last saline or sildenafil injection. (A) The typical calpain-dependent cleavage of fodrin appearing as a 145/150 kDa doublet is decreased in sildenafil-treated SAMP8 mice. (B) Representative Western blot showing hippocampal p35 and p25 protein bands and quantitative measurement of p25/p35 ratio. (C) Western blots showing Cdk5 protein bands (30 kDa) in the hippocampus of mice treated with saline or sildenafil. β-Actin was used as equal loading control. Results are expressed as mean ± standard error of the mean (n = 8–10). #p < .01 vs R1-saline; *p < .05 vs P8-saline, **p < .01 vs P8-saline, ††p < .01 main effect of treatment. Two-way analysis of variance followed by Tukey test.
As shown in Figure 4A, cathepsin B protein levels were significantly increased in 9-month-old SAMP8 mice when compared to SAMR1 ($F = 17.736, p < .01$) an effect reversed by sildenafil ($p < .01$).

When mRNA levels were analyzed, results revealed that sildenafil treatment significantly decreased cathepsin B synthesis in both SAMP8 and SAMR1 mice ($F = 27.82, p < .01$; main effect of treatment; Figure 4B).

Figure 4. Effect of sildenafil on hippocampal cathepsin B expression and Akt/GSK-3β pathway. Sildenafil (7.5 mg/kg intraperitoneally) was administered every 24 hours for 4 weeks and mice were killed 3 hours after the last saline or sildenafil injection. (A) Representative Western blot and quantitative measurement of cathepsin B levels (25 kDa) normalized to β-actin in the hippocampus of mice treated with saline or sildenafil ($n = 8–10$). (B) Sildenafil reduced cathepsin B messenger RNA (mRNA) levels in the hippocampus of senescence-accelerated mouse-resistant 1 (SAMR1) and senescence-accelerated mouse-prone 8 (SAMP8) mice. Cathepsin B mRNA was analyzed by real-time reverse transcriptase–PCR and normalized to glyceraldehyde 3-phosphate dehydrogenase mRNA as an internal control ($n = 5$). (C) Phosphorylated Akt levels (60 kDa) normalized to total Akt. (D) Phosphorylated GSK-3β (Ser9) levels (46 kDa) normalized to total GSK-3β ($n = 8–10$). Results are expressed as mean ± standard error of the mean. †p < .05 main effect of treatment, ††p < .01 main effect of treatment, #p < .05 vs R1-saline, *p < .05 vs P8-saline. Two-way analysis of variance followed by Tukey test.
Akt/GSK-3β pathway was shown to be an important regulator of APP processing via modulation of APP β-cleavage by cathepsin B (35). To examine whether the Akt/GSK-3β pathway could be mediating sildenafil-induced reduction in cathepsin B levels, we measured the phosphorylation levels of Akt at Ser473 and its downstream target, GSK-3β at Ser9. As shown in Figure 4C, sildenafil markedly increased Akt phosphorylation ($F = 4.956, p < .05$; main effect of treatment). This result was paralleled with a decrease in GSK-3β activity, as seen by the enhancement of inactive GSK-3β protein levels (pSer9-GSK-3β/total GSK-3β; $F = 5.165, p < .05$; main effect of treatment; Figure 4D).

**Sildenafil Reduces Aβ$_{42}$ Burden**

In order to investigate the functional consequences of the reduced expression in BACE1 and cathepsin B in sildenafil-treated SAMP8 mice, we next quantify hippocampal Aβ$_{42}$ levels. The results revealed a significant increase of this protein in the hippocampus of 9-month-old SAMP8 mice when compared to aged-matched SAMR1 mice ($F = 5.255, p < .05$; Figure 5). This increase was also reversed by sildenafil ($p < .05$).

**Sildenafil Decreases GFAP Expression**

Analysis of the astrocyte marker GFAP showed a significant increase in the hippocampus of SAMP8 mice in comparison to the SAMR1 control strain ($p < .05$, Student’s t-test). Interestingly, sildenafil significantly reduced hippocampal GFAP expression in both SAMP8 and SAMR1 mice ($F = 13.193, p < .01$; main effect of treatment; Figure 6A). In agreement with Western blotting data, representative images of GFAP immunofluorescence show that GFAP staining was increased in SAMP8 mice, an effect reversed by sildenafil (Figure 6B).

**Discussion**

The results of this study show that BACE1 and cathepsin B protein levels are higher in the hippocampus of 9-month-old SAMP8 mice than those of age-matched SAMR1 mice. Administration of sildenafil significantly reduced the synthesis of both β-secretase enzymes.

![Figure 5](https://example.com/figure5.png)

**Figure 5.** Sildenafil reduces Aβ$_{42}$ burden in hippocampus of 9-month old senescence-accelerated mouse-prone 8 (SAMP8) mice. Sildenafil (75 mg/kg intraperitoneally) or saline was administered every 24 hours for 4 weeks. Mice were killed 3 hours after the last saline or sildenafil injection ($n = 6–7$). Results are expressed as mean ± standard error of the mean. ## $p < .01$ vs R1-saline, * $p < .05$ vs P8-saline. Two-way analysis of variance followed by Tukey test.

![Figure 6](https://example.com/figure6.png)

**Figure 6.** Effects of sildenafil on glial fibrillary acidic protein (GFAP) expression. Sildenafil (75 mg/kg intraperitoneally) or saline was administered every 24 hours for 4 weeks. Mice were killed 3 hours after the last saline or sildenafil injection. (A) Representative Western blot and quantitative measurement of GFAP (51kDa) normalized to β-actin in the hippocampus of mice treated with saline or sildenafil. (B) Representative images obtained with the confocal microscopy of anti-GFAP antibody-stained sections. Note that sildenafil decreased GFAP levels (red) in senescence-accelerated mouse-resistant 1 (SAMR1) and senescence-accelerated mouse-prone 8 (SAMP8) mice. Results are expressed as mean ± standard error of the mean (standard error of the mean; $n = 6–8$). † $p < .05$ main effect of treatment. Two-way analysis of variance.
probably through the modulation of calpain/Cdk5/p25 and p-Akt/ GSK-3β pathways, respectively. The reduction of BACE1 and cathepsin B expression resulted in decreased Aβ42 peptide levels, which could contribute to the improvement of cognitive deficits and the decrease in GFAP levels observed in aged SAMP8 mice treated with sildenafil (Figure 7).

We previously showed that sildenafil exerts a protective effect on the learning and memory impairments shown by SAMP8 mice in the Morris Water Maze test (24). Consistent with other reports (26,36), we now report that 9-month-old SAMP8 mice show, as well, learning and memory impairments in the passive avoidance test, an effect that was markedly ameliorated by sildenafil. Strong evidence suggests that amyloid deposition plays a critical role in the development of AD (37). In fact, Aβ accumulation in brain precedes dementia, and mutations in APP or alterations in its processing are associated with familial forms of AD (38–40). In the case of SAMP8 mice, Aβ depositions have been reported in the hippocampus (13), as well as, an age-related increase in hippocampal β-secretase activity (23). Furthermore, it appears to exist a causal role for Aβ in the cognitive decline of SAMP8 mice as the reduction of Aβ burden using antibodies or antisense oligonucleotides improves learning and memory deficits in this mouse model (19–22). Based on these data, we analyzed the effect of sildenafil on amyloid pathology shown by these mice.

Our data indicate that the levels of BACE1 and cathepsin B, two key β-secretase enzymes, are increased in the hippocampus of 9-month-old SAMP8 when compared to SAMR1 mice. Interestingly, this increase was reverted by sildenafil, an effect that may contribute to the memory improvement observed in SAMP8 mice treated with this PDE-5 inhibitor. In fact, several studies have demonstrated that genetic deficiency of cathepsin B or BACE1 or inhibition of these enzymes reduces Aβ production and improves cognition in different animal models (9,33,34,41–44).

In an attempt to figure out the mechanisms underlying the decrease in both β-secretases caused by sildenafil, we next analyzed the effect of this drug on different pathways that regulate BACE1 and cathepsin B expression. For this, we first analyzed the levels of BACE1 mRNA. In agreement with (23) and with the results obtained by Western blot, we observed that BACE1 mRNA levels were increased in 9-month-old SAMP8 animals in comparison to aged-matched SAMR1 mice. Interestingly, sildenafil treatment significantly reversed this effect. We next focused our studies on calpain, a protease known to increase BACE1 levels (31) and which has been implicated in synaptic dysfunctions and neuronal death seen in AD (45). Our results show an increased activity of calpain in the hippocampus of SAMP8 versus SAMR1 mice, which is in agreement with previous studies reporting similar findings in 3- and 5-month-old SAMP8 mice (46,47). Of notice, sildenafil treatment significantly reduced the activity of calpain in the hippocampus of 9-month-old SAMP8 mice. Calpain cleavage of p35, a regulatory partner of Cdk5, generates p25, which causes the hyperactivation of Cdk5 (48–50) and the phosphorylation of many substrates, including microtubule-associated protein tau. Interestingly, p25, through the transcriptional regulation of BACE1 expression (32), promotes the production and accumulation of Aβ in vivo (51). Taking this into account, sildenafil inhibition of calpain activity, and consequently of the Cdk5/p35/p25 pathway, could participate in the mechanisms by which this PDE-5 inhibitor reduces BACE1 expression in SAMP8 mice. Further support for this contention comes from recent studies showing that the overexpression of the endogenous calpain inhibitor, calpastatin, or the administration of specific calpain inhibitors causes a remarkable decrease of amyloid plaque burden, reduces tau hyperphosphorylation, and prevents the loss of synapses in different AD models (52–55). Undoubtedly, these studies strengthen the potential of sildenafil as a therapeutic agent for AD.

On the other hand, we also found increased levels of cathepsin B, another important β-secretase, in the hippocampus of SAMP8 mice compared to age-matched SAMR1 animals. This observation, however, did not correlate with the expression levels of its mRNA, suggesting that other posttranscriptional mechanisms might account for such results. In any case, sildenafil caused a slight but significant reduction of cathepsin B mRNA levels not only in SAMP8 mice but also in SAMR1 animals, an effect that turned into a significant reduction of cathepsin B protein levels in SAMP8 mice down to those of found in control animals. To our knowledge, this is the first study to report such findings and may as well explain the beneficial effects on cognition observed in SAMP8 mice (33).

A recent study has demonstrated that cathepsin B expression is modulated by a GSK-3β (35). This contention is further supported by recent reports demonstrating the beneficial effects of GSK-3β
inhibition on APP processing in AD transgenic mice (56–58). Because GSK-3β is a downstream target of Akt and its phosphorylation at Ser9 inhibits its kinase activity (59), we analyzed the activity of this pathway in SAMR1 and SAMP8 mice treated with sildenafil. Our data show an increased phosphorylation of Akt after sildenafil, which was associated with higher levels of phosphorylated GSK-3β (ser9) in both 9-month-old SAMP8 and SAMR1 mice. According to these data, it is suggested that a decreased GSK-3β activity could contribute to the reduction of cathepsin B levels observed in sildenafil-treated SAMP8 mice.

Sildenafil inhibitory effects on calpain and GSK3β, with subsequent reductions on BACE1 and cathepsin B levels should result in changes in APP processing, with a reduction in Aβ burden in this mouse model. In agreement with this conception, we report that sildenafil decreased Aβ42 burden in the hippocampus of 9-month-old SAMP8 mice. A reduction in Aβ42 production may well play a part in cognitive improvement shown by these mice after sildenafil treatment. This result supports previous studies that also demonstrate the beneficial effects of sildenafil reducing Aβ levels in different mouse models (60–62). However, in a previous study from our group, sildenafil did not affect the Aβ burden in aged Tg2576, a transgenic mice overexpressing the Swedish APP double mutation (63). This discrepancy may be likely due to differences in the age of mice (in this study, we used 9-month-old mice, whereas the previous study was performed in 16-month-old animals) or to the different animal model used. It is worth noting that, unlike transgenic mice which express 5–14 times more than normal amount of Aβ in the brain, SAMP8 mice have an increase of about 50% more than normal (19), which is much closer to the estimated Aβ burden of AD patients (37).

Furthermore, we have previously reported that tau pathology was also improved by sildenafil (24). Interestingly, both GSK3β and Cdk5 have been identified as prime candidates mediating aberrant tau phosphorylation (64). Therefore, the inhibitory effect on these kinases observed herein after sildenafil treatment could provide a common mechanism underlying the improvements in both pathologies in 9-month-old SAMP8 mice. In any case, as the hippocampus is a functionally heterogeneous structure (65–67), further immunohistochemical analyses would be useful to elucidate which cells express the different proteins modulated by sildenafil. This would help us to understand in depth the molecular mechanisms underlying sildenafil afforded beneficial effects.

Several lines of evidence have shown that astrogliosis is one of the earliest pathological hallmarks of AD and may occur in response to the increasing number of degenerating neurons and synapses or to the accumulation of Aβ (68,69). Interestingly, mice receiving an intracerebroventricular administration of Aβ show a marked increase in hippocampal GFAP levels (70), and a recent study has demonstrated that a specific calpain inhibitor reduces the inflammatory response associated with Aβ accumulation (54). According to these data and to the findings described herein, we next analyzed the effect of sildenafil on the inflammatory marker GFAP. In agreement with a previous report (71), we observed increased GFAP levels in the hippocampus of SAMP8 compared to age-matched SAMR1 mice, an effect reversed by sildenafil. Although our findings cannot differentiate between reductions in GFAP levels resulting from less Aβ accumulation (31) or from the direct modulation of inflammatory events by sildenafil (72,73), either mechanism may be potentially beneficial in the disease.

We would like to note that sildenafil not only reversed memory impairments in SAMP8 mice, but it also enhanced memory in SAMR1 animals, an effect that was not associated with changes in most of the proteins analyzed. This result suggests that additional mechanisms underlie the improvement of passive avoidance performance in SAMR1 mice. Indeed, although not directly tested in this study, previous studies have demonstrated that sildenafil increases the expression of proteins involved in synaptic plasticity and memory such as Arc, c-Fos, p-CREB, or BDNF (63,74,75); enhances neurogenesis (76,77); and modulates antiapoptotic pathways (78,79). On the other hand, taking into account that aging is associated with an increase in PDE expression and activity and a decrease in cyclic guanosine monophosphate levels due to PDE-5 inhibition or occur independently, as demonstrated to occur with the phosphorylation of Akt (84).

In conclusion, the benefits shown by sildenafil reducing APP processing and Aβ42 levels (this work) and tau hyperphosphorylation (24), two of the main neuropathological hallmarks of AD, makes it an excellent candidate for the treatment AD and other pathologies associated to aging.

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Conflict of Interest

None of the authors have any actual or potential conflict of interest including any financial, personal, or other relationships with other people or organizations that could inappropriately influence their work.

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