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Delayed Amyloid Plaque Deposition and Behavioral Deficits in Outcrossed A β PP/PS1 Mice

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Abstract

Alzheimer's disease (AD) is a progressive neurodegenerative dementia characterized by amyloid plaque accumulation, synapse/dendrite loss, and cognitive impairment. Transgenic mice expressing mutant forms of amyloid- β precursor protein (A β PP) and presenilin-1 (PS1) recapitulate several aspects of this disease and provide a useful model system for studying elements of AD progression. A β PP/PS1 mice have been previously shown to exhibit behavioral deficits and amyloid plaque deposition between 4–9 months of age. We crossed A β PP/PS1 animals with mice of a mixed genetic background (C57BL/6 $\times$ 129/SvJ) and investigated the development of AD-like features in the resulting outcrossed mice. The onset of memory-based behavioral impairment is delayed considerably in outcrossed A β PP/PS1 mice relative to inbred mice on a C57BL/6 background. While inbred A β PP/PS1 mice develop deficits in radial-arm water maze performance and novel object recognition as early as 8 months, outcrossed A β PP/PS1 mice do not display defects until 18 months. Within the forebrain, we find that inbred A β PP/PS1 mice have significantly higher amyloid plaque burden at 12 months than outcrossed A β PP/PS1 mice of the same age. Surprisingly, inbred A β PP/PS1 mice at 8 months have low plaque burden suggesting that plaque burden alone cannot explain the accompanying behavioral deficits. Analysis of A β PP processing revealed that elevated levels of soluble A β correlate with the degree of behavioral impairment in both strains. Taken together, these findings suggest that animal behavior, amyloid plaque deposition, and A β PP processing are sensitive to genetic differences between mouse strains.

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Conflict of Interest Statement

There are no known conflicts of interest that would have inappropriately influenced this work.

Role of Authors

All authors had full access to the data in this study and take responsibility for the integrity of the data and accuracy of the data analysis. Study concept and design: BAC, MEK, & AJK. Acquisition of data: BAC, MEK, & ACK. Analysis and interpretation of data: BAC & MEK. Drafting of manuscript: BAC & MEK. Critical revision of manuscript for important intellectual content: BAC, MEK, ACK, HBN, SMS, & AJK. Statistical analysis: BAC & MEK. Obtained funding: SMS & AJK. Administrative, technical and material support: HBN, SMS, & AJK. Study supervision: AJK.

Keywords

amyloid- β precursor protein; hippocampus; novel object recognition; presenilin-1; radial-arm water maze; strain background; Alzheimer's disease

Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disease and the most common cause of dementia among the elderly. AD is characterized by aberrant production of amyloid- β (A β) peptide and accumulation of A β -containing amyloid plaques in the brains of affected individuals. AD patients exhibit distinct cognitive deficits, including memory loss, poor judgment, and compromised spatial awareness. These cognitive impairments likely result from underlying processes that occur during AD progression, including disruption of synaptic connectivity (Coleman et al., 2004; Lassmann et al., 1993), alteration of dendrite morphology (Anderton et al., 1998), and degeneration of brain structures (McEvoy et al., 2009; Uylings and de Brabander, 2002).

Structural degeneration of the hippocampus and cortex occurs early in the course of AD, and progressive impairment of these structures accompanies cognitive decline in this disease (Hempel et al., 2008). During AD, amyloid plaques form throughout the forebrain, becoming particularly abundant within the dentate gyrus molecular layer and temporal cortex (Braak and Braak, 1991; Hellström-Lindahl et al., 2009). The forebrain is also a major site of synapse loss and dendrite atrophy in AD, suggesting that synaptic connectivity is altered within this region (Flood et al., 1987; Hanks and Flood, 1991; Scheff et al., 2007; Scheff et al., 1996). Furthermore, hippocampal volume loss and cortical thinning both correlate with memory impairment in AD (Du et al., 2007; Van Petten, 2004).

Mutations in the genes encoding amyloid- β precursor protein (A β PP) or proteins involved in A β PP processing, such as presenilin-1 (PS1) and presenilin-2 (PS2), have been implicated as causal factors in familial AD (Bertram and Tanzi, 2008). The development of transgenic AD model mice based on these mutations has greatly facilitated research into the molecular mechanisms of AD (Price et al., 1998; Ryman et al., 2006). While expression of one mutant transgene reproduces several aspects of AD, co-expression of multiple transgenes has been widely used as a strategy to boost A β production and accelerate the onset of AD-like pathology in mice (Borchelt et al., 1997; Citron et al., 1997; Holcomb et al., 1998; Van Dorpe et al., 2000). Double transgenic A β PP/PS1 mice exhibit extensive amyloid plaque formation by 6 months that further increases with age (Garcia-Alloza et al., 2006; Jankowsky et al., 2004). A β PP/PS1 mice display poor performance in spatial learning tasks by 7 months with radial-arm water maze deficits arising as early as 4 months (Park et al., 2006; Reiserer et al., 2007). A β PP/PS1 mice show deficiencies in novel object recognition by 9 months (McClean et al., 2011). Dramatic reductions in brain weights and survival rates have also been observed for A β PP/PS1 mice (Delatour et al., 2006; Gimbel et al., 2010; Halford and Russell, 2009; Pugh et al., 2007).

As part of longer-term genetic studies to investigate the downstream effects of A β overproduction, we crossed A β PP/PS1 mice with wild type (WT) mice of a mixed genetic background (C57BL/6 $\times$ 129/SvJ) and investigated the development of AD-like features in the resulting mice. We found that the onset of a behavioral deficit in radial-arm water maze occurred between 16 and 18 months for outcrossed A β PP/PS1 mice, and we did not detect a defect in novel object recognition at 18 months for these mice. Strikingly, inbred C57BL/6 A β PP/PS1 mice have defects in both tasks as early as 8 months. Additionally, we found that amyloid plaque deposition was sparse at 12 months in outcrossed A β PP/PS1 mice, but

increased significantly by 18 months in regions of the hippocampus and cortex. Inbred C57BL/6 A β PP/PS1 mice have significantly higher plaque deposition at 12 months than outcrossed A β PP/PS1 mice at this same age, but have low plaque levels at 8 months. Importantly, we found that the degree of behavioral impairment coincides with increases in levels of soluble A β levels in both strains. Together, these results suggest that AD-like behavioral impairment and pathology develop considerably later in the outcrossed A β PP/PS1 transgenic mice than in A β PP/PS1 transgenic mice on an inbred C57BL/6 genetic background.

Materials and Methods

Animal use and general procedures

A β PP/PS1 mice (A β PP^{swe}, PS1-1 Δ E9) of mixed genetic background (C57BL/6 $\times$ C3H/HeJ) (Jankowsky et al., 2001) were bred with WT mice of a different mixed genetic background (C57BL/6 $\times$ 129/SvJ) (Koleske et al., 1998). The resulting outcrossed mice were propagated by inbreeding non-sibling pairs of WT and hemizygous A β PP/PS1 mice. A β PP/PS1 mice of inbred genetic background (C57BL/6) were generated by extensively backcrossing (>10 generations) A β PP/PS1 mice of mixed genetic background (C57BL/6 $\times$ C3H/HeJ) onto the C57BL/6 background, as described previously (Gimbel et al., 2010). The C57BL/6 transgenic line was maintained on this background separate from outcrossed A β PP/PS1 mice. A β PP/PS1 animals used in these studies had copies of both the A β PP and PS1 transgenes, and WT littermates were used as controls for all experiments. Animal genotypes were determined using a PCR reaction detecting both transgenes, and genotypes were confirmed at death. Both male and female animals were used for experiments.

Experiments comparing WT and A β PP/PS1 mice were conducted and scored by an experimenter blinded to animal genotype. For behavioral experiments, mice were handled 5 min each for 5 days prior to the experiment to habituate them to the tester and testing room. For experiments requiring heavy sedation, animals were administered Nembutal via intraperitoneal injection prior to the experiment. All procedures were compliant with federal regulations and approved by the Yale University Animal Care and Use Committee. Data from all experiments were analyzed using Prism software (GraphPad, San Diego, CA).

Amyloid plaque deposition

Sedated animals were transcardially perfused with 0.1M PBS pH 7.4, followed by 4% paraformaldehyde/PBS. Excised brains were postfixed in 4% paraformaldehyde/PBS overnight at 4°C, then cryoprotected in 30% sucrose/PBS overnight at 4°C. Horizontal sections (50 μ m) were cut on a sliding-freezing microtome, mounted on gelatin-coated slides, and dehydrated overnight at room temperature. Dried sections were stained for amyloid plaques with fresh, filtered 1% Thioflavin S (T1892; Sigma) as follows: ddH₂O, 2 $\times$ 2 min; 1% Thioflavin S, 60 min; 70% ethanol, 5 min; ddH₂O, 2 $\times$ 2 min. Coverslips were mounted with anti-fade medium (2% n-propyl gallate, 80% glycerol/PBS), and phase and green fluorescent images of the hippocampus were acquired on a Nikon TE2000-S microscope with NIS-Elements software (Nikon) at 4 $\times$ magnification. Images were stored as NIS-Elements files (.nd2) and analyzed for plaque deposition as described below. Representative images shown in Figures 3 and 4 were obtained using NIS-Elements software to crop, view scale bar, convert green fluorescent images to black-and-white, and increase contrast slightly for better representation. Images shown for each strain and age were obtained using identical manipulations in NIS-Elements.

Amyloid plaque deposition was quantified using ImageJ software (NIH). TIFF images were converted to 8-bit grayscale, hippocampal and cortical subregions (as defined by (Franklin

and Paxinos, 2008)) were demarcated on the phase images, and subregion boundaries were applied to the fluorescent images. Each subregion was processed with a custom macro that measured the subregion area, inverted the grayscale spectrum, applied a thresholding function, and employed particle analysis to select and measure amyloid plaque areas. Within the hippocampus, the CA1 stratum radiatum, CA1 stratum oriens, CA3 stratum radiatum, CA3 stratum oriens, and dentate gyrus molecular layer subregions were quantified independently and plaque burden values were combined to obtain a weighted average for overall hippocampal plaque burden. Mean values for amyloid plaque burden was calculated for each mouse and analyzed by Kruskal-Wallis tests, followed by Mann-Whitney post-tests for significance.

Radial-arm water maze

The radial-arm water maze testing protocol was based on (Alamed et al., 2006), including modifications from (Park et al., 2006). The maze consisted of an inflatable pool 1.2 m in diameter with six 18 cm wide swim alleys radiating from a 35 cm wide central area. Spatial cues 60 cm tall were placed on the walls of the room adjacent to the end of each arm. A hidden escape platform was submerged at the end of the goal arm, which remained constant for each mouse across trials. The main task consisted of six learning blocks of five trials spread equally over two consecutive days. For each trial, the animal was placed into a start arm and allowed to swim to the escape platform. Start arms were assigned semi-randomly so that all five non-goal arms were represented during each learning block. After entering an incorrect arm or failing to select an arm for 1 min, the mouse was returned to the start arm and charged an error. Each trial ended when the animal either located the platform or committed six errors, at which point it was guided to the platform. Once on the platform, the animal was left for 15 sec before being returned to its cage.

Two measurements were taken to account for potential differences between genotypes in escape motivation, swimming ability, and vision. First, during the main task, the time was recorded for each excursion that occurred during the first five trials. An excursion was defined as the individual period from when the animal is placed in the start arm to when it either commits an error or locates the escape platform. Second, following the main task, the maze was modified so that the non-goal and non-start arms were blocked. Animals were placed into the start arm, and escape latency was timed for each of the five start arms.

For 12-month outcrossed animals, a reversal task consisting of three learning blocks of four trials was administered the day after the main task. A new goal arm located at least two arms away from the previous goal arm was assigned randomly for each mouse and remained constant across reversal trials. Start arms, which excluded the previous and current goal arms, were assigned semi-randomly so that all four remaining arms were represented during each learning block. The reversal task was conducted similarly to the main task, and mice were scored for total errors. A perseverant error was additionally scored when an animal entered its previous goal arm.

For total errors and escape latencies, mean values for each learning block were calculated for each mouse and analyzed by two-factor ANOVAs (genotype $\times$ trial) with repeated measures (trial), followed by post hoc Student's *t*-tests for significance.

Novel object recognition

The novel object recognition testing protocol was based on (Frick and Gresack, 2003), including adaptations from (Sfakianos et al., 2007). On the last day of pre-test handling, animals were habituated to the empty testing arena (45 cm $\times$ 24 cm $\times$ 20 cm) for 30 min. The following day, two identical objects (rubber ducks, bottle caps, or conical tubes) were

placed into the testing arena, and the animals were allowed to amass 30 sec of total object exploration. Two days later, one familiar object was placed into the arena with one novel object, and the animals were allowed to amass 30 sec of total object exploration. The type and location of the novel object was counterbalanced between mice. The sample and choice phases were video recorded and scored for object exploration, defined as time spent in directed nasal or oral contact with the objects. Exploration times for each mouse were analyzed by two-factor ANOVAs (genotype $\times$ object), followed by post hoc Student's *t*-tests for significance.

Animal survival

Outcrossed animals were monitored over a period of 18 months for survival. Animal deaths were only counted if they could not be attributed to other causes, such as disease or aggression-related injury. Kaplan-Meier curves for each genotype were generated by calculating the cumulative percent of animals surviving to each month. Animal survival curves were analyzed using the Mantel-Haenszel log-rank test for significance.

Brain tissue collection and immunoblot analysis

Protein levels were determined using a previously described protocol (Gimbel et al., 2010). Briefly, mice were sacrificed and perfused with ice-cold 0.1M PBS pH 7.4 for 2 min. Each brain was extracted, and the right hemisphere was homogenized in TBS (50 mM Tris, 150 mM NaCl, pH 7.6) containing a protease inhibitor cocktail (Thermo Scientific, Rockford, IL). Tissue was homogenized using a Polytron tissue disruptor (Kinematica, Bohemia, NY), homogenates were centrifuged at 100,000 $\times g$ for 20 min at 4°, and supernatants were frozen for subsequent analysis of the soluble fraction. Pellet fractions were then resuspended in TBS with 2% Triton X-100, 0.1% SDS, and the protease inhibitor cocktail, homogenized, and centrifuged at 100,000 $\times g$ for 20 min at 4°. Supernatants were frozen for subsequent analysis of the detergent-soluble fraction. The final detergent-insoluble pellet was resuspended in 0.1M formic acid, homogenized, and centrifuged at 100,000 $\times g$ for 20 min at 4° to obtain the insoluble fraction. Prior to freezing, the insoluble fraction samples were neutralized with 1M Trizma-base. Lysate samples were run on SDS-PAGE gels or pre-cast 10–20% Tris-tricine gels (BioRad), transferred to nitrocellulose membranes, and probed with antibodies to the following proteins: full length A β PP, soluble A β PP α (sA β PP α), A β PP β -C-terminal fragment (β -CTF), soluble A β (sA β), insoluble A β , presenilin-1 (PS1), HSP70, and actin (Table 1). Immunoblots were quantified using Quantity One software (Bio-Rad), and protein signals were normalized to loading control levels (HSP70 or actin). Mean expression levels were analyzed with Kruskal-Wallis tests followed by post hoc Mann-Whitney tests for significance.

Antibody characterization

The rabbit anti-A β PP polyclonal antibody specifically recognizes residues 695, 751, and 770 of the human form of A β PP. This antibody detects a ~110 kDa band in immunoblots of transgenic A β PP/PS1 mouse brain lysates.

The mouse anti-A β PP (6E10) antibody specifically recognizes the human form of A β PP. This antibody detects an ~100 kDa band in immunoblots of transgenic A β PP/PS1 mouse brain lysates as the sA β PP α cleavage product. This antibody recognizes soluble and insoluble A β peptide (~3.8 kDa) on immunoblots from gradient gels optimized for detection of small proteins. This antibody also recognizes the A β PP β -C-terminal fragment (~16 kDa) from detergent-extracted mouse brain lysates.

The rabbit anti-PS1 polyclonal antibody specifically recognizes the human form of PS1. This antibody detects the ~22 kDa PS1 C-terminal fragment in immunoblots of transgenic

A β PP/PS1 mouse brain lysates. Additionally, this antibody detects full length PS1 protein at ~55 kDa to a lesser extent.

Antibodies to HSP70 (5A5), and actin (C4) were used as loading controls. The mouse anti-HSP70 antibody recognizes the murine form of HSP70 and detects an ~70 kDa band in immunoblots of mouse brain lysates. The mouse anti-actin antibody recognizes the murine form of actin and detects an ~42 kDa band in immunoblots of mouse brain lysates.

Results

Inbred C57BL/6 A β PP/PS1 mice have an earlier onset of radial-arm water maze deficits than outcrossed mice

To determine if and when A β PP/PS1 mice exhibit deficits in forebrain function, we tested them at different ages in a radial-arm water maze using a protocol previously shown to detect spatial learning and reference memory defects in AD transgenic mice (Alamed et al., 2006; Park et al., 2006; Wilcock et al., 2006). For this task, animals placed into one arm of a six-arm water maze used spatial cues located on the walls of the testing room to locate a hidden platform, which remained constant for each animal across 30 trials (Fig. 1A). An error was charged each time an animal entered an incorrect arm or failed to select an arm after 1 min. Errors due to failing to select an arm comprised only two percent of all errors and mostly occurred during the first two learning blocks.

At 12 months, there were no significant differences in water maze performance between WT and A β PP/PS1 outcrossed animals (Fig. 1C). There was a main effect of trial, indicating that animal performance improved across successive trials, as they went from making 4 errors per trial in the first five trials to only 1 error per trial in the last five trials. At 16 months, outcrossed A β PP/PS1 mice do not perform significantly different than their WT counterparts (Fig. 1D). These results were striking as previously published work measured behavioral impairments in A β PP/PS1 mice as early as 9 months (Park et al., 2006). By 18 months, outcrossed A β PP/PS1 animals performed significantly worse in the radial-arm water maze than WT animals, maintaining a rate of 3 errors per trial for their last 15 trials, while WT mice improved to 1 error per trial during that same time (Fig. 1E). These results indicate that outcrossed A β PP/PS1 mice have a significant delay in behavioral impairment relative to previously published reports (Garcia-Alloza et al., 2006; McClean et al., 2011; Park et al., 2006).

We hypothesized that the delayed behavioral phenotype could be due to genetic elements introduced during the initial outcrossing. Therefore, we measured radial-arm water maze behavior in inbred C57BL/6 A β PP/PS1 mice at an earlier age. Inbred A β PP/PS1 mice perform significantly worse than their WT littermates at 8 months, maintaining a rate of 2 errors per trial for the last 15 trials, while WT mice make on average less than 1 error per trial during this same time (Fig. 1F). These results indicate that outcrossed A β PP/PS1 mice are delayed in their development of behavioral deficits relative to their inbred C57BL/6 A β PP/PS1 counterparts.

Two measurements were taken to account for potential differences between genotypes in escape motivation, swimming ability, and vision. During the main task, each time the animal was placed in the start arm, we determined how long it took for it to make an excursion (either make an error or locate the platform), which could reveal if an animal was unequally motivated to perform the task. There were no differences between genotypes for any of the experimental groups during the first 5 trials (Fig. 1B). Following the main task, the maze was modified so that non-goal arms were blocked, and the animal needed only to traverse a closed channel to reach its goal arm. For five trials, there were no significant differences

between genotypes in escape latencies for outcrossed 12-month mice (WT, 14 ± 2 sec versus A β PP/PS1, 14 ± 1 sec; mean $\pm$ SE; Student's *t*-test, $p = 0.90$), outcrossed 18-month mice (WT, 17 ± 4 sec versus A β PP/PS1, 23 ± 4 sec; $p = 0.28$), or inbred C57BL/6 8-month mice (WT, 8.9 ± 2 sec versus A β PP/PS1, 9.9 ± 2 sec; $p = 0.74$). Together, these results suggest that the poor performance of outcrossed A β PP/PS1 animals at 18 months and inbred A β PP/PS1 mice at 8 months cannot be attributed to reduced escape motivation, swimming ability, or vision.

Since both genotypes in outcrossed mice learned the task similarly at 12 months, we also tested behavioral flexibility with a reversal task in which the location of the goal platform was changed. For an animal to succeed in this reversal task, it must be able to remember the new platform location as well as inhibit its inclination to return to the previous goal arm. WT and A β PP/PS1 animals performed similarly in the reversal task when scored for either total errors or perseverant errors (returns to the previous goal arm) (Fig. 1G and 1H). There were main effects of trial for both total errors and perseverant errors, signifying that animal performance improved across reversal trials.

Inbred C57BL/6 A β PP/PS1 mice have an early deficit in novel object recognition while outcrossed animals show no deficit in novel object recognition

To assess the degree of functional impairment of the outcrossed strain at 18 months, we tested A β PP/PS1 mice in a novel object recognition task. Novel object recognition requires proper hippocampal function, but lacks the spatial challenge of the radial-arm water maze (Clark et al., 2000; Zola et al., 2000). We followed a protocol used previously to detect deficits in animals with hippocampal impairment (Sfakianos et al., 2007). This task takes advantage of the innate tendency mice have to explore novel objects placed into familiar environments. During the sample phase, the animal is allowed to become familiar with two identical objects (Fig. 2A). During the choice phase, which takes place 48 hours later, the animal is presented with a familiar object and a novel object, and the time the animal spends exploring each object is measured.

During the sample phase, we found that 18-month-old outcrossed WT and A β PP/PS1 animals explored both familiar objects for equivalent amounts of time (Fig. 2B). During the choice phase, WT animals spent significantly more time exploring the novel object than the familiar one, as expected (Fig. 2C). Outcrossed A β PP/PS1 mice also spent significantly more time exploring the novel object, indicating that they do not have a deficit discriminating the novel object from the familiar one. Thus, while outcrossed A β PP/PS1 mice have a clear deficit in radial-arm water maze performance, they are still able to perform a novel object recognition task at 18 months.

We then tested 8-month inbred C57BL/6 WT and A β PP/PS1 animals in the novel object recognition task. We found that during the sample phase each genotype explored both familiar objects equally (Fig. 2D). During the choice phase, while inbred WT mice spend significantly more time with the novel object as expected, inbred A β PP/PS1 mice do not spend more time exploring the novel object (Fig. 2E). These results indicate that inbred A β PP/PS1 mice fail to discriminate the novel from the familiar object at 8 months and that genetic elements present in the outcrossed strain may help protect outcrossed A β PP/PS1 mice from developing defects in novel object recognition.

Inbred C57BL/6 A β PP/PS1 mice have higher amyloid plaque burden than outcrossed mice

To better understand the timing and extent of neurodegeneration in outcrossed A β PP/PS1 animals, we analyzed amyloid plaque deposition at 12 and 18 months. To visualize amyloid plaques, we stained brain sections from WT and A β PP/PS1 mice with the amyloid-binding

dye Thioflavin S (Westermarck et al., 1999). We quantified overall plaque burden in various subfields of the hippocampus, including the CA1 stratum radiatum and stratum oriens, the CA3 stratum radiatum and stratum oriens, and the dentate gyrus molecular layer (Fig. 3A). We then combined the plaque burden from each region to obtain a value for total hippocampal plaque burden (Fig. 3B), which is a function of both plaque density and plaque size. At 12 months, we found that the A β PP/PS1 hippocampus had only sparse amyloid plaques (Fig. 3C), while by 18 months amyloid plaque deposition had increased dramatically (Fig. 3D). WT mice did not have any detectable amyloid deposition at either of these ages (data not shown). Similarly, when we quantified plaque burden within another brain region, the temporal cortex, outcrossed A β PP/PS1 transgenic mice had low amyloid plaque burden at 12 months, which significantly increased by 18 months (Fig. 4B-D).

Consistent with the behavioral measurements, the development of AD-like pathology in outcrossed A β PP/PS1 mice is delayed considerably relative to published phenotypes, which display robust amyloid plaque deposition and behavioral impairments as early as 9 months (Garcia-Alloza et al., 2006; McClean et al., 2011; Park et al., 2006). We hypothesized that the delayed phenotype may be due to genetic elements introduced during the initial outcrossing. Therefore, we compared plaque deposition in the hippocampus and cortex of outcrossed A β PP/PS1 mice to A β PP/PS1 mice on a uniform C57BL/6 background at both 8 and 12 months. In both the hippocampus (Fig. 3B and 3F) and cortex (Fig. 4B and 4F), inbred mice had higher plaque burden at 12 months than outcrossed mice of the same age. Interestingly, 8-month-old inbred A β PP/PS1 mice have low hippocampal and cortical plaque burden (Fig. 3E and 4E). Thus, while outcrossed mice have a delayed onset of plaque deposition, this is not sufficient to explain their delayed behavioral deficits, as 8-month-old inbred mice also have low amyloid plaque burden, yet display significant behavioral impairments.

Normal brain weight, body weight, and survival in outcrossed A β PP/PS1 mice

During the course of our studies, we recorded gross body parameters and survival rates for the outcrossed A β PP/PS1 mice. Brain weight reductions of 10 percent and body weight reductions of 30–40 percent have been reported for aged A β PP/PS1 mice (Delatour et al., 2006; Pugh et al., 2007). We found no differences in brain weight, body weight, or brain/body weight ratios in outcrossed A β PP/PS1 mice compared to WT mice at 18 months (Table 2). Furthermore, reduced survival rates have also been documented in inbred A β PP/PS1 mice (Gimbel et al., 2010; Halford and Russell, 2009), possibly due to neuron hyperexcitability and epileptic seizures (Minkeviciene et al., 2009; Palop et al., 2007). In our study, outcrossed A β PP/PS1 animals showed no differences from WT animals in survival rates up to 18 months (Fig. 5A). Only one outcrossed animal in our study was witnessed to be having a seizure, but this was a WT animal.

Soluble A β levels are increased in inbred C57BL/6 transgenic mice compared to outcrossed mice

Based on the reduced amyloid plaque burden observed in outcrossed A β PP/PS1 mice, we wanted to determine if the levels of A β PP and its various processed forms were different in these mice. Since 8-month inbred A β PP/PS1 mice display a strong behavioral deficit despite a low overall plaque burden in both the hippocampus and cortex, we hypothesized that these mice may have differences in A β PP transgene expression or processing that would not be detectable by histological staining methods. In particular, soluble A β species are thought to play a toxic role in AD and have been shown to correlate with levels of cognitive impairment in AD transgenic mice (Zhang et al., 2011). First, we measured levels of full length A β PP by immunoblot in fractionated brain lysate from outcrossed A β PP/PS1 mice at 12 and 18 months and inbred C57BL/6 A β PP/PS1 mice at 8 and 12 months. We found that

full length A β PP expression increases with age in both transgenic strains, with 12-month inbred A β PP/PS1 mice showing a robust increase above each of the other groups (Fig. 5B and 5G). Full length A β PP is processed by one of two secretase pathways, which produce different soluble and membrane-bound cleavage products (O'Brien et al., 2001). We quantified the levels of three cleavage products from these same samples. Soluble A β PP α is released after cleavage of full length A β PP by the α -secretase complex. We found no differences in soluble A β PP α levels in outcrossed transgenic mice relative to their inbred counterparts at any age (Fig. 5C and 5G). Full length A β PP can also be processed first by the β -secretase complex, which produces a membrane-associated C-terminal fragment (β -CTF). This fragment is subsequently cleaved by the PS1-containing γ -secretase complex, leading to the production of soluble A β peptide, which can aggregate to form toxic soluble species and insoluble amyloid plaques. The levels of the β -CTF and PS1 from detergent solubilized extracts are unchanged across each strain and age (Fig. 5D and 5H). Soluble A β levels increase with age in both outcrossed and inbred mice and are significantly higher in 8-month inbred mice compared to 12-month outcrossed mice (Fig. 5E and 5H), which mirrors the differential impairments in behavioral tasks we observed in these two mouse populations. Consistent with Thioflavin S staining of insoluble plaques, 12-month outcrossed and 8-month inbred transgenic mice have low insoluble A β levels, while both 18-month outcrossed mice and 12-month inbred mice have significantly higher insoluble A β levels (Fig. 5F and 5H).

Discussion

AD is characterized by distinct features including high levels of amyloid plaque formation, progressive cognitive impairment, widespread synapse/dendrite loss, and atrophy in selected brain regions. Transgenic mice expressing mutant forms of A β PP and PS1 have proven useful for investigating the molecular mechanisms that give rise to these clinical features. We crossed A β PP/PS1 mice with mice of a mixed genetic background (C57BL/6 $\times$ 129/SvJ) and investigated the development of AD-like pathology in the resulting mice. While we did reproduce many of the phenotypes reported for expression of these transgenes, we found that the onset of these traits was delayed considerably in the outcrossed mice relative to A β PP/PS1 mice on an inbred C57BL/6 background.

In the radial-arm water maze, outcrossed A β PP/PS1 mice performed similarly to WT mice at 12 and 16 months, but develop a clear deficit by 18 months. This onset of impairment is delayed considerably relative to other A β PP/PS1 mouse studies. Park et al. (2006) measured radial-arm water maze performance in mice expressing the same A β PP/PS1 transgenes on a mixed genetic background (C57BL/6 $\times$ C3H/HeJ) using a very similar protocol for animals between 4–13 months. In this study, A β PP/PS1 animals made more errors at 8 months than mice in this study did at 12 months and made as many errors at 10 months as the mice in this study did at 18 months. Our finding that inbred C57BL/6 A β PP/PS1 mice develop water maze deficits as early as 8 months further supports our hypothesis that the delayed onset of behavioral deficits is due to differences in strain background.

To determine the extent of functional impairment at 18 months, we tested outcrossed A β PP/PS1 mice in a hippocampal-dependent novel object recognition task. Outcrossed A β PP/PS1 mice are able to discriminate between the novel and familiar objects and perform this task similarly to WT animals. Using a similar hippocampal-dependent protocol, McClean et al. (2011) found that C57BL/6 mice expressing the same A β PP/PS1 transgenes had a deficit in novel object recognition as early as 9 months. We found that C57BL/6 A β PP/PS1 mice have deficits in novel object recognition at 8 months, further supporting the idea that genetic elements in the outcrossed A β PP/PS1 mice are likely responsible for the stark behavioral differences.

A discrepancy for the outcrossed mice exists in their differential success at 18 months in novel object recognition and the radial-arm water maze, both of which have been shown to depend on hippocampal function (Clark et al., 2000; Diamond et al., 1999). An important difference between these two behavioral tasks is that novel object recognition lacks the spatial element of the radial-arm water maze. Within the hippocampus, dentate gyrus granule neurons have been shown to play a critical role in spatial behavior, but these same neurons are not required for novel object discrimination (Lee et al., 2005; Xavier and Costa, 2009). Thus, it is possible that functional impairment in the outcrossed mice is restricted to particular regions that affect spatial behavior, but has not yet affected neurons in the dentate gyrus needed for novel object recognition.

Outcrossed A β PP/PS1 mice had minimal amyloid plaque deposition at 12 months, but overall plaque burden increased significantly by 18 months in regions of the hippocampus and cortex. We found that inbred C57BL/6 A β PP/PS1 mice have a significantly higher plaque burden at 12 months compared to outcrossed mice in all brain regions examined. While quantification of amyloid plaques is sensitive to differences in staining protocols and scoring methods, plaque measurements by other groups corroborate our finding that plaque deposition is delayed in outcrossed A β PP/PS1 mice relative to other mice expressing the same transgenes. Garcia-Alloza et al. (2006) reported on the development of amyloid plaques in A β PP/PS1 mice between 4–12 months using Thioflavin S in a mixed C57BL/6 $\times$ C3H/HeJ strain background. At 12 months, they found plaque density and plaque burden to be roughly ten-fold higher than the outcrossed mice from our study at that same age, but on par with values we measure at 18 months. They also found that plaque size remained constant between 6 and 12 months with areas similar to outcrossed mice, suggesting that the staining and imaging procedures used were comparable. Taken together with these previous results, our comparison between inbred and outcrossed transgenic strains suggests that the delayed plaque deposition we observed in outcrossed mice is due to differences in strain background, particularly genetic elements of the 129/SvJ strain. Interestingly, we found that 8-month C57BL/6 A β PP/PS1 mice had low amyloid plaque deposition, despite having significant behavioral impairments. These results indicate that while plaque deposition is delayed in outcrossed mice, it cannot fully account for their late behavioral impairment.

During the course of this study, we recorded brain weights, body weights, and survival rates for A β PP/PS1 mice. We found brain and body weight values to be normal in outcrossed A β PP/PS1 mice. In contrast, Delatour et al. (2006) noted a 10 percent decrease in total brain weight and a 40 percent reduction in body weight in aged A β PP/PS1 mice bearing different mutations on a primarily C57BL/6 background. We also observed that only 10 percent of outcrossed A β PP/PS1 mice died before 12 months, which was nearly identical to WT mortality rates. Conversely, using the same line of inbred A β PP/PS1 animals as in the current study, Gimbel et al. (2010) found animal survival to be drastically shortened with over 50 percent of their transgenic mice dying by 12 months. Thus, for both brain/body weight and survival, the dramatic phenotypes seen in other reports are not recapitulated in the outcrossed A β PP/PS1 line.

Based on our own measurements and comparisons with other studies, there appear to be considerable discrepancies between the phenotypes of inbred and outcrossed A β PP/PS1 mice. In some cases, this disparity might be attributed to differences in the specific mutations used in the A β PP and PS1 transgenes, although the lack of carryover for such remarkable effects is surprising. In other cases, including this study, the same mutant A β PP and PS1 transgenes were expressed and the only major difference in these mice was the genetic background on which the transgenes were expressed. The effects of AD transgene expression have been shown previously to differ with strain background (Crawley, 1999; Ryman et al., 2006). For example, mutant A β PP expressed on an inbred C57BL/6

background produced robust amyloid plaque formation and deficits in Morris water maze performance, but FVB/N mice expressing the same transgene showed no plaque formation and early lethality (Hsiao et al., 1996; Hsiao et al., 1995). Additionally, inbred C57BL/6 A β PP transgenic mice have higher amyloid plaque deposition than A β PP transgenic mice of either an A/J or a mixed C57BL/6 $\times$ DBA/2 genetic background (Ozmen et al., 2007; Sebastiani et al., 2006). Strain background has also been shown to have a strong influence on the performance of A β PP transgenic mice in a variety of behavioral assays (Glazner et al., 2010; Lassalle et al., 2008; Rustay et al., 2010).

The primary genetic element introduced by our initial outcrossing was the presence of the 129/SvJ genetic background. Interestingly, 129/SvJ mice harboring mutant A β PP transgenes have been shown previously to exhibit reduced A β PP processing as well as resistance to A β PP transgene-induced early lethality (Krezowski et al., 2004; Lehman et al., 2003). Based on our findings that amyloid plaques levels do not correlate with behavioral impairment, we hypothesized that the delayed behavioral phenotypes could be due to differences in A β PP production or processing. We found that full length A β PP levels increase with age in both in outcrossed and inbred A β PP/PS1 mice. Processing of full length A β PP via the non-amyloidogenic α -secretase pathway is not affected by strain or age based on soluble A β PP α levels. Full length A β PP is also processed via an amyloidogenic pathway, mediated by β -secretase and PS1-containing γ -secretase complexes (O'Brien et al., 2001). We found that soluble A β increases with age in each strain and that soluble A β levels mirror the degree of behavioral impairment in both strains. We found low insoluble A β levels in 8-month C57BL/6 mice when compared to 18-month outcrossed mice or 12-month inbred mice, indicating that while soluble A β is produced at high levels in 8-month inbred mice, it is not efficiently incorporated into insoluble plaques.

The presence of soluble A β species prior to amyloid plaque formation has been associated with synaptic impairment and cognitive decline associated with Alzheimer's disease (Hsiao, et al., 1999; Mucke, et al., 2000). Additionally, soluble A β oligomers have toxic effects on neurons when introduced in culture (Um, et al., 2012; Walsh, et al., 2002). In our study, we found that 8-month C57BL/6 mice have a low overall plaque burden, yet significant behavioral impairments in memory tasks. One explanation for this discrepancy is that inbred mice express high levels of soluble A β , which may lead to impaired neuronal signaling and subsequent behavioral deficits. Based on our findings, we conclude that soluble A β levels represent the major pathological difference between these strains. However, 129/SvJ mice also show greater resistance to various neural insults, including ischemia and spinal cord injury (Dimou et al., 2006; Fujii et al., 1997; Wellons et al., 2000). Thus, the delayed phenotypes observed in the outcrossed A β PP/PS1 mice in this study could be the product of differences in both soluble A β levels and decreased susceptibility of the 129/SvJ strain to neural insult.

In conclusion, we crossed A β PP/PS1 mice with mice of a mixed genetic background and investigated the development of AD-like features in the resulting mice. We observed a delayed onset of spatial learning impairment and amyloid plaque deposition in the outcrossed mice, through both direct comparisons with an inbred C57BL/6 strain and indirect comparisons to published reports of A β PP/PS1 mice on other genetic backgrounds. Furthermore, the different level of soluble A β peptide present in these two strains provides a potential mechanism underlying their behavioral differences. Strain background introduces a potential confound for interpreting the effects of transgene expression across studies, and these findings suggest that genetic elements present in certain mouse strains can significantly delay the onset and severity of AD-like pathology and AD-related behaviors in different transgenic mouse lines.

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Abbreviations

Aβ	amyloid- β peptide
AβPP	amyloid- β protein precursor
AD	Alzheimer's disease
ANOVA	analysis of variance
NOR	novel object recognition
PBS	phosphate-buffered saline
PS1	presenilin-1
PS2	presenilin-2
RAWM	radial-arm water maze
TBS	Tris-buffered saline
WT	wild type.

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Fig. 1.

Onset of radial-arm water maze deficit occurs between 16 and 18 months for outcrossed AβPP/PS1 mice and as early as 8 months in inbred C57BL/6 AβPP/PS1 mice. (A) Diagram of radial-arm water maze. Maze contained six arms with large visual cues positioned on the walls of the room adjacent to the distal end of each arm. A hidden platform (HP) was submerged at the end of one arm. (B) Controls assessing escape motivation, swimming ability, and vision across trials in outcrossed WT and AβPP/PS1 mice at 12, 16, and 18 months and inbred C57BL/6 WT and AβPP/PS1 mice at 8 months. Symbols represent mean seconds per excursion during first five trials $\pm$ SE. Two-factor ANOVAs (genotype $\times$ group): no interaction, $F < 1$. Errors made across trials by WT and AβPP/PS1 mice at (C-E)

12, 16, and 18 months in outcrossed mice and (F) 8 months in inbred C57BL/6 mice. Symbols represent mean errors made per trial during groups of five trials $\pm$ SE. Two-factor ANOVAs (genotype $\times$ trial) with repeated measures (trial): 12 months outcrossed: main effect of trial, $F_{(5,27)} = 22.08$, $p < 0.001$; 16 months outcrossed: main effect of trial, $F_{(5,10)} = 15.63$, $p < 0.001$; 18 months outcrossed: interaction, $F_{(5,14)} = 4.35$, $p < 0.01$; 8 months inbred C57BL/6: interaction, $F_{(5,19)} = 4.63$, $p < 0.001$. Post hoc Student's t -tests, $*p < 0.05$, $***p < 0.001$. Excursion time and water maze analyses: 12 months outcrossed: WT, $n = 15$ mice; A β PP/PS1, $n = 14$ mice; 16 months outcrossed: WT, $n = 5$ mice; A β PP/PS1, $n = 7$ mice; 18 months outcrossed: WT, $n = 8$ mice; A β PP/PS1, $n = 8$ mice; 8 months inbred C57BL/6: WT, $n = 11$ mice; A β PP/PS1, $n = 10$ mice. (G-H) Performance in radial-arm water maze reversal does not differ between WT and A β PP/PS1 mice at 12 months. (G) Total errors and (H) perseverant errors made per trial by WT and A β PP/PS1 mice during reversal task at 12 months. Symbols represent mean errors made per trial during groups of four trials $\pm$ SE. Two-factor ANOVAs (genotype $\times$ trial) with repeated measures (trial): total errors: main effect of trial, $F_{(2,27)} = 29.36$, $p < 0.001$; perseverant errors: main effect of trial, $F_{(2,27)} = 15.09$, $p < 0.001$. Water maze reversal analyses: 12 months: WT, $n = 15$ mice; A β PP/PS1, $n = 14$ mice.

Fig. 2.

Novel object recognition does not differ between outcrossed WT and AβPP/PS1 mice at 18 months while inbred C57BL/6 AβPP/PS1 mice have defects in this task at 8 months. (A) Diagram of object placement during sample and choice phases. L = left object, R = right object, F = familiar object, N = novel object. Object exploration times by outcrossed WT and AβPP/PS1 mice at 18 months during the (B) sample phase and (C) choice phase. Bars represent mean seconds spent exploring object $\pm$ SE. Two-factor ANOVAs (genotype $\times$ object): sample phase: no interaction, $F < 1$; choice phase: interaction, $F_{(1,14)} = 6.05$, $p < 0.05$. Post hoc Student's *t*-tests, $**p < 0.01$, $***p < 0.001$. Object exploration times by inbred C57BL/6 WT and AβPP/PS1 mice at 8 months during the (D) sample phase and (E)

choice phase. Bars represent mean seconds spent exploring object $\pm$ SE. Two-factor ANOVAs (genotype $\times$ object): sample phase: no interaction, $F < 1$; choice phase: interaction, $F_{(1,19)} = 3.89$, $p < 0.05$. Post hoc Student's t -tests, *** $p < 0.001$. Novel object recognition analyses: outcrossed mice: WT, $n = 8$ mice; A β PP/PS1, $n = 8$ mice; inbred C57BL/6: WT, $n = 11$ mice; A β PP/PS1, $n = 10$ mice.

Fig. 3.

Amyloid plaque burden increases significantly with age in outcrossed and inbred C57BL/6 A β PP/PS1 mouse hippocampus and is highest in 12-month inbred C57BL/6 mice. (A) Diagram of mouse hippocampus showing regions scored for plaque deposition, adapted from (Franklin and Paxinos, 2008). CA1 = CA1 region, CA3 = CA3 region, Rad = stratum radiatum, Or = stratum oriens, DG = dentate gyrus, Mol = molecular layer. Scale bar = 200 μ m. (B) Plaque burden in the A β PP/PS1 hippocampus in outcrossed mice at 12 and 18 months and in inbred C57BL/6 mice at 8 and 12 months. Bars represent means $\pm$ SE. Kruskal-Wallis test: plaque burden: $p < 0.01$. Post hoc Mann-Whitney, $*p < 0.05$, $**p < 0.01$. Plaque deposition analyses: 12 months outcrossed, $n = 5$ mice (6 sections per mouse);

18 months outcrossed, $n = 8$ mice (6 sections per mouse); 8 months inbred C57BL/6, $n = 3$ mice (6 sections per mouse); 12 months inbred C57BL/6, $n = 6$ mice (4–6 sections per mouse). (C-F) Representative fluorescent images of Thioflavin S stained sections from the A β PP/PS1 hippocampus at (C) 12 months in outcrossed mice, (D) 18 months in outcrossed mice, (E) 8 months in inbred C57BL/6 mice, and (F) 12 months in inbred C57BL/6 mice. Bright puncta indicate Thioflavin S -reactive amyloid deposits. Scale bars = 200 μ m.

Fig. 4.

Amyloid plaque burden increases significantly with age in outcrossed and inbred C57BL/6 mouse temporal cortex and is higher in inbred C57BL/6 mice than outcrossed mice at 12 months. (A) Diagram of mouse cortex showing region scored for plaque deposition, adapted from (Franklin and Paxinos, 2008). TeA = temporal cortex, Ent = entorhinal cortex. Scale bar = 200 μ m. (B) Plaque burden in the temporal cortex in 12- and 18-month outcrossed A β PP/PS1 mice and 8- and 12-month inbred C57BL/6 mice. Bars represent means $\pm$ SE. Kruskal-Wallis test: plaque burden: $p < 0.01$. Post hoc Mann-Whitney tests of plaque burden, * $p < 0.05$, ** $p < 0.01$. Plaque deposition analyses: 12 months outcrossed, $n = 5$ mice (6 sections per mouse); 18 months outcrossed, $n = 8$ mice (6 sections per mouse); 8 months

inbred C57BL/6, $n = 3$ mice (6 sections per mouse); 12 months inbred C57BL/6, $n = 6$ mice (4–6 sections per mouse). (C-F) Representative fluorescent images of Thioflavin S -stained sections from the A β PP/PS1 cortex at (C) 12 months in outcrossed mice, (D) 18 months in outcrossed mice, (E) 8 months in inbred C57BL/6 mice, and (F) 12 months in inbred C57BL/6 mice. Bright puncta indicate Thioflavin S -reactive amyloid deposits. Scale bars = 200 μ m.

Fig. 5. Measurements of animal survival, PS1 levels, AβPP levels, and AβPP processing. (A) Kaplan-Meier curve showing cumulative survival percentage for outcrossed WT and AβPP/PS1 mice up to 18 months. Symbols represent cumulative percent of animals surviving to each month. Mantel-Haenszel log-rank test: $p = 0.61$. Animal survival analysis: WT, $n = 83$ mice (9 deaths total); AβPP/PS1, $n = 81$ mice (11 deaths total). Immunoblots of (B) full length (FL) AβPP, (C) soluble AβPPα (sAβPPα), (D) AβPP β-C-terminal fragment (β-CTF) and presenilin-1 (PS1), (E) soluble Aβ (sAβ), and (F) insoluble Aβ levels in outcrossed WT and AβPP/PS1 mice on outcrossed at 12 and 18 months and inbred C57BL/6WT and AβPP/PS1 at 8 and 12 months. HSP70 and actin levels are shown as loading controls. Numbers on left of immunoblots represent approximate molecular weights (kDa). (G) Quantification of non-amyloidogenic pathway including full length AβPP and sAβPPα levels normalized to respective loading controls. Bars represent means $\pm$ SE. Kruskal-Wallis test: Full length AβPP: $p < 0.001$; sAβPPα: no significance, $p > 0.05$. Post hoc Mann-Whitney tests, ** $p < 0.01$, *** $p < 0.001$. Full length AβPP analysis: $n = 3$ mice/group.

sA β PP α analysis: outcrossed mice: 12M, $n=9$ mice; 18M, $n=3$ mice; inbred C57BL/6: 8M, $n=3$ mice; 12M, $n=16$ mice. (H) Quantification of amyloidogenic pathway, including β -CTF, PS1, sA β , and insoluble A β normalized to respective loading controls, when possible. Bars represent means $\pm$ SE. Kruskal-Wallis test: PS1: no significance, $p > 0.05$; β -CTF: no significance, $p > 0.05$; sA β : $p < 0.05$; insoluble A β : $p < 0.05$. Post hoc Mann-Whitney tests, $*p < 0.05$, $**p < 0.01$. PS1, β -CTF, sA β , and insoluble A β analysis: $n=3$ mice/group.

Table 1

Primary antibodies

Antibody Name	Immunogen	Source, Catalog No.	Species
A β PP (6E10)	A β peptide residues 1–17	Millipore, MAB1560	Mouse monoclonal
A β PP, C-term	A β PP residues 695, 751, 770	Sigma-Aldrich, A8717	Rabbit polyclonal
Presenilin 1	Residues around V293	Cell Signaling, 3622	Rabbit polyclonal
HSP70 (5A5)	HSP70 residues 122–264	Abcam, 2787	Mouse monoclonal
Actin (C4)	Actin residues 50–70	Millipore, MAB1501R	Mouse monoclonal

Table 2

Brain and body weight at 18 months

Genotype	Brain Weight (g)	Body Weight (g)	(Brain/Body Ratio)×100
WT	0.50 ± 0.01	51.7 ± 2.6	0.99 ± 0.05
A β PP/PS1	0.50 ± 0.01	51.4 ± 2.8	1.01 ± 0.07
Student's <i>t</i> -test	<i>p</i> = 0.97	<i>p</i> = 0.94	<i>p</i> = 0.81

Values represent means ± SE.

WT, *n* = 13 mice; A β PP/PS1, *n* = 10 mice.