Caffeine Reverses Cognitive Impairment and Decreases Brain Amyloid-β Levels in Aged Alzheimer’s Disease Mice

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Abstract. We have recently shown that Alzheimer’s disease (AD) transgenic mice given a moderate level of caffeine intake (the human equivalent of 5 cups of coffee per day) are protected from development of otherwise certain cognitive impairment and have decreased hippocampal amyloid-β (Aβ) levels due to suppression of both β-secretase (BACE1) and presenilin 1 (PS1)/γ-secretase expression. To determine if caffeine intake can have beneficial effects in “aged” APP\textsuperscript{sw} mice already demonstrating cognitive impairment, we administered caffeine in the drinking water of 18–19 month old APP\textsuperscript{sw} mice that were impaired in working memory. At 4–5 weeks into caffeine treatment, those impaired transgenic mice given caffeine (Tg/Caff) exhibited vastly superior working memory compared to the continuing impairment of control transgenic mice. In addition, Tg/Caff mice had substantially reduced Aβ deposition in hippocampus (↓40%) and entorhinal cortex (↓46%), as well as correlated decreases in brain soluble Aβ levels. Mechanistically, evidence is provided that caffeine suppression of BACE1 involves the cRaf-1/NFκB pathway. We also determined that caffeine concentrations within human physiological range effectively reduce active and total glycogen synthase kinase 3 levels in SweAPP N2a cells. Even with pre-existing and substantial Aβ burden, aged APP\textsuperscript{sw} mice exhibited memory restoration and reversal of AD pathology, suggesting a treatment potential of caffeine in cases of established AD.

Keywords: Alzheimer’s disease, Alzheimer’s transgenic mice, amyloid-β, caffeine, cognitive impairment, memory, treatment

INTRODUCTION

Caffeine is the most widely consumed psychoactive substance known to man, being primarily consumed in coffee, tea, and soft drinks. As a brain stimulant, its primary actions are to increase cerebral energy metabolism, cortical activity, and extracellular levels of acetylcholine, collectively resulting in enhanced alert-
ness [1]. In addition to these well-documented stimulatory effects of caffeine, epidemiology/longitudinal studies in humans have suggested that caffeine is protective against a variety of diseases associated with aging including Parkinson’s disease, Type 2 Diabetes, and liver disease [2–5]. More recently, several longitudinal studies have reported that daily caffeine intake, equivalent to 3 or more cups of coffee, reduces cognitive decline in “non-demented” elderly men and women [6, 7]. Of direct relation to Alzheimer’s disease (AD), another recent study involving a 21 year follow-up period found that midlife intake of 3–5 cups of coffee substantially reduced the risk of AD in later life [8]. Moreover, AD patients were found to have consumed markedly less caffeine during the 20 years preceding AD diagnosis when compared to age-matched individuals without AD [9]. These cognition-based human studies suggest that long-term caffeine intake may protect against aged-associated memory impairment and AD [10].

Although human epidemiologic/longitudinal studies are insightful, they cannot definitively isolate caffeine intake from other lifestyle choices that potentially affect cognition. Therefore, we recently performed a highly controlled study in AD transgenic (APPsw) mice, which develop high levels of brain amyloid-β (Aβ) and widespread cognitive impairment as they age [11,12]. Long-term caffeine administration that began in young adulthood protected these APPsw mice against otherwise certain cognitive impairment, while also limiting their brain Aβ levels [13]. We further found that this long-term caffeine administration suppressed brain Aβ production by reducing expression of both Presenilin 1 (PS1) and β-secretase (BACE1). Because the beneficial effects were achieved by a moderate daily level of caffeine in drinking water (the human equivalent of 5 cups of coffee or 500 mg caffeine per day), we have suggested that a modest amount of daily caffeine may delay or reduce the risk of AD [13].

In the present study, we first aimed to determine if caffeine intake can have beneficial effects in “aged” APPsw mice already exhibiting cognitive impairment. Therefore, at several months into caffeine treatment, we re-evaluated cognitive performance in aged APPsw and determined the impact on both Aβ levels/Aβ deposition and synaptophysin immunostaining in cognitively important brain areas.

Second, we wished to develop greater insight into the mechanism(s) of the ability of caffeine to reduce Aβ and thus explored the potential involvement of the Raf-1 inflammatory pathway and glycogen synthase kinase 3 (GSK-3) in caffeine’s mechanism of action in APPsw mice. Raf-1 is hyperactivated in AD brains [14,15], evidently due to an Aβ-induced decrease in the activity of proteins (e.g., Ras, PKA) that regulate Raf-1 activity. GSK-3 is a constitutively active serine/threonine kinase that is highly expressed in the central nervous system (CNS) [16–18]. Dysregulation of GSK-3 is an essential component of AD pathogenesis, playing an integral role in PS1/γ-secretase activity, spatial learning deficits, Aβ-induced toxicity, and tau phosphorylation [19–21].

Third, we administered caffeine throughout adult life to non-transgenic (NT) mice, with cognitive assessment in older age to determine if: 1) life-long caffeine intake can provide cognitive benefits to NT (normal) mice and consequently, 2) generalized, non-amyloidogenic mechanisms of caffeine action (such as increased cerebral blood flow or enhanced brain glucose utilization) could be important for its therapeutic effects in APPsw mice.

MATERIAL AND METHODS

Animals

A total of 55 mice were included in these studies. Each mouse had a mixed background of 56.25% C57, 12.5% B6, 18.75% SJL, and 12.5% Swiss-Webster. All mice were derived from a cross between heterozygous mice carrying the mutant APPK670N, M671L gene (APPsw) with heterozygous PS1 (Tg line 6.2) mice to obtain F4 and F5 generation mice consisting of APP/PS1, APPsw, PS1, and NT genotypes. After weaning and genotyping, only APPsw and NT mice were selected for behavioral testing and/or caffeine administration. All mice were maintained on a 12 h dark and 12 h light cycle with ad libitum access to rodent chow and water or caffeineated water. All animal procedures were performed in AAALAC-certified facilities under protocols approved by Institutional Animal Care and Use Committees at the University of South Florida.

General protocol

Three separate in vivo studies were performed as follows:

Study 1: At 18–19 months of age, APPsw and NT controls were pre-tested in the radial arm water maze (RAMW) task, wherein APPsw mice were shown to be impaired in working memory. Impaired transgenic (Tg) mice were divided into two groups, equally bal-
anced by behavioral performance, with half of Tg mice starting on caffeine administered in their drinking water (0.3 mg/ml; Sigma, St. Louis, MO) and the other half remaining on standard tap water. At 4–5 weeks into caffeine treatment, Tg caffeine-treated (Tg/Caff; \( n = 6 \)), as well as Tg control (Tg; \( n = 7 \)), and NT control (NT; \( n = 8 \)) mice were re-tested in the RAWM, as well as in several other behavioral tasks. All animals were euthanatized immediately following completion of behavioral testing at 20–21 months of age. Their brains were processed for determination of total Aβ deposition, soluble Aβ1–40 and Aβ1–42, and synaptophysin immunohistochemistry.

Study 2: Beginning at 9 months of age, APPsw mice \(( n = 6 )\) were gavaged with caffeine (1.5 mg/200 µl) twice daily for two weeks. Gavage administration of caffeine was done in this study because: 1) the treatment period was relatively short-term compared to that of Studies 1 and 3, and 2) gavage treatment is more precise in providing exactly the amount of caffeine desired compared to ad libitum administration of caffeine in drinking water. Control APPsw mice \(( n = 5 )\) and NT mice \(( n = 4 )\) of the same age received gavage with water vehicle instead. All mice were sacrificed immediately following the treatment period, their hippocampus dissected out and stored at \(-80^\circ C\) until neurochemical assay of pRaf-1 and PKA activity analyses.

Study 3: In a “behavior-only” study, NT (normal) mice began receiving caffeine in their drinking water (0.3 mg/ml) at 5½ months of age (NT/Caff; \( n = 8 \)) or were continued on standard water (NT; \( n = 11 \)). Between 15–16 months of age, and at 10 months into caffeine treatment, all animals were tested in our comprehensive 6-week behavioral battery that included five cognitive-based tasks.

Caffeine treatment

For Studies 1 and 3, caffeine was administered in the drinking water (0.3 mg/ml) of Tg or NT mice. On average, mice drink 5 ml per day, yielding a daily dose of 1.5 mg caffeine to each mouse. According to our prior study’s calculations accommodating for size and metabolic rate differences [13], this daily dose in a mouse is equivalent to approximately 500 mg (5 cups of coffee) daily caffeine intake by a human. The caffeinated water was changed two times a week to ensure caffeine remained fully dissolved at the appropriate concentration. Control APPsw and NT mice were given ad libitum access to untreated tap water that was also changed twice weekly to ensure freshness. Caffeine treatment was continued throughout behavioral testing and until euthanasia. All groups consisted of approximate equal numbers of male and female mice.

Behavioral testing

In Study 1, aged 18–19 month mice were pretreatment tested in the RAWM task, and then behaviorally tested again at 4–5 weeks into treatment in the following tasks: open field activity, balance beam, string agility, Y-maze, elevated plus maze, RAWM, and platform recognition. In Study 3, NT mice were tested in our full behavioral battery beginning at 15–16 months of age (e.g., at 10 months into treatment). Although detailed protocols for all behavioral tasks have been described previously [22–24], a brief description of each task is provided below:

Open Field Activity (Sensorimotor-based task; 1 day). As a test of activity and exploratory behavior, each animal was placed in an open black box painted with perpendicular lines demarcating 16 squares. The total number of line crossings over a single 5-minute trial was recorded through visual observation.

Balance Beam (Sensorimotor-based task; 1 day). As a test of vestibular and general motor function, each animal was placed at the center of a suspended beam and released. The average balance time from three successive trials was recorded. The surface below the beam had a thick sponge cushion to protect mice that fall off the beam.

String Agility (Sensorimotor-based task; 1 day). As a test of agility and grip capacity, animals were permitted to grasp a suspended string only by their forepaws and then released. During a single 60-second trial, each animal was assessed using a 0–5 rating system (0 = animal unable to remain on string; 1 = hangs by two forepaws; 2 = attempts to climb onto string; 3 = two forepaws and one or both hind paws around string; 4 = four paws and tail around string, with lateral movement; 5 = escape to one of the two poles suspending the string).

Y-maze spontaneous alternation (Sensorimotor- and Cognitive-based task; 1 day). To measure general activity and basic hippocampal-dependent mnemonic function, mice were allowed to explore a black Y-maze with 3 arms. Each arm measures 21 × 4 cm with 40 cm high walls. Mice were placed in the center of the maze facing the center area and allowed to explore for 5 minutes, with the number and sequence of arm choices being recorded. General activity was measured as the total number of arm entries, while basic mnemonic...
function was determined as a percent spontaneous alternation (the ratio of arm choices differing from the previous two choices divided by the total number of entries).

**Elevated Plus Maze (Anxiety-based task; 1 day).** To measure anxiety/emotionality, mice were placed in the center of an elevated plus maze 82 cm above the floor. The maze consists of two opposite “open” and two opposite “closed” arms, each 30 × 5 cm; 15 cm high black aluminum walls surround the closed arms. The mice were placed in the 5 × 5 cm maze center, facing a closed arm, and were allowed to explore for 5 minutes. The total number of closed arm entries, open arm entries and total time (seconds) spent in the open arms was recorded. We have found that time spend in open arms is the purest measure of anxiety in this task [22].

**Morris Water Maze (Cognitive-based task measuring spatial learning and reference memory; 15 days).** The floor of a 100 cm circular pool was divided into four quadrants, with a 9 cm platform submerged in the goal quadrant. Surrounding the pool were a variety of 3-D and 2-D visual cues. Fourteen consecutive days of acquisition were performed, with each of four trials per day beginning with the animal being placed into each of the four quadrants to initiate a 60-second trial. Thus, a single training session consisted of four successive trials, with a 30 second stay period (e.g., the animal is allowed to remain on the platform for 30 seconds after finding it or being guided to it at the end of the trial). Average latency to find the submerged platform was obtained through visual observation. On the day following acquisition testing, memory retention was evaluated in a single 60-second probe trial in which the submerged platform was removed and the animal released from the quadrant opposite the former platform-containing quadrant. Percent of time spent in each quadrant, number of annulus crossings, and swim speed were all determined from video tracking records.

**Circular Platform (Cognitive-based task measuring spatial reference learning/memory; 8 days).** A walled 69 cm circular platform, with 16 equidistantly spaced holes along its periphery, was encircled by a black curtain. Visual cues, located on the black curtain and platform walls can be used by the animal to find the one hole through which it can escape the platform surface to avoid the aversive stimuli of bright lights and fan wind. During a single 5-minute maximum daily trial, the total number of errors (head pokes into non-escape holes) and latency to find the escape hole were recorded. Although the escape hole remained constant for any given animal over the 8 days of testing, it was located after each animal’s trial to control for olfactory cues. Also, to control for olfactory cues, the maze was cleaned with a dilute vinegar solution following each animal’s trial.

**Platform Recognition (Cognitive-based task measuring identification and recognition; 4 days).** The platform recognition task measures the ability to search for and identify/recognize a variably placed elevated platform. It was run after the Morris Maze task (Study 2), or immediately following the RAWM task (Study 1) and in the same pool as these two tasks. Thus, the platform recognition task requires animals to ignore the spatial cues present around the pool and switch from a spatial to an identification/recognition strategy – it is not a task of visual acuity alone in our paradigm, with cognitive-based performance directly linked to levels of brain Aβ [25]. Mice were given four successive trials per day over a 4-day period. Latencies to find an elevated platform (9 cm diameter), bearing a prominent cone-shaped styrofoam ensign on a wire pole, were determined. For each trial (60 second maximum), animals were placed into the pool at the same location and the platform was moved to a different one of four possible locations. For statistical analysis, escape latencies for all four daily trials were averaged.

**Radial arm water maze (RAWM) (Cognitively-based task measuring working memory).** For the radial arm water maze task of spatial working memory, an aluminum insert was placed into a 100 cm circular pool to create 6 radially distributed swim arms emanating from a central circular swim area. An assortment of 2-D and 3-D visual cues surrounded the pool. For Study 1 involving aged Tg mice, the number of errors prior to locating which one of the 6 swim arms contained a submerged escape platform (9 cm diameter) was determined for 5 trials/day over 6 days of pre-treatment testing and 9 days of post-treatment testing: 3-day blocks were used to facilitate statistical analysis. For Study 3 (involving NT mice), 6 days of testing were evaluated over two 3-day blocks. There was a 30-min time delay between the 4th trial (T4; final acquisition trial) and 5th trial (T5; memory retention trial). The platform location was changed daily to a different arm, with different start arms for each of the 5 trials semi-randomly selected from the remaining 5 swim arms. During each trial (60 second maximum), the mouse was returned to that trial’s start arm upon swimming into an incorrect arm and the number of seconds required to locate the submerged platform was recorded. If the mouse did not find the platform within a 60-second trial, it was guided to the platform for the 30-second stay. The numbers
of errors during trials 4 and 5 are both considered indices of working memory and are temporally similar to the standard registration/recall testing of specific items used clinically in evaluating AD patients. Analysis of the number of seconds taken per arm choice (an index of swim speed) over all days of post-treatment testing revealed no differences in swim speed between groups in both Studies 1 and 2.

**Brain tissue preparation**

Following completion of behavioral testing in Study 1, 20–21 month-old mice were deeply anesthetized with sodium pentobarbital and transcendally perfused with 100 ml of 0.9% saline. Postmortem brains were immediately removed and bisected sagitally. The hippocampus and cerebral cortex were dissected from the right hemisphere and processed for soluble Aβ_{1–40} and Aβ_{1–42} determinations by ELISA. The left hemisphere was placed in 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4) overnight, wherein tissues remained until routine paraffin embedding for immunohistochemical analyses.

**Immunohistochemistry and image analysis**

At the level of the hippocampus (bregma −2.92 mm to −3.64 mm), five 5 µm sections (150 µm apart) were made from each mouse brain using a sliding microtome. Immunohistochemical staining was performed following the manufacturer’s protocol using a Vectastain ABC Elite kit (Vector Laboratories, Burlingame, CA) coupled with the diaminobenzidine reaction, except that the biotinylated secondary antibody step was omitted for Aβ immunohistochemical staining. The following primary antibodies were used for immunohistochemical staining: a biotinylated human Aβ monoclonal antibody (clone 4G8; 1:200, Covance Research Products, Emeryville, CA) and rabbit synaptophysin polyclonal antibody (undiluted, DAKO, Carpinteria, CA). For Aβ immunohistochemical staining, brain sections were treated with 70% formic acid prior to the pre-blocking step. Phosphate-buffered saline (0.1 mM, pH 7.4) or normal rabbit serum (isotype control) was used instead of primary antibody or ABC reagent as a negative control.

Quantitative image analysis was done based on previous methods with modifications [26,27]. Images were acquired using an Olympus BX60 microscope with an attached digital camera system (DP-70, Olympus, Tokyo, Japan), and the digital image was routed into a Windows PC for quantitative analysis using SimplePCI software (Compix Inc., Imaging Systems, Cranberry Township, PA). Images of five 5-µm sections (150 µm apart) through both anatomic regions of interest (hippocampus and entorhinal cortex) were captured from each animal, and a threshold optical density was obtained that discriminated staining from background. Each region of interest was manually edited to eliminate artifacts. For Aβ burden analysis, data are reported as percentage of immunolabeled area captured (positive pixels) relative to the full area captured (total pixels).

To evaluate synaptophysin immunoreactivity, after the mode of all images was converted to gray scale, the average intensity of positive signals from each image was quantified in the CA1 and CA3 regions of hippocampus as a relative number from zero (white) to 255 (black). Each analysis was done by a single examiner blinded to sample identities.

**Aβ ELISA**

Hippocampal and cortical levels of human soluble Aβ_{1–40} and Aβ_{1–42} were measured by ELISA. Briefly, 150 mg brain samples were homogenized in 400 µl RIPA buffer (100 mM Tris [pH 8.0], 150 mM NaCl, 0.5% DOC, 1% NP-40, 0.2% SDS, and 1 tablet proteinase inhibitor per 100 ml (S8820, Sigma, St. Louis, MO), and sonicated for 20 seconds on ice. Samples were then centrifuged for 30 min at 27,000 g at 4°C, and supernatants were transferred into new screw cap tube. The supernatants obtained from this protocol were then stored at −80°C for determination of soluble Aβ levels using ELISA kits (KHB3482 for 40, KHB3442 for 42, Invitrogen, Carlsbad, CA). Standard and samples were mixed with detection antibody and loaded on the antibody pre-coated plate as the designated wells. HRP-conjugated antibody was added after wash, and substrates were added for colorimetric reaction, and then stopped with sulfuric acid. Optical density was obtained and concentrations were calculated according a standard curve.

**Analysis of pcRaf-1 and Protein Kinase A (PKA)**

For hippocampal tissue from Study 2, equal amounts of protein were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (BA83 0.2 µm; Bio-Rad, Richmond, CA). The membranes were blocked in buffered saline with 0.05% Tween 20 (PBST) containing 10% dry milk. Membranes were incu-
bated with primary antibodies in PBST with 3% dry milk (Sigma) overnight at 4°C and with secondary antibodies for 2 h. Rabbit polyclonal antibodies (Cell Signaling Technology, Inc., Danvers, MA) were directed against pcRaf-1(Ser338; 1:250) or pcRaf-1(Ser259; 1:500). A monoclonal mouse antibody anti α-tubulin (Promega, Madison, WI) was used as a control of protein loading. The bands were detected using ECL detection kits (ECL, Pharmacia Biotech, Piscataway, NJ), visualized using the Kodak Image Station 440CF, and analyzed using the molecular Imaging Software, version 4.0 (Rochester, NY) and NIH image J software. All data was normalized against tubulin immunoreactivity and expressed as percent of control values. PKA activity from the same hippocampal tissue lysates were measured using a non-Radioactive PKA activity assay kit (Assay Designs, Ann Arbor, MI), which is based on a solid phase enzyme-linked immuno-absorbent assay (ELISA) using a synthetic peptide as a substrate for PKA and a polyclonal antibody against the phosphorylated form of the substrate. All determinations of PKA activity were performed at least in quintuplicate, repeated twice in separate experiment, and expressed as percentage of NT control values.

N2a cell cultures and caffeine treatment

Following dibutyryl cAMP-induced neuronal differentiation, cultured SweAPP N2a cells were treated with caffeine in either a concentration- or time-dependent manner. For the concentration-dependent treatment, cells were administered a serial dilution of caffeine ranging from 0–20 µM to establish the optimal range of caffeine action on GSK-3 activity. After Western blot analysis established this range as 0–20 µM concentration of caffeine C for 1 h prior to lysis with ice-cold lysis buffer. For the time-dependent application, cells were given a 20 µM concentration of caffeine and incubated at 37°C for 1 h prior to lysis with ice-cold lysis buffer. For the time-dependent application, cells were given a 20 µM concentration of caffeine and incubated at 37°C for 1 h prior to lysis.

For Western blotting, aliquots containing up to 50 µg of total protein were electrophoretically separated. Electrophoresed proteins were then transferred to nitrocellulose membranes and blocked for 1 h at room temperature in Tris-buffered saline. After blocking, membranes were hybridized overnight at 4°C with primary antibodies (Sigma-Aldrich, St. Louis, MO) against total GSK-3α/β or active GSK-3α/β [pTyr279/286 residues]; diluted 1:500 or 1:1000, respectively]. Membranes were then washed 3 × for 5 min each and incubated for 1 h at room temperature with the appropriate HRP-conjugated secondary antibody. Blots were developed using a luminol reagent, and densitometric analysis of the blots ensued.

Statistical analysis

Behavioral performance was statistically evaluated to determine any group difference based on transgenicity or caffeine treatment. For the single day tasks, one-way ANOVAs were used. For multi-day tasks, both one-way ANOVAs and two-way repeated measure ANOVAs were performed. Prior to analysis, Morris water maze (MWM) data was broken down into 2-day blocks and the RAWM data was divided into 3-day blocks, to aid in data presentation and analysis. After ANOVA analysis, post hoc pair-by-pair differences between groups (planned comparisons) were resolved using the Fisher LSD test. Pre-treatment versus during-treatment RAWM performance was evaluated for each group separately, using paired t-tests. All group comparisons were considered significant at p < 0.05. While very few in number, any outliers or non-performers (e.g., repeated circulars, consistent floaters) in any given task were eliminated from statistical analysis of that task. Data analyses involving Aβ burden, Aβ levels, pcRaf-1 levels, and synaptophysin immunoreactivity were performed using ANOVA. All other statistical analyses are as indicated in the text. In order to test if relationships were present between neurohistologic, neurochemical, and behavioral measures, correlation analysis was performed using the Systat analytical software package (Systat Software, Inc., Chicago, IL).

To determine if the three groups of Study 1 (NT, Tg, Tg/Caff) or the two groups of Study 2 (NT, NT/Caff) could be distinguishable from one another based on “overall” behavioral performance, discriminant function analysis (DFA) was performed using Systat software. For Study 1, direct entry DFAs were performed using seven behavioral measures collected from the three cognitive-based tasks administered during treatment. For Study 2, direct entry DFAs were also performed, using 19 behavioral measures collected from the entire behavioral battery. A complete description of DFA multi-metric statistical analyses relevant to AD transgenic mouse studies can be found in Leighty et al. [25,28].
RESULTS

Caffeine treatment reverses working memory impairment and normalizes identification/recognition ability in aged Tg mice

We have previously shown that long-term caffeine treatment in young adult APPsw Tg mice protected them against otherwise inevitable cognitive impairment in older age [13]. To determine if the same caffeine treatment could benefit aged Tg mice already demonstrating cognitive impairment, caffeine treatment was given in drinking water to 19 month-old APPsw mice. Prior to caffeine administration, mice were evaluated in the RAWM of working memory and verified to be cognitive-impaired, as evidenced by many more errors than NT littermate controls during delayed retention Trial 5 (T5) for the last block of pre-treatment testing (Fig. 1A). At 4–5 weeks into caffeine treatment, however, those impaired Tg mice given caffeine (Tg/Caff) exhibited vastly superior working memory on Trials 4 and 5 compared to the continuing impairment of control
Tg mice (Fig. 1B). Indeed, the performance of Tg/Caff mice was identical to that of NT controls during the last block of testing. We further characterized the cognitive benefits of long-term caffeine treatment by comparing pre-treatment RAWM performance to “during treatment” performance (Fig. 1C). In sharp contrast to the inability of Tg controls to improve upon their poor overall T5 working memory, Tg/Caff mice showed a precipitous drop in errors during RAWM testing at 4–5 weeks into treatment. Even NT controls significantly improved upon their pre-treatment performance level, although not to an error level lower than Tg/Caff mice.

We have consistently found that AD Tg mice are impaired in their ability to switch from the spatial strategy of RAWM to the identification/recognition strategy required for platform recognition testing [13,29,30]. To determine if caffeine treatment can improve the “strategy switching” ability of Tg mice, we evaluated mice in the platform recognition task immediately following completion of RAWM testing. As expected, Tg controls were impaired overall ($p < 0.01$), and particularly during the last two days of testing (Fig. 1D). However, Tg/Caff mice were not different from NT mice on any of the four test days or overall, signifying that caffeine improved strategy switching ability in aged Tg mice (Fig. 1D).

There were no group differences in tasks of sensorimotor skill (open field activity, balance beam, string agility, Y-maze choices) and anxiety (elevated-pluss maze), indicating that neither genotype or caffeine treatment impacted these non-cognitive measures (data not shown). Additionally, there were no group differences in swim speed, as indexed by time taken per choice during RAWM testing.

**Aged Tg mice show “overall” cognitive benefit with caffeine treatment**

Although evaluation of the effect of caffeine on individual cognitive measures is informative and important, we have found that a collective evaluation across multiple cognitive measures/tasks is a more insightful approach for determining if a given AD therapeutic has broad-based value in multiple cognitive domains [22–24]. In this context, we have previously utilized discriminant function analysis (DFA) in reporting that caffeine treatment from young adulthood through older age “protects” APPsw mice from overall cognitive impairment across multiple cognitive domains [13,28]. To elucidate whether caffeine treatment in aged Tg mice provides “overall” cognitive ben-

Caffeine treatment markedly reduces brain Aβ deposition in aged Tg mice

Our prior work has demonstrated that oral caffeine administration in young adult Tg mice for $5\frac{1}{2}$ months suppresses levels of both BACE1 and PS1 in hippocampus, resulting in lower hippocampal levels of both soluble and insoluble Aβ [13]. This suppression of both major enzymes involved in brain Aβ production would suggest that caffeine administration, even to aged Tg mice exhibiting robust Aβ pathology, could reduce/reverse that pathology. Therefore, we next performed a neurohistologic analysis of total Aβ im-
Caffeine treatment reduces brain levels of "soluble" Aβ in aged Tg mice, which correlate with extent of Aβ deposition

We have previously reported that long-term oral administration of caffeine to young adulthood Tg mice induces a significant 37% reduction in hippocampal Aβ1–40 levels, as measured by ELISA [13]. In the present study, we wished to determine if brain soluble Aβ levels could also be reduced by caffeine treatment in aged Tg mice bearing high brain levels of both Aβ1–40 and Aβ1–42. In the same aged Tg mice wherein rever-

munostaining in the same aged Tg mice wherein caffeine administration reversed memory impairment. For both entorhinal cortex and hippocampus, total Aβ deposition was strikingly reduced in aged Tg mice following two months of oral caffeine treatment compared to Tg controls (Fig. 3). Reductions of 46% and 40% in Aβ deposition were evident within entorhinal cortex and hippocampus, respectively, at 20–21 months of age. These caffeine-induced reductions in Aβ deposition are clearly evident in representative photomicrographs of Aβ immunostaining from entorhinal cortex and hippocampus (Fig. 3).

Fig. 3. Caffeine treatment reduces the robust Aβ deposition present in aged Tg mice. Total Aβ deposition was strikingly reduced in both the entorhinal cortex and hippocampus in aged Tg mice following two months of oral caffeine treatment. (Upper) Photomicrographs of Aβ deposition staining in the entorhinal cortex and hippocampus exemplifying the reduction in Aβ deposition induced by caffeine treatment. Scale bar = 50 µm (Lower) Caffeine induced reductions of 46% and 40% in Aβ deposition within the entorhinal cortex and hippocampus, respectively. *p < 0.001. (Colours are visible in the electronic version of the article at www.iospress.nl.)
Fig. 4. Long-term caffeine treatment to aged Tg mice reduces soluble Aβ levels in both cortex and hippocampus; Hippocampal soluble Aβ levels correlate with hippocampal Aβ deposition. (A) At two months into oral caffeine treatment, hippocampal and cortical levels of both Aβ_1−40 and Aβ_1−42 were reduced in comparison to values from Tg controls. *p < 0.05 versus Tg control mice. Abbreviations: N.D., non-detectable.

(B) In hippocampus (but not in cortex), significant correlations were evident between soluble Aβ and deposited Aβ, indicative of a dynamic equilibrium between these two pools of Aβ in the hippocampus. Each group consisted of 5–8 mice. (Colours are visible in the electronic version of the article at www.iospress.nl.)

sal of cognitive impairment was exhibited, measurement of brain soluble Aβ levels at 20–21 months of age (approximately 2 months into treatment) revealed significant decreases in both cortical and hippocampal Aβ levels for caffeine-treated mice (Fig. 4A). Aβ_1−42 levels were significantly reduced in both cortex (↓51%) and hippocampus (↓59%) of caffeine-treated Tg mice compared to Tg controls. Similarly, Aβ_1−40 levels in Tg mice were significantly reduced in cortex (↓25%) and nearly reduced (p = 0.09) in hippocampus (↓37%) by caffeine treatment.

To determine if an association exists between soluble and deposited Aβ pools in the brains of these aged Tg mice, correlation analysis was performed between extent of hippocampal Aβ deposition and Aβ_1−40 or Aβ_1−42 levels, irrespective of treatment. The results of these correlation analyses indicate a clear relationship between extent of hippocampal Aβ deposition and hippocampal levels of Aβ_1−40 (Fig. 4B) and Aβ_1−42 (Fig. 4C). These correlations underscore a dynamic re-
Synaptophysin immunostaining in hippocampus of aged Tg mice is not affected by caffeine treatment

Synaptophysin immunostaining/immunoreactivity is a marker for total synaptic area – not number of synaptic terminals – in a given brain region. As such, we have observed increases in hippocampal synaptophysin immunoreactivity following several therapeutics that provide cognitive benefit to AD Tg mice such as environmental enrichment [11,24] and T-cell immunotherapy [30]. Therefore, we subsequently wished to determine if the beneficial cognitive effects of caffeine in aged Tg mice was associated with changes in hippocampal synaptophysin immunostaining. As shown in Fig. 5, caffeine treatment did not impact synaptophysin immunostaining in the hippocampal CA1 and CA3 regions of Tg mice. Irrespective of treatment, Tg mice exhibited enhanced synaptophysin immunostaining in comparison to NT controls (Fig. 5), which is consistent with our prior work [31] and probably indicative of the intense synaptophysin immunostaining associated with dystrophic nerve terminals in Tg mice.

Caffeine treatment down-regulates the active form of cRaf-1 in hippocampus from Tg mice: Possible linkage to BACE1 suppression

Because we have previously reported that long-term caffeine treatment decreases BACE1 expression
in brains of AD Tg mice [13] and also found caffeine-induced reductions in brain Aβ levels in the present study, we wished to develop insight into possible mechanism(s) involved in BACE1 suppression by caffeine. The cRaf-1 is a cytosolic protein kinase and is an integral part of the Ras/cRaf/ERK pathway that regulates cell proliferation, survival, and senescence. When persistently activated through phosphorylation at its Serine 338 site, cRaf-1 stimulates the transcription factor nuclear factor κappa B (NFκB) [32], which then can stimulate expression of BACE1 and other AD-linked proteins [33]. Phosphorylation of cRaf-1 at its Serine 259 site stabilizes cRaf-1 in an inactive conformation, thus suppressing BACE1 expression. These relationships are diagrammed in Fig. 10.

In a second caffeine treatment study, we therefore examined the hippocampus of 9.5 month-old Tg mice that had been given oral caffeine treatment via gavage for two weeks prior to euthanization. Analysis of hippocampal levels of the inactive form of cRaf-1 (pcRaf-1[Ser259]) by Western blot revealed a significant down-regulation of this inactive form in Tg controls to 42% of NT control levels (Fig. 6A). In sharp contrast, Tg mice that had been treated with caffeine for only two weeks exhibited active cRaf-1 (pcRaf-1[Ser338]) levels that were significantly lower than Tg controls (47%; Fig. 6B) and inactive cRaf-1 (pcRaf-1[Ser259]) that were significantly higher than Tg controls (141%; Fig. 6A). Moreover, we also determined from the same hippocampal tissue lysates that a significant decrease (29%) in protein kinase A (PKA) activity was present in Tg controls and that caffeine treatment significantly increased PKA activity (125%) in Tg mice to levels exhibited by NT mice (Fig. 6C). Since PKA converts active to inactive cRaf-1, these results are consistent with a PKA/Raf-1 mediated mechanism of BACE1 suppression by caffeine (see Fig. 10).

Caffeine induces concentration- and time-dependent decreases in both active and total GSK-3 levels in SweAPP N2a cells

We have reported that chronic caffeine treatment reduces PS1 expression/γ-secretase activity in AD Tg mice, presumably contributing to the decreased Aβ production also observed [13]. In the present study, we next examined the potential involvement of GSK-3 in caffeine’s reduction of γ-secretase-mediated Aβ production, as GSK-3 is reported to regulate Aβ production by interfering with γ-secretase cleavage [20, 34]. To identify the possible actions of caffeine on this essential component of the AD pathogenic pathway, Western blot analysis was performed on SweAPP N2a cell lysates using antibodies against both active and total GSK-3. A concentration-dependent decrease in both GSK-3α and GSK-3β was evident, with significant reductions achieved at caffeine concentrations of 10 and 20 µM (Fig. 7). This effect was observed on active GSK-3 (pTyr216/279) protein levels (A), as well as on GSK-3 holoprotein levels (C). Caffeine also exerted a time-dependent decrease in both GSK-3 isoforms, as shown in Fig. 7. Significant reductions in active protein levels were seen in both GSK3 isoforms at time-points exceeding 120 minutes (B). However, significant alterations in holoprotein levels were isoform-specific, with GSK-3/β reduction by 30 minutes and GSK-3/α reduction by 90 minutes (D). It is noteworthy that, while active GSK-3 levels declined as caffeine concentration was raised from 0 to 16 µM, active GSK-3 levels increased as caffeine concentration was elevated from 31.2 µM to 1 mM (data not shown). Thus, approximately 20 µM was determined to be the optimal caffeine concentration for active GSK-3/α and GSK-3/β inhibition, which corresponds to that typically observed in the blood and brain of humans following 1–2 cups of coffee [1].

Long-term caffeine treatment from adulthood through old age does not provide cognitive benefit to normal mice

It is possible that the caffeine-induced reversal of cognitive impairment that we observed in aged Tg mice involved non-specific, generalized mechanisms such as increased glucose utilization [35], resulting in the well-known ability of caffeine to increase alertness/attention. If such generalized, non-amyloidogenic mechanisms were critical for the cognitive benefits seen in Tg mice, we reasoned that NT mice should also benefit from long-term caffeine treatment. Therefore, in a third caffeine treatment study, we began providing 5 1/2 month-old NT (normal) mice with caffeine in their drinking water and behaviorally evaluated them at 10 months into caffeine treatment (e.g., 15–16 months of age). Parenthetically, no truly long-term caffeine administration studies (over months) have been done previously in rodents that might relate to habitual caffeine use in humans.

As shown in Fig. 8, life-long caffeine administration had no effect on cognitive performance of NT mice across 13 measures in five cognitively-based tasks (Y-maze percent alternation, standard Morris water maze,
Fig. 6. In the hippocampus of Tg mice, caffeine treatment increases the “inactive” form (A) and decreases the active form (B) of cRaf-1, while also increasing protein kinase A (PKA) activity back to normal levels. Two weeks of oral caffeine treatment was given to 9 month-old Tg mice and their hippocampus analyzed. (A) The significant decrease in inactive cRaf-1 (pcRaf-1[Ser259]) that was evident in Tg controls was reversed by caffeine treatment to the level of NT controls. *p < 0.05 versus other two groups (B) Caffeine treatment decreased the levels of active cRaf-1 (pcRaf-1[Ser338]) in Tg mice treated with caffeine compared to Tg control mice. Tubulin or GADPH served as non-specific protein controls. *p < 0.05 versus Tg group. (C) The significant decrease in hippocampal PKA activity present in Tg mice was reversed by caffeine treatment back to levels present in NT mice. *p < 0.02 for Tg controls versus other two groups. (Colours are visible in the electronic version of the article at www.iospress.nl.

circular platform, platform recognition, and radial arm water maze). It should be noted that cognitive performance of the control NT mice at 15–16 months of age was not outstanding, thus allowing for possible therapeutic improvement. Chronic caffeine administration did not affect sensorimotor (open field activity, balance beam, string agility) or anxiety (elevated-plus maze) measures, nor did it affect swim speed in water-based
Fig. 7. Caffeine induces concentration- and time-dependent decreases in both GSK-3α and GSK-3β expression in vitro. SweAPP N2a cells were treated with a range of caffeine concentrations for 1 h (A,C) or with a 20 µM concentration of caffeine over a range of time points (B,D). Prepared lysates were subjected to Western blot (WB) analysis using antibodies against active GSK-3α (pTyr216 residue) and GSK-3β (pTyr279 residue) isoforms (A,B) or against total GSK-3α and GSK-3β (C and D). Anti-actin antibodies were used as an internal reference control for each WB. Histograms depict densitometric analysis of three independent experiments, with a representative WB of each experiment displayed. Isoform-specific band to actin ratios are delineated on histogram y-axes, and concentration- or time-points are depicted on x-axes. Asterisks denote significant differences (p < 0.05) between specific concentration- or time-group and the control group (0 µM or 0 minutes), using a Mann-Whitney test for pair wise comparisons of non-parametric data. (Colours are visible in the electronic version of the article at www.iospress.nl.

tasks (data not shown). A direct entry DFA performed across all 19 behavioral measures of the test battery also indicated no significance discrimination between caffeine-treated and control NT mice in overall behavioral performance.

**DISCUSSION**

This study provides the first evidence that caffeine treatment can reverse cognitive impairment and AD neuropathology in a model for the disease. Aged,
cognitively-impaired APPsw mice given a moderate amount of daily caffeine (the human equivalent of 5 cups of coffee) exhibited a restoration of working memory to the level of normal, aged mice. In these same aged AD mice, which had pre-existing and substantial Aβ burdens, caffeine treatment reduced both soluble and deposited (insoluble) brain Aβ levels. Caffeine-induced cognitive restoration is likely due to its profound ability to reduce Aβ production by reducing expression of both BACE1 and PS1/γ-secretase [13]. The present study provides mechanistic insight into both of these caffeinergic actions by showing that 1) caffeine administration to APPsw mice was able to correct their dysregulation of the Raf-1/NFκB inflammatory pathway, which is linked to BACE expression, and 2) caffeine suppressed both α and β isoforms of GSK-3 in vitro, which are linked to PS1/γ-secretase activity and tau hyperphosphorylation, respectively. The extent to which these caffeinergic effects involve adenosine receptor antagonism are unknown and currently being investigated.

We recently reported that caffeine, when administered from young adulthood through older age, protected APPsw mice from otherwise certain impairment in multiple cognitive domains and reduced their brain Aβ levels [13]. These results, suggestive that moderate daily intake of caffeine could delay or reduce the risk of AD, are supportive of an earlier epidemiologic study showing that AD patients consumed much less caffeine in the 20 years prior to disease onset compared to same-aged individuals who were non-demented [9]. More recent longitudinal studies have also reported protective effects of caffeine/coffee intake against cognitive impairment during normal aging [6,7] and AD [8]. The evidence for the protective effects of caffeine against aged-associated cognitive impairment and AD, which
is nicely reviewed by Rosso et al. [10], is in contrast to the reality that human intake of caffeine usually decreases during aging – this, despite the fact that the metabolism and physiologic responses to caffeine are the same in elderly and younger individuals [36].

Accompanying the caffeine-induced cognitive restoration in aged APPsw mice was a reversal of Aβ deposition and decrease in soluble Aβ levels within their brains. These reductions in both soluble and deposited (insoluble) forms of Aβ would be expected, given that caffeine decreases Aβ production through suppression of both BACE and PS1/γ-secretase expression [13]. Newly produced Aβ enters a dynamic equilibrium between soluble and deposited Aβ in the brain, with continual transport of soluble Aβ out of the brain and into plasma down a concentration gradient (Fig. 9, Unmodulated). We hypothesize that caffeine suppression of Aβ production results in lower brain levels of soluble Aβ, which then induces a flux of insoluble Aβ out of the deposited form and into the soluble form (Fig. 9, Caffeine). This newly-solubilized Aβ would then be cleared into the plasma via Aβ transport. Supportive of this dynamic equilibrium hypothesis are the strong correlations that we report in aged APPsw mice between soluble Aβ levels and deposited Aβ in hippocampus. Moreover, the additional correlations that we report between both soluble and deposited forms of Aβ versus cognitive performance clearly underscore a direct impact of this dynamic equilibrium on cognitive performance. Indeed, caffeine’s reversal of cognitive dysfunction in aged APPsw mice most likely involves lowering of both soluble and deposited Aβ through this equilibrium.

The present study presents new mechanistic insight into how caffeine may suppress both BACE1 and PS1/γ-secretase expression to decrease Aβ production. Whether or not this caffeine-induced decrease in Aβ production is direct or involves adenosine receptor blockade/mediation is currently unknown. There certainly exists considerable evidence that the actions of caffeine against Aβ are mediated through antagonism of A2a and/or A1 adenosine receptors, particularly in the context that 1) caffeine and selective A2a receptor antagonists both provide protection against Aβ neurotoxicity in vitro [37]; 2) caffeine and/or selective A2a receptor antagonists protect against the cognitive impairment induced by intracerebral Aβ1−42 or Aβ25−35 infusion in mice [38,39]; and 3) acetylcholine neurotransmission in hippocampus/cortex is increased through pre-synaptic A1 receptor blockade [40]. Although we have determined that brain adenosine receptor levels are unaffected by long-
term caffeine treatment in AD transgenic mice [13], a rich body of scientific findings suggests that the possibility of adenosine-receptor-mediated actions needs further investigation [41].

Regarding BACE1 suppression, we provide evidence that caffeine is able to correct a dysregulation of the cRaf-1 inflammatory pathway in APPsw mice by stimulating PKA activity, thus increasing brain levels of “inactive” cRaf-1 (pcRaf-1[Ser259]) and reducing the active form of cRaf-1 phosphorylated at Serine 338 (pcRaf-1[Ser338]). Serine(s) 259 and S621 bind to 14-3-3, a protein that maintains cRaf-1 in an inhibited state (phosphorylated at Serine 259) in basal conditions. After stimulation, active Ras displaces 14-3-3 proteins, cRaf-1 is trans-located to the plasma membrane, de-phosphorylated by phosphatases at this site and phosphorylated at the kinase domain at the Serine 338 activation site [42]. As shown in Fig. 10, “active” Raf stimulates NFκB intracellularly, resulting in increased expression of reporter genes such as BACE1 [43,44]. It should be noted that cRaf-1 has been reported to be over-activated in AD brains [15,45].

In this study, we demonstrate that caffeine stimulates PKA activity in the hippocampus of treated AD Tg mice, which is consistent with similar effects of caffeine in peripheral tissues [46,47]. This enhanced PKA activity then inhibits (inactivate) cRaf-1 by phosphorylation at serine259 [48], decreasing NFκB activity and the expression of NFκB-controlled genes such as BACE1; thus, decreased Aβ production results (Fig. 10). Supportive of the idea that the cRaf-1/NFκB pathway plays a key role in Aβ production and toxicity in the brain is our recent discovery that the Raf-1 inhibitor Sorafenib inhibits NFκB signaling and ameliorates memory impairment in aged APPsw mice [49]. Caffeine treatment has often been associated with the inhibition of phosphodiesterases (PDEs); indeed, we and others have reported that treatment with the PDE inhibitor Rolipram improves working memory of AD transgenic mice [50, 51]. However, caffeine’s inhibition of PDE occurs at mM concentrations [1], well above blood levels of caffeine that are achievable in rodents/humans. We therefore believe that the profound ability of caffeine to reduce brain Aβ pathology is due to its suppression of both β- and γ-secretases [13]. Consistent with this premise, Rolipram was unable to decrease Aβ levels in vivo [51].

A potential mechanism through which caffeine suppresses PS1/γ-secretase (to reduce Aβ production) was also explored in the present study. Dysregulation of GSK-3 is a key feature of AD pathogenesis, with the activity of both α and β isoforms playing critical roles [52]. While GSK-3/β directly binds PS1 [53], GSK-3α is most notable for enhancing PS1/γ-secretase activity and consequently increasing Aβ production [20]. In vitro studies demonstrate
that siRNA inhibition of GSK-3β drastically reduces Aβ production by interfering with γ-secretase cleavage of APP [20], likely through disruption of APP-PS1 association [34]. Furthermore, conditional over-expression of GSK-3β results in increased tau phosphorylation, neuronal apoptosis, reactive gliosis, and spatial learning deficits in the Tet/GSK-3β mouse model for AD [19,54]. This AD-like phenotype is completely reversed once GSK-3β overexpression is ceased and GSK-3β activity returns to normal [55]. In the present study, we report that caffeine suppresses GSK-3α and GSK-3β levels in SweAPP N2a cell cultures in both a dose- and time-dependent fashion. Moreover, caffeine impacted both active and holoprotein (total) GSK-3β levels, suggesting that caffeine does not diminish GSK-3 activity through direct substrate-level phosphorylation, but rather through alternations in protein production or degradation.

Because optimal GSK-3β inhibition was obtained with a 20 µM concentration of caffeine in vitro, actions of caffeine that only occur at much higher concentrations are unlikely candidates to mechanistically explain both our in vitro and in vivo observations [1]. Indeed, a 10–20 µM plasma and brain concentration of caffeine would be quickly reached (1–2 hours) in humans following ingestion of 1–2 cups of coffee [1]. This underscores that the effects of caffeine on GSK-3β are readily attained at physiologically relevant concentrations – in fact, the same 10–20 µM concentration of caffeine effectively inhibits both Aβ40 and Aβ42 production by SweAPP N2a cell cultures [13]. Reductions in GSK-3β thus represent a further mechanism through which caffeine may ameliorate AD pathology and restores cognitive function. Accordingly, reduction of GSK-3 levels/activity in the brain is in itself a pursued AD therapy [52].

In addition to the aforementioned mechanisms of caffeine action directly impacting Aβ production, there are multiple “non-amyloidogenic” mechanisms that could contribute to the ability of caffeine to protect against or reverse memory impairment. Since inflammation is an essential part of the AD pathogenic pathway [56] and AD is characterized by increased oxidative stress, it is possible that the anti-inflammatory [46] and antioxidant [57] actions of caffeine contribute to its pro-cognitive affects. It is important to emphasize that generalized mechanisms of caffeine action (e.g., increased glucose utilization) may also be contributory [35] to the cognitive reversal observed. However, our finding that life-long caffeine treatment to non-transgenic mice did not provide any cognitive bene-

fits in old age does not support this possibility – particularly given that there was ample room for cognitive improvement when testing occurred in older age. Nonetheless, if the same caffeine treatment paradigm had been given to aged “normal” mice as was given to aged Tg mice, beneficial cognitive effects may have been revealed. Indeed, generalized mechanisms (such as increased glucose utilization) may play a role because the substantial brain Aβ levels in aged APPsw mice reduce their cerebral blood flow [58].

Although caffeine has been reported to have effects on dendritic morphology during post-natal development [59], we presently found no effect of long-term caffeine treatment on synaptic area in hippocampus of aged APPsw mice. Regarding caffeine effects on synaptic function, several electrophysiologic studies involving hippocampal slice recordings from the hippocampal CA1 region have reported that caffeine increases the amplitude of EPSPs [60,61] and induces a novel pre-synaptic form of long-term potentiation (LTP) [62]. However, because all such studies have involved caffeine concentrations well above physiologic levels (e.g., 0.1–10 mM), electrophysiologic studies involving caffeine concentrations relevant to tissue levels of caffeine achieved with human or rodent caffeine consumption (e.g., 10–30 µM) are necessary to determine the impact of caffeine impact on synaptic transmission. Finally, caffeine has very recently been shown to block disruptions in the blood-brain barrier induced by a high cholesterol diet in rabbit [63]. Since breakdown of the blood-brain barrier has been reported in AD [64] and appears to precede Aβ plaque formation in APPsw transgenic mice [65], blood-brain barrier stabilization by caffeine is a viable mechanism for consideration.

All of the aforementioned non-amyloidogenic and generalized mechanisms of caffeine action notwithstanding, the cognitive restoration provided to aged AD transgenic mice by caffeine treatment in the present study would appear to be mostly due to the profound ability of caffeine to reduce Aβ production via secretase suppression, and in the process result in brain clearance of both soluble and deposited/insoluble Aβ. Given the safety, availability, and brain bioavailability of caffeine, its ability to suppress both β- and γ-secretases would suggest it is preferable to synthetic secretase inhibitors currently under development. Based on the robust protective and treatment effects of caffeine that we have observed in AD transgenic mice, we have initiated clinical trials with caffeine.
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