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Neurobiology of Disease

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Neurobiology of Disease 24 (2006) 506–515

Pomegranate juice decreases amyloid load and improves behavior in a mouse model of Alzheimer's disease

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Received 6 June 2006; revised 3 August 2006; accepted 15 August 2006
Available online 28 September 2006

Although there are no proven ways to delay onset or slow progression of Alzheimer's disease (AD), studies suggest that diet can affect risk. Pomegranates contain very high levels of antioxidant polyphenolic substances as compared to other fruits and vegetables. Polyphenols have been shown to be neuroprotective in different model systems. We asked whether dietary supplementation with pomegranate juice (PJ) would influence behavior and AD-like pathology in a transgenic mouse model. Transgenic mice (APP_{sw}/Tg2576) received either PJ or sugar water control from 6 to 12.5 months of age. PJ-treated mice learned water maze tasks more quickly and swam faster than controls. Mice treated with PJ had significantly less (~50%) accumulation of soluble $A\beta_{42}$ and amyloid deposition in the hippocampus as compared to control mice. These results suggest that further studies to validate and determine the mechanism of these effects, as well as whether substances in PJ may be useful in AD, should be considered.

Keywords: Alzheimer; Transgenic; Mouse; Diet; Pomegranate; Amyloid; Plaque; Behavior; Spatial learning; Polyphenol; Antioxidant

Introduction

Alzheimer's disease (AD) is the most common cause of dementia and affects more than 10% of individuals over the age of 65. Although there are currently no proven ways to delay the onset or slow the progression of AD, epidemiological and experimental evidence suggests that diet can affect the risk for AD and alter amyloid-β (Aβ) levels. For example, a high cholesterol diet has been shown to increase levels of Aβ and apoE (key constituents of the plaques deposited in the brains of AD patients) in the brains of rabbits (Wu et al., 2003) and APP transgenic mice (Refolo et al., 2000). It has been hypothesized that diets high in carbohydrates may alter metabolism of cellular membrane proteins (e.g., APP) and trigger excessive cell signaling cascades, leading to neuronal damage (Henderson, 2004). Other research suggests that dietary intake of aluminum may increase the risk of developing AD (Newman, 1992; Roberts et al., 1998; Rogers and Simon, 1999) and that diets deficient in magnesium can produce cognitive deficits in mice (Bardgett et al., 2005). Importantly, mounting evidence suggests that diet can also decrease the risk for developing AD (Mattson, 2000; Pope et al., 2003). Caloric restriction appears to be neuroprotective in mouse models of AD (Love, 2005; Wang et al., 2005), perhaps by decreasing the accumulation of Aβ deposits (Patel et al., 2005). Another recent study suggests that increased dietary intake of niacin may slow the progression of cognitive decline in AD (Morris et al., 2004).

Foods containing high levels of antioxidants may also slow the progression of AD, possibly by preventing or neutralizing the damaging effects of free radicals (Kostrzewa and Segura-Aguilar, 2003; Polidori, 2003). The essential fatty acids contained in fish oil (e.g., docosahexaenoic acid/DHA) may be neuroprotective in humans (Grant, 2000, 2003; Horrocks and Yeo, 1999; Peers, 1990). Recent studies have also shown beneficial effects of DHA on learning in a rat model of AD (Hashimoto et al., 2002, 2005) and on both plaque deposition and dendritic pathology in aged APP_{sw} transgenic mice (Calon et al., 2004; Lim et al., 2005). Chronic dietary administration of the antioxidant vitamin E has been shown to reduce Aβ deposits in APP_{sw} mice (Sung et al.,

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Available online on ScienceDirect (www.sciencedirect.com).

0969-9961/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.nbd.2006.08.006

2004), and epidemiological evidence suggests that high intake of food-based vitamin E is associated with a lower incidence of AD in humans (Morris et al., 2005).

Phytochemicals are nonnutritive bioactive chemicals found in plants (especially pigments) that can have beneficial effects on health. Phytochemicals like polyphenols (including the phenolic acids and flavonoids) have been shown to have antioxidant properties and to suppress inflammatory and other pathways (Aggarwal and Shishodia, 2004; Joseph et al., 2005). Quercetin, a flavonoid polyphenol found in several fruits and vegetables, was recently shown to protect against oxidative stress *in vitro* (Heo and Lee, 2004), and curcumin, a polyphenol found in the curry spice turmeric, was shown to lower levels of oxidized proteins and plaque burden in APP_{sw} mice (Lim et al., 2001). Green tea, another food high in polyphenols, may also be neuroprotective (Weinreb et al., 2004), and one of its flavonoid components, epigallocatechin-3-gallate, decreased Aβ levels in APP_{sw} mice (Rezai-Zadeh et al., 2005). Dietary supplementation with blueberries, also rich in polyphenols, has been shown to improve Y-maze performance, but not plaque deposition, in APP+PS1 transgenic mice (Joseph et al., 2003).

Pomegranates contain very high levels of polyphenols as compared to other fruits and vegetables (Kelawala and Anantha-narayan, 2004; Wang et al., 2004; Xu et al., 2005). Dietary supplementation of pregnant mice with pomegranate juice was recently shown by our laboratory to protect against neurodegeneration in neonatal mice subjected to hypoxic–ischemic brain injury (Loren et al., 2005). Therefore, we asked whether dietary supplementation with pomegranate juice would influence AD-like pathology and behavior in a mouse model of AD.

Materials and methods

Animals—Transgenic experiments

Beginning at 6 months of age, transgenic mice expressing a form of the amyloid precursor protein (APP) that causes early-onset familial AD (APP_{sw}/Tg2576) (Hsiao et al., 1996) received in their drinking bottles pomegranate juice (PJ) from a single lot of PJ concentrate (PomWonderful; Los Angeles, CA) diluted 1:160 or 1:80 in filtered water. Since the PJ concentrate is 4 times more concentrated than regular strength PJ sold commercially, the dilutions of concentrate are approximately equivalent to dilutions of 1:40 or 1:20 of non-concentrated PJ. The mice drank an average of 5 ml of fluid per day. This amount of PJ at these dilutions is roughly equivalent on a mg/kg basis to a human drinking one versus two 8-ounce glasses of PJ per day. The amount of polyphenols consumed per day was estimated to be ~0.3–0.6 mg. Control APP_{sw} mice received sugar water that mimicked the sugar content of the 1:40 PJ (85% sucrose, 7.5% D-(+)-glucose, 7.5% D-fructose). Previous data from our laboratory have shown that polyphenolic substances in PJ are detectable in the plasma of mice pups whose mothers drank PJ but not in pups whose mothers drank sugar water (Loren et al., 2005). Because there were no statistically significant differences between the 1:40 and 1:20 PJ groups for any of the behavioral or neuropathological assessments, the two PJ groups were grouped together for purposes of data analysis. Additionally, there were no weight differences between any of the groups. Behavioral testing began at 11.5 months of age, and the mice continued their treatment regimen throughout testing.

Animals—Wildtype experiments

Wildtype (non-transgenic) control littermates of the APP_{sw} mice were fed PJ (1:40 dilution) or vehicle (sugar water control) beginning at 3–5 months of age. There were no weight differences between the groups. Behavioral testing began after 3 weeks of treatment, and the mice continued their treatment regimen throughout testing.

Pomegranate juice preparation

Pomegranates (Wonderful variety) were picked by hand, washed, and stored in tanks. The fruit was crushed and squeezed. The juice was filtered, pasteurized, concentrated and stored at −18°C. The composition of PJ that was used in these experiments was assessed as follows. PJ was fractionated by low-pressure chromatography on Sephadex LH-20. Five different fractions were obtained by sequential elution with aqueous/organic solvents and then analyzed by high-performance liquid chromatography (HPLC), diode array ultraviolet detection (DAD), electrospray ionization-mass spectrometry (ESI-MS), and matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) (J. Reed, University of Wisconsin, Madison, personal communication). PJ consisted of 84% water, 14% carbohydrates, 0.48% ash, 0.4% citric acid, 0.1% protein, 0.02% fat and 1% other, including polyphenols (phenolic acids and flavonoids). Phenolic acids included 115 ppm ellagic acid and 5 ppm gallic acid. Flavonoids included 1880 ppm hydrolysable tannins (e.g., gallotannins, ellagitannins, punicalagin) and 369 ppm anthocyanins and their glycosides (e.g., cyanidin, delphinidin, pelargonidin).

Water maze

The learning and memory abilities of the mice were tested in the Morris water maze, as described (Hartman et al., 2005). Briefly, this test of spatial navigation learning requires the mouse to find a hidden (submerged) platform in a pool of water using visual cues from around the room. As performance improves, escape latency and swim path length generally decrease. The water maze consisted of a metal pool (118 cm diameter) in a well-lit room filled to within 10 cm of the upper edge with water made opaque by the addition of white non-toxic tempera paint. The pool contained a round platform (22 cm diameter) that the mice could step on to escape the water. For each trial, a mouse was released nose against the wall into the pool at one of four release points and allowed to find the platform. All trials lasted a maximum of 60 s, at which point the mouse was manually guided to the platform. An overhead camera recorded the animals' swim paths, allowing for quantification of distance, latency, proximity to target and swimming speed by a computer (Polytrack; San Diego Instruments, San Diego, CA).

CUED water maze

The CUED (visible platform) task was used to assess sensorimotor and/or motivational deficits that could affect performance during the SPATIAL water maze task. For this task, the surface of the escape platform was visible (5 mm above the surface of the water), and a 10 cm tall pole capped by a red tennis ball was placed on top of the platform to make its location even more obvious. The walls of the room were kept bare, although the room geometry, experimenter and computer system were obvious

spatial cues. The mice were given four consecutive trials per day, each time with the platform in the middle of a different pool quadrant. The mouse was released into the pool opposite the location of the platform for that trial. After each trial, the mouse was placed into a holding cage for 30 s while the platform was moved to its next location. For the APP_{sw} mice, CUED testing continued for 5 days, giving each mouse a total of 20 CUED trials. For the wildtype mice, CUED testing continued for 3 days, giving each mouse a total of 12 CUED trials. Mice that displayed behaviors inappropriate for SPATIAL water maze testing (including spinning, thygmotaxic navigation around the perimeter of the pool and inability to swim) were removed from the study (APP_{sw}: 3 of 16 control mice and 4 of 32 PJ mice; wildtype: 2 of 14 control and 3 of 13 PJ mice). This left sample sizes of 13 controls/28 PJ APP_{sw} mice and 12 vehicle/10 PJ wildtype mice for continued spatial testing.

SPATIAL water maze

Three days after the conclusion of CUED testing, SPATIAL (hidden platform) testing began. For this task, the surface of the escape platform was submerged 1 cm below the surface of the water, and several spatial cues were added to the walls of the room, which required the mice to find the platform based on its relationship to the cues rather than direct visualization. Four consecutive trials were administered per day for 5 days. Each day, the mouse was released once from each of four release points. After a 2-day break, the mice were given a "probe" trial in which the platform was removed from the water maze, and the mice were allowed to search the pool for 60 s. The amount of time spent searching the quadrant that had contained the platform was measured, as well as the total number of times that the mouse crossed over the former location of the platform. An hour later, the platform was placed back into the pool in its former location, and 5 more days of SPATIAL testing were administered. APP_{sw} mice were tested in the SPATIAL condition for a total of 15 days/60 trials, and 3 probe trials were administered. Wildtype mice were tested for 5 days/20 trials, with 1 probe trial administered.

Histology and tissue dissection

At 12.5 months of age, the APP_{sw} mice were perfused through the heart with PBS, and their brains were removed. The left hemisphere of each brain was immersed in 4% paraformaldehyde in 0.1 M PBS at 4°C for 24 h and then soaked in a 30% sucrose solution at 4°C for 24 h, followed by freezing in powdered dry ice. The brain was then cut coronally in 50 μm sections from the genu of the corpus callosum to the end of the hippocampal formation. A subset of the sections (every 6th) was stained with pan anti-Aβ antibody (Biosource International; Camarillo, CA), and another subset of every 6th section was stained for fibrillar Aβ (amyloid) using thioflavine-S, as described (Hartman et al., 2005). The hippocampus and dorsal cortex (defined as the cortical tissue overlying the hippocampus) of each animal were assessed for Aβ and fibrillar Aβ load (i.e., percent area covered by deposits) using an unbiased stereological method (area fraction fractionator; Stereo Investigator, Microbrightfield; VT) and a Nikon E800 microscope. To examine the effects of PJ on cerebral amyloid angiopathy (CAA; a common neuropathological condition in humans with AD and in the APP_{sw} mouse (Fryer et al., 2005)), thioflavine-S positive blood vessels were quantified in the dorsal cortex. To further assess whether PJ had a protective effect on neuronal processes near fibrillar Aβ deposits, thioflavine-S stained brain sections were double-labeled with a silver stain as described. Numbers of dystrophic/swollen neurites (>2.5 μm in diameter) were counted at the site of each thioflavine-S positive plaque to attain an average number of dystrophic neurites per plaque. The hippocampus and cortex were dissected from the right hemisphere of each brain and frozen at −80°C for later biochemical analyses.

Aβ ELISAs

Tissue was prepared for ELISA as described (DeMattos et al., 2002). Cortices and hippocampi were homogenized in carbonate buffer, spun down, and the supernatant was collected. The remaining pellet was re-homogenized in 5 M guanidine buffer, spun down, and the supernatant was collected. $Aβ_{40}$ and $Aβ_{42}$ levels were assessed with Biosource International Aβ ELISA kits (Camarillo, CA).

APP Western blots

To assess APP/Aβ processing, cortices were processed as described (Wahrle et al., 2005). Briefly, the tissue was sonicated in 10 μl/mg RIPA buffer with protease inhibitors and spun at 20,000×g for 25 min. The supernatant was collected, and protein levels were measured with a bicinchoninic acid (BCA) assay (Pierce; Rockford, IL). The samples were then run on 4–12% Bis–Tris gels with MES running buffer (Invitrogen; Carlsbad, CA). Following electrophoresis, proteins in the gels were transferred to nitrocellulose membranes, blocked in 4% milk in PBS and probed with a polyclonal antibody specific to the C-terminal 22 amino acids of APP (Zymed Laboratories; South San Francisco, CA). The blots were also probed with an anti-tubulin antibody (Sigma; St. Louis, MO) as a control. The blots were quantified using Kodak 1D Image Analysis software.

Data analysis

Statistica 6.0 (StatSoft, Inc.) was used to analyze the collected data. An α-level of 0.05 was used for all statistical significance tests. There were no gender × treatment interactions. Histological and biochemical data were analyzed using a one-way ANOVA with one between-subjects factor (group: control vs. PJ). Correlations between behavioral and biological variables were determined using the Pearson product–moment coefficient. To account for the pseudorandom nature of the distance to the escape platform from any given release point, swim path distance, escape latency, proximity to target and swim speed data were analyzed by averaging trials into blocks of 4 daily trials. These blocks were analyzed with two-way ANOVAs that included one between-subjects variable (group: control vs. PJ) and one within-subjects variable (test day). To avoid violating the assumptions of compound symmetry and sphericity that underlie univariate statistics for repeated measures factors with more than two levels (i.e., differences between levels of repeated measures must not be correlated across subjects), the reported *p*-values for every repeated-measures analysis reflect the Huynh–Feldt adjustment to the degrees of freedom. Probe trial data (time spent searching the probe quadrant and number of platform location crossings) for each phase and histological analyses were

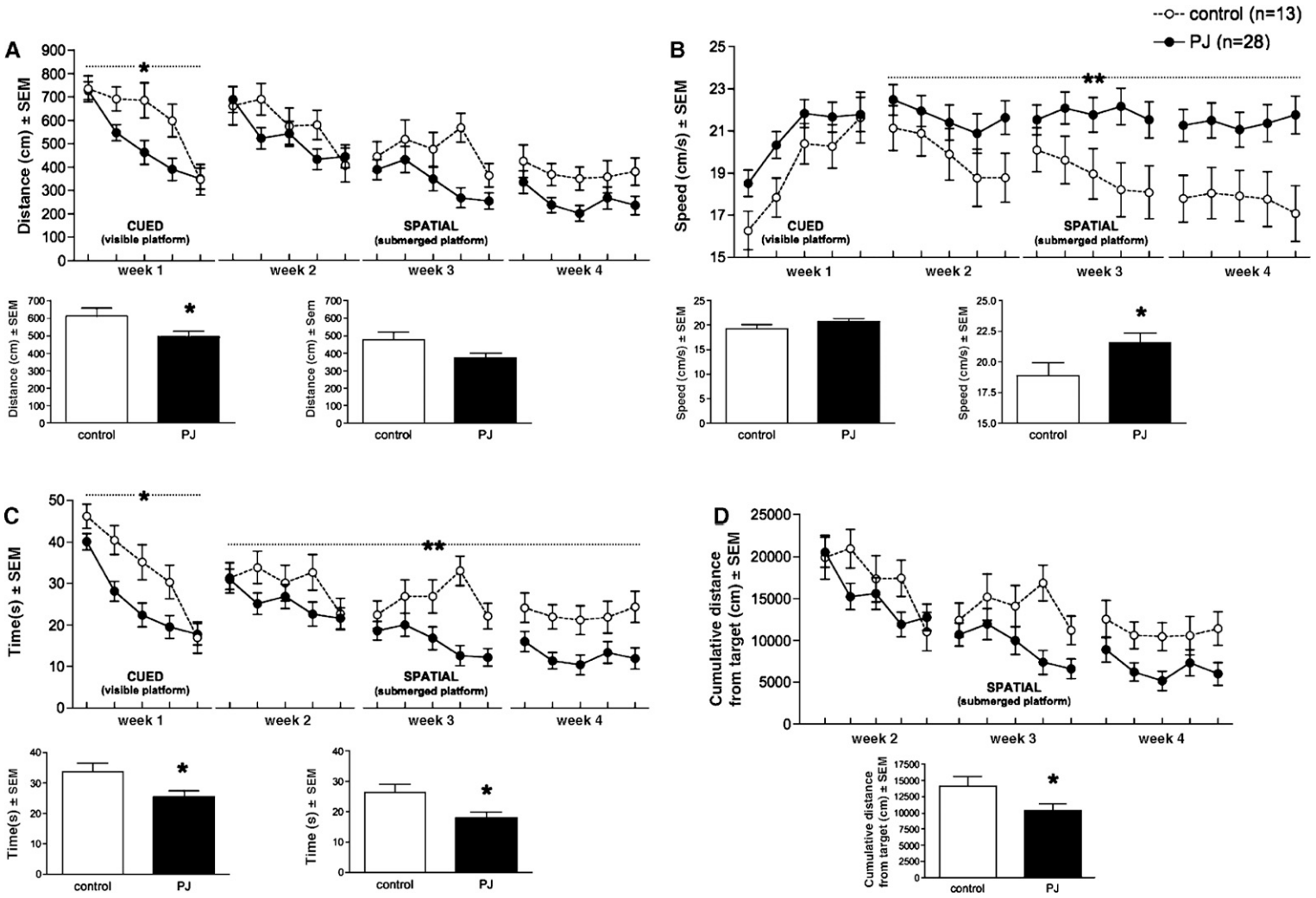

Fig. 1. APP_{sw} mice water maze performance. Each point represents the average of 4 daily trials. The bar graphs represent average performance (main effects) over each phase of testing. (A) Swim distance. PJ-treated mice learned the CUED task significantly more quickly (∗ treatment main effect $p<0.05$;–∗– treatment × day interaction $p<0.02$). A similar trend was observed for the SPATIAL task, but the difference was not significant. (B) Swim speed. Both PJ-treated and control mice swam progressively faster during the CUED task, but the difference between the groups was not significant. Control mice swam progressively slower during the SPATIAL task, whereas PJ mice maintained a significantly higher rate of speed (∗ treatment main effect $p<0.04$; –∗∗– treatment × day interaction $p<0.003$). (C) Escape latency. PJ-treated mice found the platform significantly more quickly in both the CUED task (∗ treatment main effect $p<0.02$; –∗– treatment × day interaction $p<0.04$) and the SPATIAL task (∗ treatment main effect $p<0.02$; –∗∗– treatment × day interaction $p<0.002$). (D) Proximity to platform. These data were gathered during the SPATIAL, but not the CUED, task. Mice treated with PJ spent more of each trial swimming closer to the target platform (∗ treatment main effect $p<0.05$).

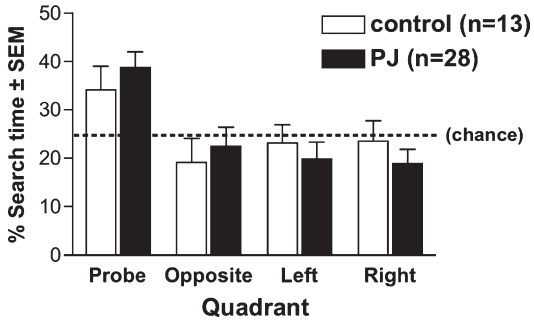

Fig. 2. APP_{sw} mice probe trial performance. The bar graph shows the average of the three probe trials and represents the percent of probe trial search time spent searching each quadrant. Both groups demonstrated evidence of spatial memory, and there were no significant group differences.

analyzed with one-way ANOVAs that included one between-subjects variable (group).

Results

PJ improved behavioral performance of APP_{sw} mice in the water maze

APP_{sw} mice have demonstrated normal performance in the water maze at 3 months of age and impaired performance by 9–10 months of age as compared to wildtype littermates (Hsiao et al., 1996). We compared APP_{sw} mice that were treated from 6 to 12.5 months. Thus, we studied PJ and control-treated APP_{sw} mice after the onset of cognitive changes that are detectable by the water maze test (Fig. 1). The PJ-treated APP_{sw} mice learned the CUED (visible platform) task significantly faster than the control APP_{sw} mice as assessed by both swim distance ($p<0.05$) and escape latency ($p<0.02$), although both groups had learned the task equally well by the final day of testing. Swim speed did not differ significantly between the groups on the CUED task. In the SPATIAL (hidden platform) task, PJ mice escaped the maze more quickly than controls ($p<0.02$), and swim distance data revealed a similar, but non-significant, learning curve. The discrepancy in significance between escape latency and swim distance data was partially explained by the overall faster swim speeds of the PJ mice during the SPATIAL trials ($p<0.04$). During the CUED task, both groups swam progressively faster on each day of testing. During the SPATIAL phase, control mice began to swim progressively slower on each test day, whereas PJ mice maintained a relatively constant swim speed across the 15 days. It has been shown that differences in search strategies can underlie differences in water maze performance (Janus, 2004) and that different strategies are associated with different swimming speeds (Brody and Holtzman, 2006). Therefore, "proximity to target", a measure that has been used to indirectly assess water maze strategies (Magnusson, 1998), was analyzed. For each trial, the distance of the mouse to the center of the escape platform was measured every 0.055 s and summed until the trial ended. PJ mice had significantly lower cumulative proximity to target scores ($p<0.05$), suggesting better performance on the SPATIAL task. The groups did not differ on putative measures of spatial memory during the probe trials (i.e., quadrant search time and platform annulus crossings). Both groups exhibited evidence of intact spatial memory, as probe trial search times were above chance levels, and probe trial performance improved across all three probe trials for both groups (Fig. 2). There were no differences between wildtype mice fed PJ or vehicle in CUED or SPATIAL task performance or in swim speed (Fig. 3).

PJ decreased Aβ and amyloid levels in the hippocampus

APP_{sw} mice develop large increases of Aβ in brain tissue at about the time that amyloid plaque deposition begins at 7–8 months of age (Hsiao et al., 1996). Thus, APP_{sw} mice were treated with PJ or control treatment before plaque deposition (6 months of age) and assessed for Aβ levels and plaque load at a time point (12.5 months of age) after which plaques would be expected to be present. APP_{sw} mice treated with PJ had significant reductions in Aβ deposition (53%; $p<0.03$; Fig. 4), thioflavine-S positive fibrillar Aβ (amyloid) deposition (50%; $p<0.008$; Fig. 4) and hippocampal soluble $Aβ_{42}$ (51%; $p<0.004$; Fig. 5) as compared to control mice. Soluble and insoluble $Aβ_{42}$:$Aβ_{40}$ ratios were both significantly reduced in PJ-treated mice ($p<0.04$ and $p<0.009$, respectively). There were similar reductions in the dorsal cortex, although these decreases were not statistically significant. Levels of CAA in the dorsal cortex did not differ between groups. There was a non-significant, but consistent, trend for fewer dystrophic neurites associated with amyloid plaques in the dorsal cortex, hippocampus and corpus callosum. An analysis of amyloid plaque size revealed significantly smaller thioflavine-S positive plaques in the hippocampus, but not the dorsal cortex or corpus callosum, of the PJ group ($p<0.004$; data not shown).

PJ treatment did not affect APP processing

There may be several reasons why Aβ levels and Aβ deposition were lower in PJ-treated mice. One possibility is that PJ treatment resulted in decreased Aβ production via increased α-secretase and/or decreased β-secretase processing of APP. We found that there were no statistically significant differences between the groups in

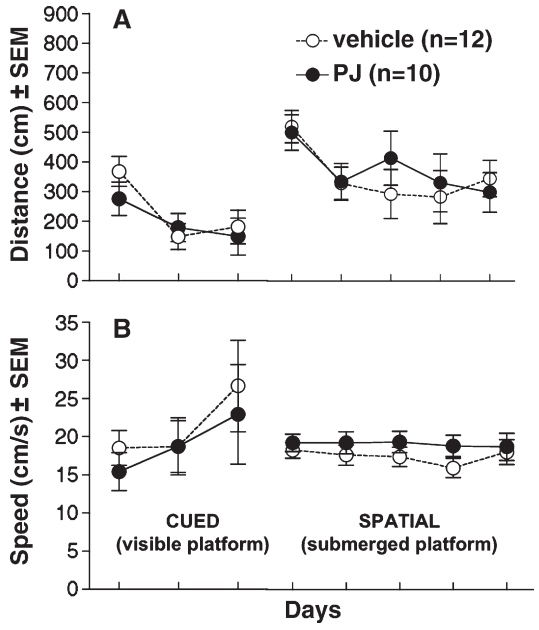

Fig. 3. Wildtype mice water maze performance. There were no differences between wildtype mice fed PJ or vehicle in (A) CUED and SPATIAL task performance, (B) swim speed or probe trial performance (not shown).

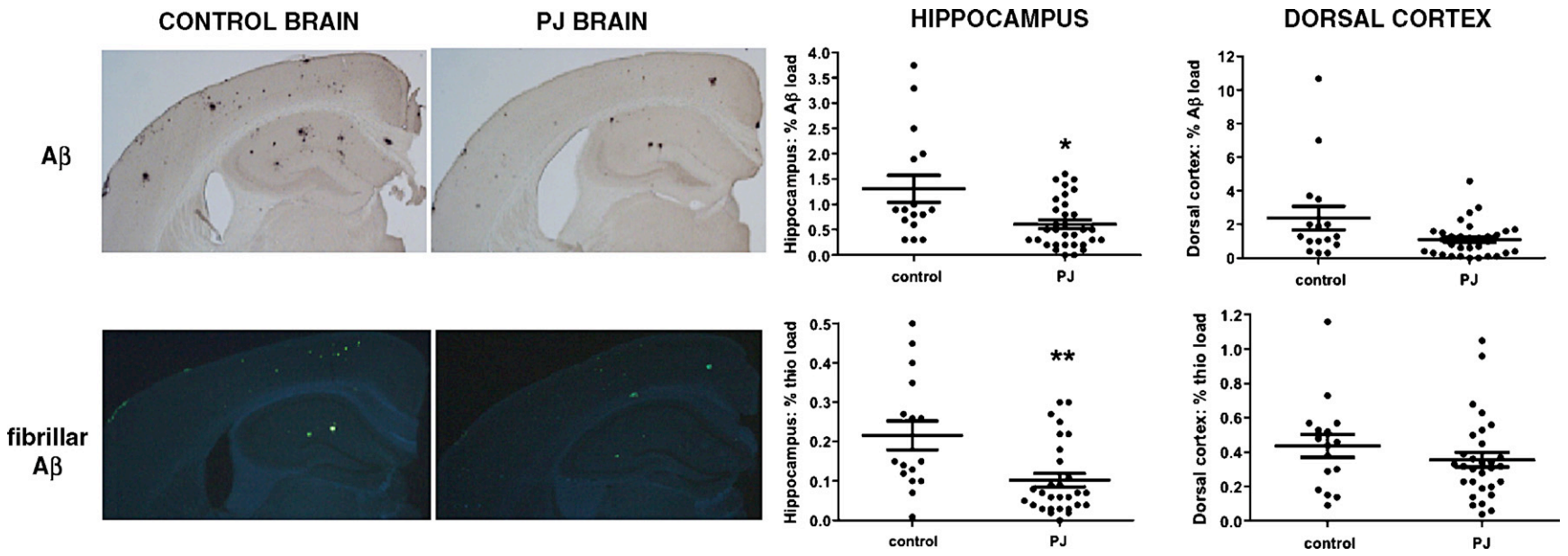

Fig. 4. Brain Aβ load. Representative photomicrographs (2×) of brains from mice treated with control and PJ. Mice treated with PJ had significantly less Aβ (*$p<0.03$) and fibrillar (thioflavine-S+) Aβ (**$p<0.008$) in the hippocampus. There were similar reductions in the dorsal cortex, although these decreases were not statistically significant.

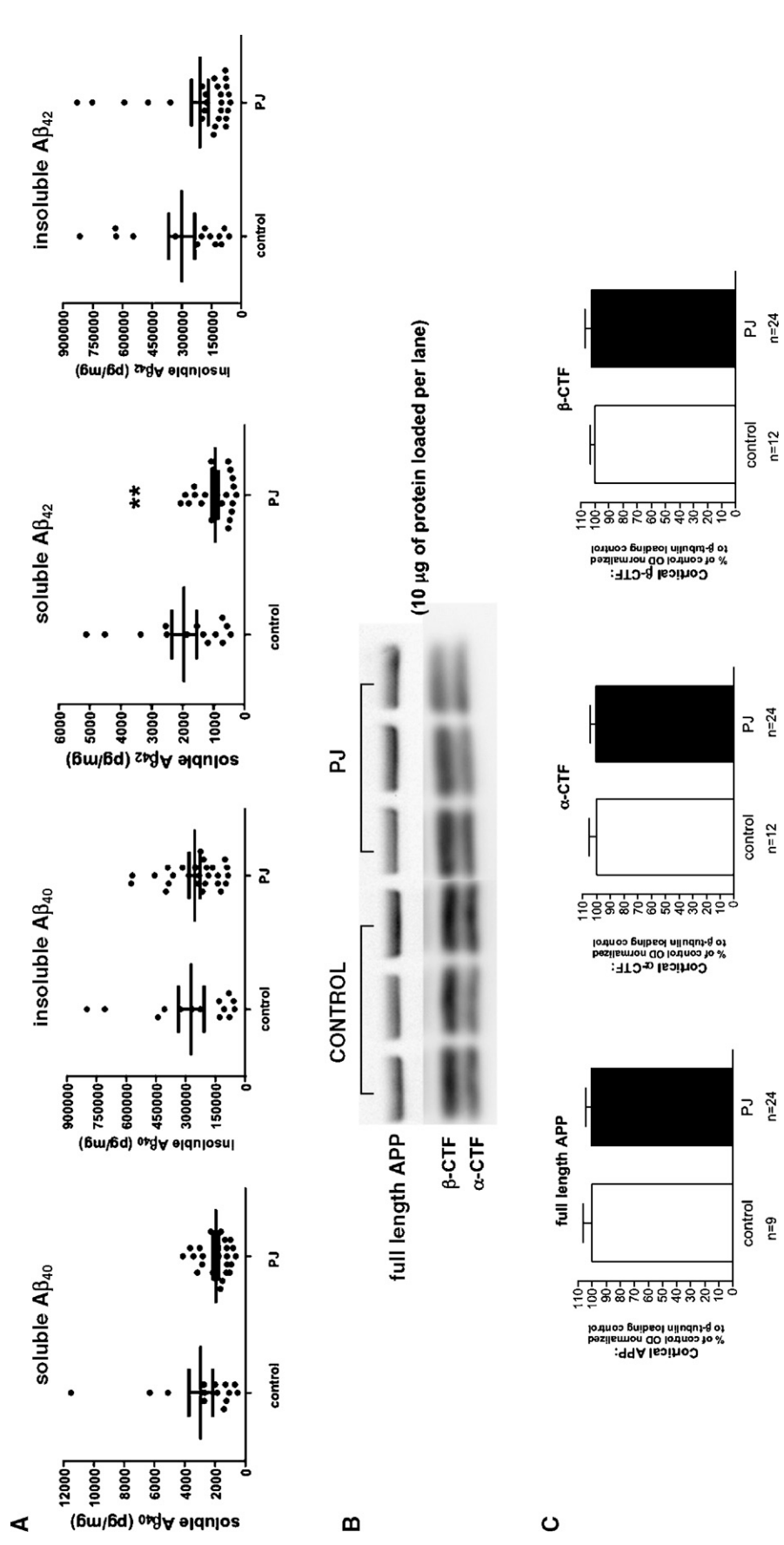

Fig. 5. Aβ and APP biochemistry. (A) Aβ ELISAs. Mice treated with PJ had significantly less hippocampal soluble $A\beta_{42}$ ($^{**}p < 0.004$) as compared to control mice. There were similar reductions in the dorsal cortex, but these decreases were not statistically significant. (B) Representative Western blots. Western blots were performed on 10 µg of total protein from cortical lysates of APP_{sw} mice treated with PJ or control. (C) APP and APP C-terminal fragments. The percent optical density of the Western bands representing APP or APP fragments relative to tubulin is shown.

measurements of full-length APP, α-C terminal fragments (CTFs) or β-CTFs in the cortex (Fig. 5). These data suggest that the mechanism by which PJ influences behavior and amyloid load is not by altering APP processing or Aβ production.

Discussion

In this study, Tg2576/APP_{sw} mice, commonly used as a model of Aβ deposition and associated AD-like pathology, were given pomegranate juice in their drinking water starting at 6 months of age. When their learning behavior was assessed at 1 year of age, PJ-treated mice exhibited improvements on cued and spatial learning tasks as well as faster overall swim speeds. Additionally, plaque load (both Aβ and fibrillar Aβ/amyloid) and soluble $Aβ_{42}$ were significantly reduced in the hippocampus. These results strongly suggest that pomegranates and/or their constituent substances should be further explored as potentially useful therapeutic agents.

PJ-treated APP_{sw} mice learned both the cued and spatial components of the water maze more quickly than control mice. The superior performance of the PJ mice on the cued learning task suggests that there were some PJ-induced differences between the groups in general learning ability, swimming ability, visual acuity or motivation to escape the water, but these differences had dissipated by 20 trials over 5 days of CUED testing. Although the PJ mice found the platform more quickly during the SPATIAL trials, they were also swimming more quickly, which confounds interpretation of these data. A similar trend was seen for swim distance, a measure often used to control for the effect of swim speed on water maze performance. Swim distance, however, is not immune to the effects of swim speed. Given two trials in which the mice did not find the platform by the 60-second cut-off, the slower swimming mouse would actually swim a shorter distance for that trial. Even though both mice had performed equally poorly, the swim distance data would give the appearance of better performance to the slower mouse. The "cumulative proximity to target" measure is correlated with both escape latency and swim distance, but also partially controls for the search strategy used by the mouse. For example, consider two trials in which neither mouse found the platform, but one was searching in general vicinity of the platform, and the other was simply swimming around the perimeter of the pool. Even if they both swam the same total distance, the cumulative proximity score of the mouse that was searching the correct area would be smaller, indicating better performance. The fact that PJ-treated APP_{sw} mice exhibited better performance in the SPATIAL task as assessed by the proximity score, in combination with the non-significant trend for better swim distance performance and the significant difference in swim speed, suggests that PJ-treated mice may have used more efficient search strategies than control mice. Performance on the probe trials, often reported as a putative measure of spatial memory, did not differ significantly between the groups. Both groups performed well above chance on all three probe trials, suggesting that PJ may have more of an effect on learning processes than on the facilitation of memory. Regardless of the effects of pomegranate juice on spatial learning, the effects on overall swim speed and performance during the CUED phase of testing were profound. We also assessed the effects of PJ versus vehicle in 3- to 5-month-old wildtype mice treated for 5 weeks. There was no difference in water maze performance between the PJ and vehicle-treated mice. This suggests that the effects of PJ in APP_{sw} mice may be acting to slow or prevent a disease process. However, an experiment treating wildtype mice from 6 to 12.5 months of age would be required to make this conclusion.

PJ also reduced levels of soluble $Aβ_{42}$, Aβ deposition and fibrillar Aβ/amyloid deposition in the hippocampi of APP_{sw} mice. Other foods containing high levels of antioxidants and polyphenols have also been shown to reduce plaque deposition in APP_{sw} mice. For example, both the essential fatty acid DHA (Calon et al., 2004; Lim et al., 2005) and chronic administration of the dietary antioxidant vitamin E (Morris et al., 2005) reduced plaque deposition in aged APP_{sw} mice. Curcumin, a polyphenol found in the curry spice turmeric, was also shown to lower levels of oxidized proteins and plaque burden in APP_{sw} mice (Lim et al., 2001). Another polyphenol found in green tea, epigallocatechin-3-gallate (EGCG), was recently shown to reduce production of Aβ and elevate levels of α-CTFs *in vitro* and to decrease levels of Aβ and plaque deposition in the brains of APP_{sw} mice (Rezai-Zadeh et al., 2005). Tannic acid, also found in tea, has been shown to inhibit amyloid fibril formation *in vitro* (Ono et al., 2004). Interestingly, resveratrol, a polyphenol found in grapes and red wine, was shown to decrease levels of Aβ *in vitro* by increasing clearance, rather than inhibiting production, of Aβ (Marambaud et al., 2005). Thus, it appears that various naturally occurring polyphenols can decrease levels of Aβ and Aβ deposition in the brains of APP transgenic mice, possibly by inhibiting production, enhancing clearance or altering Aβ fibrillogenesis. Decreasing levels of Aβ may also ameliorate the tau pathology observed in the AD brain (Oddo et al., 2004). Recent data suggest that extracts from pomegranate husk contain β-secretase inhibitors (Kwak et al., 2005). In our experiments, analyses of APP CTFs suggest that PJ did not affect β-secretase cleavage of APP in the cortex. However, the pomegranate juice that we used did not contain material from the husk. It will be interesting for future studies to characterize the effects of pomegranate juice and husk on Aβ and APP processing, as well as its effects on production, clearance and fibrillogenesis in the brains of younger APP transgenic mice, both before and during the process of Aβ deposition. Whether PJ would decrease the amount of Aβ deposition, gliosis and neuritic dystrophy in older APP_{sw} mice that already have abundant pathology will be of interest. If the effects of PJ are on Aβ fibrillogenesis or clearance, one might expect an effect since Aβ deposition continues over time even in old APP_{sw} mice. In addition to effects on Aβ, it will be useful to determine if PJ affects other process such as inflammation and reactive oxygen species. Such studies will assist in understanding the mechanism of the effects of PJ.

Several other lines of research have shown beneficial effects of PJ intake on both humans and animal models of disease. A recent paper from our laboratory showed that dietary supplementation of pregnant mice with PJ resulted in significantly less brain damage to postnatal day 7 pups that were exposed to hypoxia–ischemia (Loren et al., 2005). Aβ is presumably not involved in the mechanism of protection in this neonatal model. This suggests that although the beneficial behavioral effects that we observed in adult APP_{sw} mice may be due to an effect on Aβ, they may also be due to other mechanisms. Because recent evidence suggests that exercise may also reduce plaque load in a mouse model of AD (Adlard et al., 2005), it is interesting to speculate about whether increased swim speed in the PJ mice affected levels of Aβ in the brain.

Pomegranate juice has been shown to have a variety of actions in disease models, including reduced low-density lipoprotein (LDL) aggregation, oxidative stress (Aviram et al., 2000), serum angio-

tensin converting enzyme (ACE) and systolic blood pressure in hypertensive patients (Aviram and Dornfeld, 2001), and reduced LDL oxidation, atherosclerosis and systolic blood pressure in patients with carotid artery stenosis (Aviram et al., 2004). Numerous studies have found similar results in animals. PJ was found to reduce LDL aggregation and oxidative stress in apoE−/− mice (Aviram et al., 2000), and other studies have shown that PJ reduced atherosclerosis in hypercholesterolimic apoE−/− mice (Kaplan et al., 2001) and LDLR−/− mice (de Nigris et al., 2005) and improved cardiac lipid metabolism in a rat model of diabetes (Huang et al., 2005). It is possible that some of the vascular effects of PJ are via its effects on nitric oxide synthase in the vasculature (de Nigris et al., 2005). Perhaps surprisingly, levels of CAA were not affected by pomegranate consumption in this study. However, given the potential roles of cholesterol (Wellington, 2004) and vascular amyloidosis (Sadowski et al., 2004) in AD, these lines of research should be more fully explored. Pomegranates may have anti-cancer properties as well as anti-atherogenic properties (Albrecht et al., 2004; Burton, 2003; Kohno et al., 2004; Longtin, 2003; Mehta and Lansky, 2004), and it is likely that several polyphenols act synergistically to prevent tumor growth and contribute to its beneficial anti-atherogenic effects (Seeram et al., 2005). Interestingly, pomegranates also contain phytoestrogens, and PJ has improved depressed behavior and bone properties in an ovariectomized mouse model of menopause (Mori-Okamoto et al., 2004). The vast number of compounds in PJ, along with the evidence that these compounds may act together in a synergistic fashion, suggests that isolated components of pomegranate may not be as effective as dietary supplementation with either the whole fruit or its juice.

This study is the first to show beneficial effects (both behavioral and neuropathological) of pomegranate juice in an animal model of AD. These data suggest that further studies to validate and determine the mechanism of these effects, as well as whether PJ can protect against AD in humans, may be warranted.

Acknowledgment

This study was supported by funds from the Stewart and Lynda Resnick Revocable Trust. The Resnick Trust/Foundation did not participate in the design, implementation, analysis or interpretation of this study.

References

Adlard, P.A., Perreau, V.M., Pop, V., Cotman, C.W., 2005. Voluntary exercise decreases amyloid load in a transgenic model of Alzheimer's disease. J. Neurosci. 25, 4217–4221.

Aggarwal, B.B., Shishodia, S., 2004. Suppression of the nuclear factor-kappaB activation pathway by spice-derived phytochemicals: reasoning for seasoning. Ann. N.Y. Acad. Sci. 1030, 434–441.

Albrecht, M., Jiang, W., Kumi-Diaka, J., Lansky, E.P., Gommersall, L.M., Patel, A., Mansel, R.E., Neeman, I., Geldof, A.A., Campbell, M.J., 2004. Pomegranate extracts potently suppress proliferation, xenograft growth, and invasion of human prostate cancer cells. J. Med. Food 7, 274–283.

Aviram, M., Dornfeld, L., 2001. Pomegranate juice consumption inhibits serum angiotensin converting enzyme activity and reduces systolic blood pressure. Atherosclerosis 158, 195–198.

Aviram, M., Dornfeld, L., Rosenblat, M., Volkova, N., Kaplan, M., Coleman, R., Hayek, T., Presser, D., Fuhrman, B., 2000. Pomegranate juice consumption reduces oxidative stress, atherogenic modifications to LDL, and platelet aggregation: studies in humans and in atherosclerotic apolipoprotein E-deficient mice. Am. J. Clin. Nutr. 71, 1062–1076.

Aviram, M., Rosenblat, M., Gaitini, D., Nitecki, S., Hoffman, A., Dornfeld, L., Volkova, N., Presser, D., Attias, J., Liker, H., Hayek, T., 2004. Pomegranate juice consumption for 3 years by patients with carotid artery stenosis reduces common carotid intima-media thickness, blood pressure and LDL oxidation. Clin. Nutr. 23, 423–433.

Bardgett, M.E., Schultheis, P.J., McGill, D.L., Richmond, R.E., Wagge, J.R., 2005. Magnesium deficiency impairs fear conditioning in mice. Brain Res. 1038, 100–106.

Brody, D.L., Holtzman, D.M., 2006. Morris water maze search strategy analysis in PDAPP mice before and after experimental traumatic brain injury. Exp. Neurol. 197, 330–340.

Burton, A., 2003. Chemoprevention: eat ginger, rub on pomegranate. Lancet Oncol. 4, 715.

Calon, F., Lim, G.P., Yang, F., Morihara, T., Teter, B., Ubeda, O., Rostaing, P., Triller, A., Salem Jr., N., Ashe, K.H., Frautschy, S.A., Cole, G.M., 2004. Docosahexaenoic acid protects from dendritic pathology in an Alzheimer's disease mouse model. Neuron 43, 633–645.

DeMattos, R.B., O'Dell, M.A., Parsadanian, M., Taylor, J.W., Harmony, J.A., Bales, K.R., Paul, S.M., Aronow, B.J., Holtzman, D.M., 2002. Clusterin promotes amyloid plaque formation and is critical for neuritic toxicity in a mouse model of Alzheimer's disease. Proc. Natl. Acad. Sci. U. S. A. 99, 10843–10848.

de Nigris, F., Williams-Ignarro, S., Lerman, L.O., Crimi, E., Botti, C., Mansueto, G., D'Armiento, F.P., De Rosa, G., Sica, V., Ignarro, L.J., Napoli, C., 2005. Beneficial effects of pomegranate juice on oxidation-sensitive genes and endothelial nitric oxide synthase activity at sites of perturbed shear stress. Proc. Natl. Acad. Sci. U. S. A. 102, 4896–4901.

Fryer, J.D., Simmons, K., Parsadanian, M., Bales, K.R., Paul, S.M., Sullivan, P.M., Holtzman, D.M., 2005. Human apolipoprotein E4 alters the amyloid-beta 40:42 ratio and promotes the formation of cerebral amyloid angiopathy in an amyloid precursor protein transgenic model. J. Neurosci. 25, 2803–2810.

Grant, W.B., 2000. Fish consumption, cancer, and Alzheimer disease. Am. J. Clin. Nutr. 71, 599–600.

Grant, W.B., 2003. Diet and risk of dementia: does fat matter? The Rotterdam study. Neurology 60, 2020–2021.

Hartman, R.E., Izumi, Y., Bales, K.R., Paul, S.M., Wozniak, D.F., Holtzman, D.M., 2005. Treatment with an amyloid-beta antibody ameliorates plaque load, learning deficits, and hippocampal long-term potentiation in a mouse model of Alzheimer's disease. J. Neurosci. 25, 6213–6220.

Hashimoto, M., Hossain, S., Shimada, T., Sugioka, K., Yamasaki, H., Fujii, Y., Ishibashi, Y., Oka, J., Shido, O., 2002. Docosahexaenoic acid provides protection from impairment of learning ability in Alzheimer's disease model rats. J. Neurochem. 81, 1084–1091.

Hashimoto, M., Tanabe, Y., Fujii, Y., Kikuta, T., Shibata, H., Shido, O., 2005. Chronic administration of docosahexaenoic acid ameliorates the impairment of spatial cognition learning ability in amyloid beta-infused rats. J. Nutr. 135, 549–555.

Henderson, S.T., 2004. High carbohydrate diets and Alzheimer's disease. Med. Hypotheses 62, 689–700.

Heo, H.J., Lee, C.Y., 2004. Protective effects of quercetin and vitamin C against oxidative stress-induced neurodegeneration. J. Agric. Food Chem. 52, 7514–7517.

Horrocks, L.A., Yeo, Y.K., 1999. Health benefits of docosahexaenoic acid (DHA). Pharmacol. Res. 40, 211–225.

Hsiao, K., Chapman, P., Nilsen, S., Eckman, C., Harigaya, Y., Younkin, S., Yang, F., Cole, G., 1996. Correlative memory deficits, Abeta elevation, and amyloid plaques in transgenic mice. Science 274, 99–102.

Huang, T.H., Peng, G., Kota, B.P., Li, G.Q., Yamahara, J., Roufogalis, B.D., Li, Y., 2005. Pomegranate flower improves cardiac lipid metabolism in a diabetic rat model: role of lowering circulating lipids. Br. J. Pharmacol. 145 (6) (Jul.), 767–774.

Janus, C., 2004. Search strategies used by APP transgenic mice during navigation in the Morris water maze. Learn. Mem. 11, 337–346.

Joseph, J.A., Denisova, N.A., Arendash, G., Gordon, M., Diamond, D., Shukitt-Hale, B., Morgan, D., 2003. Blueberry supplementation

enhances signaling and prevents behavioral deficits in an Alzheimer disease model. Nutr. Neurosci. 6, 153–162.
Joseph, J.A., Shukitt-Hale, B., Casadesus, G., 2005. Reversing the deleterious effects of aging on neuronal communication and behavior: beneficial properties of fruit polyphenolic compounds. Am. J. Clin. Nutr. 81, 313S–316S.
Kaplan, M., Hayek, T., Raz, A., Coleman, R., Dornfeld, L., Vaya, J., Aviram, M., 2001. Pomegranate juice supplementation to atherosclerotic mice reduces macrophage lipid peroxidation, cellular cholesterol accumulation and development of atherosclerosis. J. Nutr. 131, 2082–2089.
Kelawala, N.S., Ananthanarayan, L., 2004. Antioxidant activity of selected foodstuffs. Int. J. Food Sci. Nutr. 55, 511–516.
Kohno, H., Suzuki, R., Yasui, Y., Hosokawa, M., Miyashita, K., Tanaka, T., 2004. Pomegranate seed oil rich in conjugated linolenic acid suppresses chemically induced colon carcinogenesis in rats. Cancer Sci. 95, 481–486.
Kostrzewa, R.M., Segura-Aguilar, J., 2003. Novel mechanisms and approaches in the study of neurodegeneration and neuroprotection. a review. Neurotox. Res. 5, 375–383.
Kwak, H.M., Jeon, S.Y., Sohng, B.H., Kim, J.G., Lee, J.M., Lee, K.B., Jeong, H.H., Hur, J.M., Kang, Y.H., Song, K.S., 2005. beta-Secretase (BACE1) inhibitors from pomegranate (*Punica granatum*) husk. Arch. Pharm. Res. 28, 1328–1332.
Lim, G.P., Chu, T., Yang, F., Beech, W., Frautschy, S.A., Cole, G.M., 2001. The curry spice curcumin reduces oxidative damage and amyloid pathology in an Alzheimer transgenic mouse. J. Neurosci. 21, 8370–8377.
Lim, G.P., Calon, F., Morihara, T., Yang, F., Teter, B., Ubeda, O., Salem Jr., N., Frautschy, S.A., Cole, G.M., 2005. A diet enriched with the omega-3 fatty acid docosahexaenoic acid reduces amyloid burden in an aged Alzheimer mouse model. J. Neurosci. 25, 3032–3040.
Longtin, R., 2003. The pomegranate: nature's power fruit? J. Natl. Cancer Inst. 95, 346–348.
Loren, D.J., Seeram, N.P., Schulman, R.N., Holtzman, D.M., 2005. Maternal dietary supplementation with pomegranate juice is neuroprotective in an animal model of neonatal hypoxic–ischemic brain injury. Pediatr. Res. 57, 858–864.
Love, R., 2005. Calorie restriction may be neuroprotective in AD and PD. Lancet Neurol. 4, 84.
Magnusson, K.R., 1998. Aging of glutamate receptors: correlations between binding and spatial memory performance in mice. Mech. Ageing Dev. 104, 227–248.
Marambaud, P., Zhao, H., Davies, P., 2005. Resveratrol promotes clearance of Alzheimer's disease amyloid-beta peptides. J. Biol. Chem. 280 (45) (Nov. 11), 37377–37382.
Mattson, M.P., 2000. Existing data suggest that Alzheimer's disease is preventable. Ann. N.Y. Acad. Sci. 924, 153–159.
Mehta, R., Lansky, E.P., 2004. Breast cancer chemopreventive properties of pomegranate (*Punica granatum*) fruit extracts in a mouse mammary organ culture. Eur. J. Cancer Prev. 13, 345–348.
Mori-Okamoto, J., Otawara-Hamamoto, Y., Yamato, H., Yoshimura, H., 2004. Pomegranate extract improves a depressive state and bone properties in menopausal syndrome model ovariectomized mice. Eur. J. Cancer Prev. 92, 93–101.
Morris, M.C., Evans, D.A., Bienias, J.L., Scherr, P.A., Tangney, C.C., Hebert, L.E., Bennett, D.A., Wilson, R.S., Aggarwal, N., 2004. Dietary niacin and the risk of incident Alzheimer's disease and of cognitive decline. J. Neurol. Neurosurg. Psychiatry 75, 1093–1099.
Morris, M.C., Evans, D.A., Tangney, C.C., Bienias, J.L., Wilson, R.S., Aggarwal, N.T., Scherr, P.A., 2005. Relation of the tocopherol forms to incident Alzheimer disease and to cognitive change. Am. J. Clin. Nutr. 81, 508–514.
Newman, P.E., 1992. Could diet be one of the causal factors of Alzheimer's disease? Med. Hypotheses 39, 123–126.
Oddo, S., Billings, L., Kesslak, J.P., Cribbs, D.H., LaFerla, F.M., 2004. Abeta immunotherapy leads to clearance of early, but not late, hyperphosphorylated tau aggregates via the proteasome. Neuron 43, 321–332.
Ono, K., Hasegawa, K., Naiki, H., Yamada, M., 2004. Anti-amyloidogenic activity of tannic acid and its activity to destabilize Alzheimer's beta-amyloid fibrils in vitro. Biochim. Biophys. Acta 1690, 193–202.
Patel, N.V., Gordon, M.N., Connor, K.E., Good, R.A., Engelman, R.W., Mason, J., Morgan, D.G., Morgan, T.E., Finch, C.E., 2005. Caloric restriction attenuates Abeta-deposition in Alzheimer transgenic models. Neurobiol. Aging 26, 995–1000.
Peers, R.J., 1990. Alzheimer's disease and omega-3 fatty acids: hypothesis. Med. J. Aust. 153, 563–564.
Polidori, M.C., 2003. Antioxidant micronutrients in the prevention of age-related diseases. J. Postgrad. Med. 49, 229–235.
Pope, S.K., Shue, V.M., Beck, C., 2003. Will a healthy lifestyle help prevent Alzheimer's disease? Annu. Rev. Public Health 24, 111–132.
Refolo, L.M., Malester, B., LaFrancois, J., Bryant-Thomas, T., Wang, R., Tint, G.S., Sambamurti, K., Duff, K., Pappolla, M.A., 2000. Hypercholesterolemia accelerates the Alzheimer's amyloid pathology in a transgenic mouse model. Neurobiol. Dis. 7, 321–331.
Rezai-Zadeh, K., Shytle, D., Sun, N., Mori, T., Hou, H., Jeanniton, D., Ehrhart, J., Townsend, K., Zeng, J., Morgan, D., Hardy, J., Town, T., Tan, J., 2005. Green tea epigallocatechin-3-gallate (EGCG) modulates amyloid precursor protein cleavage and reduces cerebral amyloidosis in Alzheimer transgenic mice. J. Neurosci. 25, 8807–8814.
Roberts, N.B., Clough, A., Bellia, J.P., Kim, J.Y., 1998. Increased absorption of aluminium from a normal dietary intake in dementia. J. Inorg. Biochem. 69, 171–176.
Rogers, M.A., Simon, D.G., 1999. A preliminary study of dietary aluminium intake and risk of Alzheimer's disease. Age Ageing 28, 205–209.
Sadowski, M., Pankiewicz, J., Scholtzova, H., Li, Y.S., Quartermain, D., Duff, K., Wisniewski, T., 2004. Links between the pathology of Alzheimer's disease and vascular dementia. Neurochem. Res. 29, 1257–1266.
Seeram, N.P., Adams, L.S., Henning, S.M., Niu, Y., Zhang, Y., Nair, M.G., Heber, D., 2005. In vitro antiproliferative, apoptotic and antioxidant activities of punicalagin, ellagic acid and a total pomegranate tannin extract are enhanced in combination with other polyphenols as found in pomegranate juice. J. Nutr. Biochem. 16, 360–367.
Sung, S., Yao, Y., Uryu, K., Yang, H., Lee, V.M., Trojanowski, J.Q., Pratico, D., 2004. Early vitamin E supplementation in young but not aged mice reduces Abeta levels and amyloid deposition in a transgenic model of Alzheimer's disease. FASEB J. 18, 323–325.
Wahrle, S.E., Jiang, H., Parsadanian, M., Hartman, R.E., Bales, K.R., Paul, S.M., Holtzman, D.M., 2005. Deletion of Abca1 increases Abeta deposition in the PDAPP transgenic mouse model of Alzheimer's disease. J. Biol. Chem. 280 (52) (Dec. 30), 43236–43242.
Wang, R.F., Xie, W.D., Zhang, Z., Xing, D.M., Ding, Y., Wang, W., Ma, C., Du, L.J., 2004. Bioactive compounds from the seeds of *Punica granatum* (pomegranate). J. Nat. Prod. 67, 2096–2098.
Wang, J., Ho, L., Qin, W., Rocher, A.B., Seror, I., Humala, N., Maniar, K., Dolios, G., Wang, R., Hof, P.R., Pasinetti, G.M., 2005. Caloric restriction attenuates beta-amyloid neuropathology in a mouse model of Alzheimer's disease. FASEB J. 19, 659–661.
Weinreb, O., Mandel, S., Amit, T., Youdim, M.B., 2004. Neurological mechanisms of green tea polyphenols in Alzheimer's and Parkinson's diseases. J. Nutr. Biochem. 15, 506–516.
Wellington, C.L., 2004. Cholesterol at the crossroads: Alzheimer's disease and lipid metabolism. Clin. Genet. 66, 1–16.
Wu, C.W., Liao, P.C., Lin, C., Kuo, C.J., Chen, S.T., Chen, H.I., Kuo, Y.M., 2003. Brain region-dependent increases in beta-amyloid and apolipoprotein E levels in hypercholesterolemic rabbits. J. Neural Transm. 110, 641–649.
Xu, J., Guo, C.J., Yang, J.J., Wei, J.Y., Li, Y.F., Pang, W., Jiang, Y.G., Cheng, S., 2005. Intervention of antioxidant system function of aged rats by giving fruit juices with different antioxidant capacities. Zhonghua Yu Fang Yi Xue Za Zhi 39, 80–83.