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Learning Decreases Aβ*56 and Tau Pathology and Ameliorates Behavioral Decline in 3xTg-AD Mice

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Transgenic mouse models of Alzheimer's disease (AD), such as the 3xTg-AD mice, are instrumental for elucidating genetic, pharmacologic, environmental, and behavioral factors that affect the cognitive phenotype. Here we present the novel findings that longitudinal water-maze spatial training produces a significant, albeit transient, improvement in subsequent learning performance and reduces amyloid β (Aβ) and tau neuropathology. The 3xTg-AD mice were trained and tested at 3 month intervals from 2 to 18 months. Separate groups of naive mice were also tested at each age. The improvement in performance seen at 6 and 12 months is dependent on spatial training, because animals that were similarly handled and exposed to swimming without a learning contingency failed to show improved performance. Training before the development of overt neuropathology is required for full expression of the training effect because we found it delays Aβ redistribution to extracellular plaques and reduces Aβ oligomers associated with cognitive decline. In addition, learning leads to decreased glycogen synthase kinase-3β activity, which likely underlies the reduced tau pathology. The previous training effects on both maze performance and neuropathology are attenuated at 15 and 18 months. These findings indicate that, in young and middle-aged 3xTg-AD mice, repeated spatial training can significantly delay the development of neuropathology and decline in spatial memory.

Key words: **tau; Alzheimer's disease; amyloid β; behavior; cognition; transgenic**

Introduction

Alzheimer's disease (AD) is the most common form of senile dementia. The affected cognitive domains in AD are initially limited to memory systems, in which the ability to retain and retrieve new information is impaired (Albert et al., 1996; Grossberg, 2003; Grundman et al., 2004). Ultimately, many domains are impacted, resulting in mood lability, anxiety, sleep disturbance, altered visuospatial perception, and impaired speech and judgment. The accompanying neuropathology primarily affects forebrain temporal structures such as the hippocampus, cortex, and amygdala and includes intraneuronal and extracellular accumulation of the amyloid β (Aβ) peptide, as well as intraneuronal aggregates of the microtubule-associated protein tau.

The generation of novel transgenic (Tg) models that recapitulate critical features of the disease has facilitated research investigating the relationship of these neuropathological lesions to the cognitive decline. The 3xTg-AD mice develop age-dependent and region-specific Aβ and tau pathology that closely mimic the disease progression in humans (Oddo et al., 2003a,b). The 3xTg-AD mice develop subtle memory deficits at 4 months of age, dependent on the appearance of intraneuronal Aβ accumulation, with memory deficits increasing with the accumulation of Aβ pathology (Billings et al., 2005). These results are consistent with previous evidence that memory deficits precede extracellular plaque pathology (Mucke et al., 1994; Hsiao et al., 1996; Moechars et al., 1999; Dodart et al., 2002; Van Dam et al., 2003) and indicate that early functional alterations rather than significant structural modifications underlie the onset of the initial cognitive decline.

Many studies indicate that memory loss in amyloid precursor protein (APP)-overexpressing transgenic mice is progressive and correlates with increasing amyloid burden (Hsiao et al., 1996; Chen et al., 2000; Billings et al., 2005). In the majority of these studies, the mice were housed in standard housing conditions, and different groups were tested at different ages. Several studies have reported that, in both rats and mice, previous learning or environmental enrichment enhances performance on cognitive tasks and alters hippocampal morphology (Greenough et al., 1972, 1979; Janus et al., 2000; Churchill et al., 2002; Fillit et al., 2002). More importantly, in relation to the present study, recent findings indicate that continual environmental enrichment from 2 to 6 months of age improves memory in female APP or APP/presenilin 1 (PS1) transgenic mice and that this improvement occurs despite significant increases in steady-state Aβ levels (Jankowsky et al., 2003, 2005). In contrast, Lazarov et al. (2005) reported that environmental enrichment from 1 to 5 months of age decreases Aβ levels in male APP/PS1 mice, which appears to be mediated by an enhancement in Aβ removal mechanisms.

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Although environmental enrichment is well known to improve performance on behavioral tasks, it is not known whether repeated exposure to a learning environment, in the absence of an enriched environment, alters the performance or neuropathology of AD mouse models. This study investigates the consequences of early and repeated learning in the 3xTg-AD mice on learning performance and Aβ and tau neuropathology. Our findings indicate that repeated spatial training induces significant, albeit transient, improvements in the cognitive and neuropathological phenotype of the 3xTg-AD mice.

Materials and Methods

Mice. A total of 403 mice were included at the beginning of the study. Thirty mice died over the course of the study (2 years). Because of the longitudinal nature of the study, only data from the remaining 373 mice are presented. Male and female mice were individually housed and kept on a 12 h light/dark schedule. All mice were given *ad libitum* access to food and water.

The 3xTg-AD mice have been characterized previously (Oddo et al., 2003a,b). Briefly, human APP cDNA harboring the Swedish mutation (KM670/671NL) and human four-repeat Tau harboring the P301L mutation were co-microinjected into single-cell embryos of homozygous $PS1_{M146V}$ knock-in mice. The background of the PS1 knock-in (PS1-KI) mice is a hybrid 129/C57BL/6. NonTg mice used were from the same strain and genetic background as the PS1-KI mice, but they harbor the endogenous wild-type mouse PS1 gene. All founder mice of the 3xTg-AD line were backcrossed to the parental PS1-KI mice, and both hemizygous (h) and homozygous (H) mice were generated. Because of the technique used to generate the 3xTg-AD mice, all of the mice used in this study are on the same genetic and strain background.

Behavioral testing. Group numbers and genotypes are provided in Table 1. All animals were handled briefly on the 4 d before each block of training, and all animals were tested on motor (gait and stepping) tasks and given a general health assessment evaluating coat, eye and nose condition, and hindleg clasping.

Hidden and cued platform Morris water maze (MWM) training and testing were conducted as described previously (Billings et al., 2005). Mice were trained to swim to a 14-cm-diameter circular clear Plexiglas platform submerged 1.5 cm beneath the surface of the water. The platform location was selected randomly for each mouse but was kept constant for each individual mouse throughout training at each age. On each trial, the mouse was placed into the tank at one of four designated start locations and allowed to find and escape onto the platform. If a mouse failed to find the platform within 60 s, it was manually guided to the platform and allowed to remain there for 5 s. After this, each mouse was placed into a holding cage under a warming lamp for 25 s until the start of the next trial. To ensure that memory differences were not attributable to a lack of task learning, mice were given four trials a day for as many days as were required to train the 3xTg-AD-H and 3xTg-AD-h mice to criterion (<25 s escape latency before the first probe trial was run). To control for overtraining, probe trials were run for the NonTg and PS1-KI mice as soon as they reached criterion and again when the 3xTg-AD mice did as well. No differences were noted between these probe trials for NonTg or PS1-KI mice; therefore, only the second set of probe trials is included.

Retention of the spatial training was assessed 1.5 h and again 24 h after the last training trial. Both probe trials consisted of a 60 s free swim in the pool without the platform. Mice were monitored by a camera, and all trials were scored during the probe trial and again after the probe trial for verification. There were no significant differences between any genotypes in the swim speeds, degree of thigmotaxis, or floating. The parameters measured during the probe trial included (1) initial latency to cross the platform location, (2) number of platform location crosses, and (3) time spent in the quadrant opposite to the target quadrant. For simplicity, the data presented are the latency to cross platform location, although for all significant findings in the probe trials, the pattern was consistently significant for all three probe measures.

At each age, the target quadrant varied for each mouse. In addition, the target quadrants varied between mice in a group to control for the salience of extramaze cues, which included three large, simple, black, block-shape cutouts on the west wall, a poster on the north wall, a shelf with the heating lamp on the east wall, and a door on the south wall.

Table 1. Experimental groups

Genotype	Behavioral test	Group	Number of animals
3xTg-AD-H	MWM	Longitudinal, "experienced"	12
3xTg-AD-h	MWM	Longitudinal, "experienced"	16
PS1-KI	MWM	Longitudinal, "experienced"	13
NonTg	MWM	Longitudinal, "experienced"	15
3xTg-AD-H	MWM	Cross-sectional, "naïve"	6 mo: 14 9 mo: 7 12 mo: 6 15 mo: 6 18 mo: 6
3xTg-AD-h	MWM	Cross-sectional, "naïve"	6 mo: 11 9 mo: 5 12 mo: 5 15 mo: 5 18 mo: 5
PS1-KI	MWM	Cross-sectional, "naïve"	6 mo: 11 9 mo: 6 12 mo: 5 15 mo: 5 18 mo: 5
NonTg	MWM	Cross-sectional, "naïve"	6 mo: 13 9 mo: 6 12 mo: 5 15 mo: 6 18 mo: 7
3xTg-AD-H	MWM	Training effect on pathology	12
NonTg	MWM	Training effect on pathology	8
3xTg-AD-H	MWM	Delayed training	10
3xTg-AD-h	MWM	Delayed training	7
NonTg	MWM	Delayed training	9
3xTg-AD-H	MWM	Yoked swim group	8
3xTg-AD-h	MWM	Yoked swim group	8
NonTg	MWM	Yoked swim group	7

□ Study 1 ▒ Study 2 ■ Study 3

No differences emerged in the cued platform until 18 months of age when the 3xTg-AD homozygous mice no longer attempted to find the platform; this was also true for hidden platform training. Thus, probe trials were not conducted for hidden platform in 18-month-old mice.

Immunohistochemistry. Mice were killed by CO_2 asphyxiation, and the brains were rapidly removed and fixed for 24–48 h in 4% paraformaldehyde followed by cryoprotection in 30% sucrose for 48 h. Free-floating sections, 50 μm thick, were processed for free-floating immunohistochemistry as described previously (Oddo et al., 2003a,b, 2004). The anti-Aβ antibody (6E10, 1:3000) and anti-tau antibody (HT7, 1:1000) antibody were applied overnight at 4°C. Sections were visualized with DAB (Vector Laboratories, Burlingame, CA) or with fluorescent secondary antibodies (Alexa 488 and 555). Quantification of Aβ was performed as described by Oddo et al. (2004) using reverse fluorescence images (fluorescent-labeled cells were black, and background was stark white).

ELISA. $A\beta_{1-40}$ and $A\beta_{1-42}$ were measured using techniques as described by Oddo et al. (2005).

Immunoblotting. Immunoblotting was performed as described previously (Oddo et al., 2003b). Antibodies and dilutions used in this study include the following: 6E10 (1:1000; Signet Laboratories, Dedham, MA) for APP; CTF20 (1:5000; Calbiochem, La Jolla, CA) for C99 and C83; HT7 (1:3000; Innogenetics, Zwÿnderecht, Antwerp, Belgium), AT8 (1:1000; Innogenetics), AT180 (1:1000; Innogenetics), anti-neprilysin (1:100; Abcam, Cambridge, MA), anti-insulin degrading enzyme (IDE)

(1:5000; a generous gift from Dr. Dennis Selkoe, Harvard Medical School, Boston, MA), and 5A6 (1:1000; Abcam) for lipoprotein receptor-related protein (LRP); anti-apolipoprotein E (ApoE) (1:1000; Abcam), anti-TGF1β (1:1000; Cell Signaling Technology, Beverly, MA), and anti-glycogen synthase kinase-3β (GSK3β) (1:3000; BD Transduction) for total GSK3β levels; anti-GSK3β-Ser9 (1:3000; Cell Signaling Technology) for Ser9 inactivated GSK3β; anti-cyclin-dependent kinase 5 (Cdk5) (1:3000; Calbiochem), anti-C-terminal p35 (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA) for p25 and p35; and α-actin (1:10,000; Sigma, St. Louis, MO). Quantitative densiometric analyses were performed on digitized images of immunoblots using Scion (Frederick, MD) Image 4.0 software.

Dot blot. Ten micrograms of protein were made up to 10 μl in H_20 and pipetted onto 0.45 μM nitrocellulose membrane (Pierce, Rockford, IL) and allowed to dry. The membrane was blocked for 45 min in 5% powder milk in TBS-T and then incubated in A11 (a generous gift from Charlie Glabe, University of California, Irvine, Irvine, CA) at 1:1000 overnight at 4°C. The membrane was then washed five times in TBS-T and incubated for 1 h in HRP goat anti-rabbit antibody (1:10,000; Sigma). After an additional five washes, the membrane was coated with ECL plus (Amersham Biosciences, Arlington Heights, IL) and then developed on photographic film. Quantitative densiometric analyses were performed on digitized images of immunoblots using Scion Image 4.0 software.

Statistics. Behavioral scores were analyzed using a multifactor or repeated-measures ANOVA including genotype, age, or experience as independent variables, and escape latencies during training and probe trial measures as dependent variables. To dissect complex interactions between factors, *post hoc* Scheffé's tests and Bonferroni's corrections were used to determine individual differences between groups. Unpaired, planned *t* tests were used to determine differences between animals in various studies. Immunohistochemistry scores were analyzed by ANOVA. Because of the numerous comparisons presented, individual *F* values are not included. For individual planned comparisons, results were reported as significant only when $p < 0.05$. For comparisons across multiple genotypes or ages, Bonferroni's correction factored into account the number of comparisons; therefore, when a single genotype was compared across ages 9, 12, 15, and 18 months or when all four genotypes were compared in a single analysis, results were considered significant only when $p < 0.01$ (0.05/4). Biochemical data were analyzed using planned Student's *t* tests.

Study 1. NonTg, PS1-KI, 3xTg-AD-h, and 3xTg-AD-H mice were tested longitudinally ("experienced" mice) at 2, 6, 9, 12, 15, and 18 months on the MWM as described previously (Billings et al., 2005). Cross-sectional studies were also conducted for all genotypes on MWM ("naive" mice) at 6, 9, 12, 15, and 18 months to be able to determine the benefit of previous exposure to this task in the longitudinal group. Some of the 12-month-old naive 3xTg-AD-H mice were killed as described above immediately after MWM testing to evaluate neuropathology via immunohistochemistry or ELISA.

Study 2: training effects on pathology. NonTg and 3xTg-AD-H mice were trained longitudinally on MWM at 2, 6, 9, and 12 months, and the 3xTg-AD-H mice were killed immediately after the 24 h MWM probe trial to evaluate the effects of longitudinal training on neuropathology. Tissue was processed for immunohistochemistry or ELISA as described above.

Study 3: post-pathology training and yoked swim. NonTg, 3xTg-AD-h, and 3xTg-AD-H mice were trained longitudinally on the hidden platform version of the MWM at 6, 9, and 12 months to evaluate the contribution of early, established pathology to the development of the observed training effect.

To evaluate the influence of exposure to the MWM testing environment without a learning contingency, NonTg, 3xTg-AD-h, and 3xTg-AD-H mice were handled as all other groups but, at 2, 6, and 9 months, were only made to swim four trials a day for the daily mean for their corresponding genotype from study 1. Thus, there was no platform in the pool when these mice were swimming ("yoked swim"), but all procedures were otherwise identical to MWM training. At 12 months of age, these mice were trained and tested in the hidden platform version of the MWM as in studies 1 and 2.

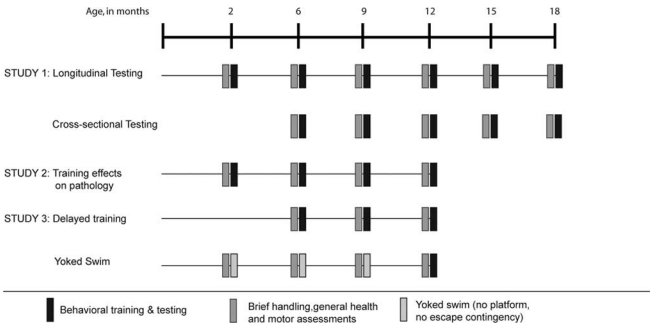

Figure 1. Study design. Separate groups of mice were trained and tested on the Morris water maze. In study 1, mice were trained either longitudinally or cross-sectionally. In study 2, a separate group of mice was trained longitudinally to 12 months of age, after which they were killed, and their brains were examined and compared with brains of mice trained only at 12 months of age for alterations in neuropathology. In study 3, mice were trained after the emergence of overt neuropathology (delayed training) to examine the ability of longitudinal training to affect established pathology. In addition, a group of yoked mice were subjected to similar handling, motor assessment, and swimming as the mice in studies 1 and 2 but were not trained to find a platform in the MWM. These mice were then trained on the MWM at 12 months of age to determine the contribution of previous learning, rather than exposure to the activity per se, on subsequent learning and memory in the MWM. For the number of animals in each study, see Table 1.

Results

Study 1: effects of longitudinal training on subsequent performance in a spatial learning and memory task

Training effect in Morris water maze acquisition

To determine the consequences of the progressive buildup of both plaques and tangles on spatial reference memory throughout their lifespan (study 1), mice of each genotype in the longitudinal (experienced mice; $n = 56$) and cross-sectional (naive mice; $n = 94$) groups were trained and tested on the hidden platform version of the MWM at 2, 6, 9, 12, 15, and 18 months (Fig. 1, Table 1). Regardless of genotype, at 2 months of age, all mice consistently located the hidden platform during training. By 6 months, however, both experienced and naive homozygous 3xTg-AD mice (designated as 3xTg-AD-H) were significantly impaired during acquisition of the MWM task (Figs. 2, 3). Notably, at the 9 month time point, experienced 3xTg-AD-H mice performed significantly better than age-matched naive 3xTg-AD-H mice on days 4–5 of training ($p \leq 0.01$ for both comparisons), and, although they still did not perform as well as experienced or naive NonTg mice, by days 4–6, all experienced 3xTg-AD mice reached criterion (<20 s escape latency) (Figs. 2*D*, 3*A*). In contrast, age-matched, naive 3xTg-AD-H mice required 7 d to reach criterion (Figs. 2*D*, 3*B*). A similar pattern was also observed in the hemizygous 3xTg-AD mice (designated as 3xTg-AD-h), whereby experienced 3xTg-AD-h mice were significantly better than their naive counterparts on days 1, 3, and 4 of MWM acquisition ($p \leq 0.01$ for all comparisons) (Fig. 2*C*). Therefore, 3xTg-AD mice trained and tested at 2 and 6 months showed a significant benefit at 9 months of age during acquisition of the MWM.

It is critical to point out that all genotypes exhibited this training effect, although the platform was moved to a completely novel location for each age of training. However, in the NonTg and PS1-KI mice, this training effect manifested, at best, as reduced latencies for experienced mice to reach the platform on the first 1–3 d of training, but naive groups quickly caught up (Fig. 2*A*,*B*). Such a training effect in PS1 mutant and NonTg animals has been reported previously (Janus et al., 2000; Vicens et al., 2003). Notably, however, a more dramatic effect of previous ex-

perience appeared in the 3xTg-AD mice at 12 months, whereby naive 3xTg-AD-H mice were severely impaired during acquisition, but, in contrast, experienced 3xTg-AD-H mice were virtually indistinguishable from experienced or naive NonTg mice, with the exception of a significant difference from experienced NonTg mice on days 1 and 3 of training ($p \leq 0.01$ vs NonTg mice on days 1 and 3) (Fig. 3*A*). Naive 3xTg-AD-H mice were significantly impaired relative to their experienced counterparts on every day of training except day 7 ($p \leq 0.001$ for all days except day 7; Bonferroni's correction for seven comparisons requires $p = 0.007$) (Fig. 2*D*). The same pattern was also true for experienced and naive 3xTg-AD-h mice, although naive hemizygous mice were significantly different from their experienced counterpart during every day of training except day 6, at which $p = 0.02$ (Bonferroni's correction requires $p < 0.007$) (Figs. 2*C*, 3). Therefore, the training effect that was evident at the 9 month testing was more pronounced at 12 months, such that experienced 3xTg-AD mice showed virtually no impairments during MWM acquisition. In contrast, naive 3xTg-AD mice exhibited progressive deterioration in MWM acquisition.

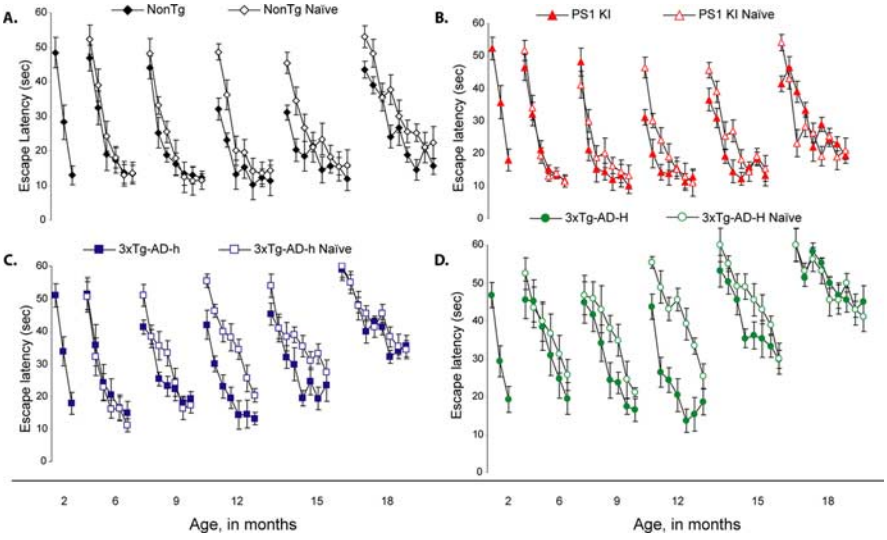

Figure 2. Longitudinal training improves MWM acquisition in the 3xTg-AD mice. Although all groups of experienced mice exhibited improved acquisition after repeated MWM training (***A–D***), this training effect was especially pronounced in 3xTg-AD mice. In particular, at 12 months of age, 3xTg-AD-h and 3xTg-AD-H mice were virtually indistinguishable from NonTg mice (***A***, ***C***, ***D***), whereas naive 3xTg-AD mice were severely impaired at every age after 6 months (***C***, ***D***). However, at 15 months of age, 3xTg-AD mice were markedly impaired in MWM acquisition regardless of whether they had been previously trained or not. Specifically, at 15 months of age, homozygous 3xTg-AD mice showed significant acquisition deficits (***D***). However, a genotype effect was still present with hemizygous 3xTg-AD mice continuing to exhibit a training effect at 15 months of age (***C***). Each cluster of results represents the daily means across each group. Error bars indicate SE.

The training effect was transient, and, by 15 months, the performance of the experienced 3xTg-AD-H mice was markedly reduced. At this age, there was only one day (day 4) during training when experienced mice were significantly better than naive 3xTg-AD-H mice ($p \leq 0.001$) (Fig. 2*D*). In contrast, experienced 3xTg-AD-h mice remained significantly better than naive 3xTg-AD-h mice during days 1, 5, and 7 of acquisition, in accord with the previously established age-dependent difference in pathology and cognitive deterioration between hemizygous and homozygous mice ($p < 0.001$) (Fig. 2*C*) (Oddo et al., 2003b; Billings et al., 2005).

By 18 months of age, neither experienced nor naive 3xTg-AD-H mice were able to consistently locate the platform location during MWM acquisition. This was also the only age at which 3xTg-AD mice exhibited deficits in the visual platform version of the MWM (data not shown). At this age, most mice floated or attempted to cling to the wall during training. Mice that did swim exhibited no discernable swim pattern, and any escapes were generally attributable to random encounters with, and subsequent clinging to, the platform. Similar to 15-month-old 3xTg-AD-H mice, both experienced and naive 3xTg-AD-h mice were markedly impaired at 18 months of age, and no training effect was apparent, although these mice eventually could find the platform in 30 s or less (Figs. 2*C*, 3).

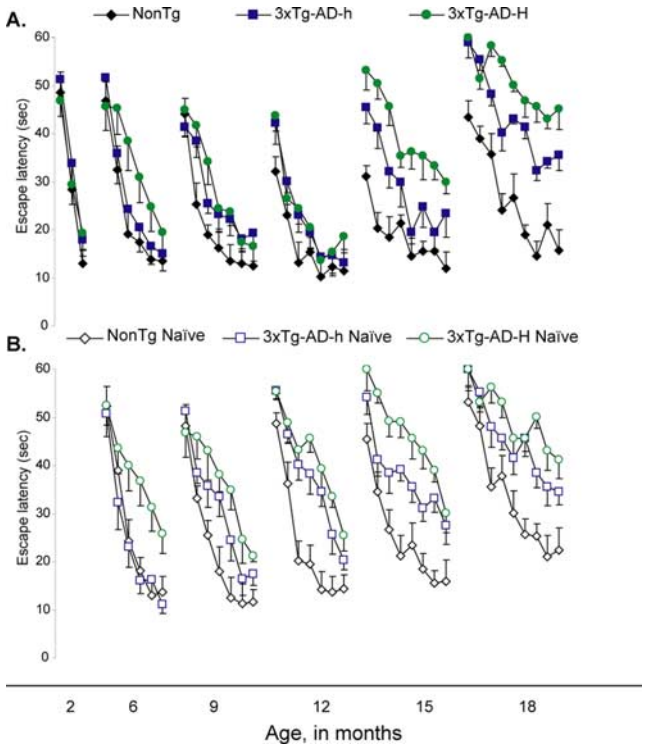

Figure 3. Previous experience on MWM induces a marked, albeit transient, improvement in acquisition. Naive 3xTg-AD mice (***A***) are markedly impaired during acquisition relative to NonTg mice, whereas experienced 3xTg-AD mice (***B***) exhibit a clear benefit of previous training that emerges at 9 months of age and gradually disappears by 15–18 months of age. Each cluster of results represents the daily means across each group. Error bars indicate SE.

Previous training improves memory for novel spatial locations

We next determined whether the training effect observed in learning also led to improved memory. Accordingly, retention was examined in probe trials 1.5 and 24 h after the last training trial. At 9 months of age, although both experienced and naive 3xTg-AD-h and 3xTg-AD-H mice were impaired on both probe trials, remarkably, we found that the experienced 3xTg-AD-H mice were significantly better than naive 3xTg-AD-H mice at both probe times ($p < 0.02$ for both comparisons). Furthermore, at 12 months of age, the influence of previous training on memory was even more striking. Neither experienced 3xTg-AD-h nor 3xTg-AD-H mice exhibited any deficit relative to NonTg mice at the 1.5 h probe trial ($p > 0.05$ relative to NonTg counterpart)

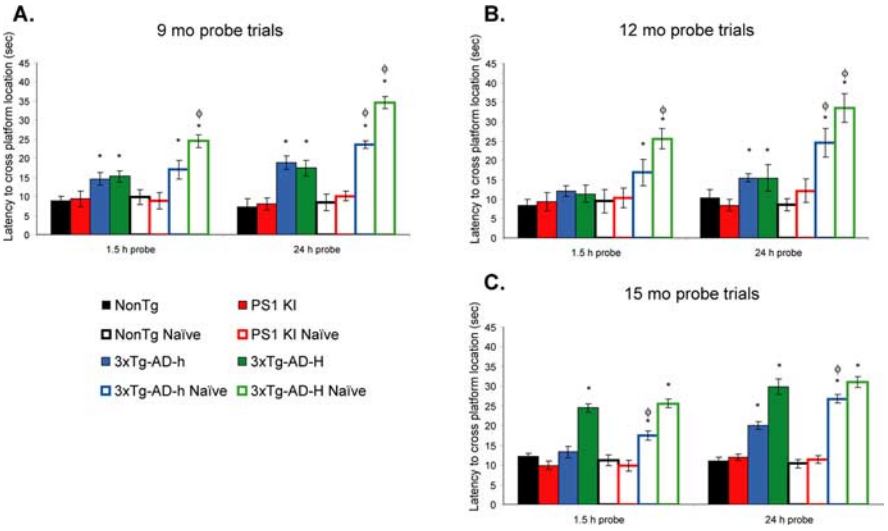

Figure 4. Previous training improves memory retention. At 9 months of age, experienced 3xTg-AD-H mice exhibit improved retention for the platform location at both 1.5 and 24 h after training (***A***). However, a more marked effect of training appeared at 12 months of age at which the 3xTg-AD mice exhibit no deficit at 1.5 h and are only impaired at 24 h (***B***), similar to 4-month-old 3xTg mice. By 15 months, however, previous training does not benefit memory because both experienced and naive 3xTg-AD mice are significantly impaired in both MWM probe trials (***C***). $^{*}p < 0.05$ with respect to NonTg mice; $^{\phi}p < 0.05$ with respect to the corresponding experienced group.

(Fig. 4, indicated by bracket). Although the 12-month-old experienced 3xTg-AD mice were impaired at 24 h, both genotypes, hemizygous and homozygous, nevertheless, still performed significantly better than the corresponding naive mice ($p < 0.02$ for both planned comparisons) (Fig. 4). To our knowledge, this is the first report to indicate that previous training in an AD transgenic model can benefit not only acquisition but retention as well.

As for the learning effect, the benefit of previous exposure on memory retention was also transient because, by 15 months, no discernible improvements on memory retention were apparent for the experienced 3xTg-AD-H mice during the probe trials ($p = 0.48$ experienced vs naive). Moreover, at this age, both experienced and naive 3xTg-AD-H mice were profoundly impaired relative to NonTg mice ($p < 0.002$ for all comparisons at 1.5 or 24 h). This finding was not surprising, because these mice were not able to reach the 20 s escape latency criterion even after 14 d of training (days 9–14 not shown). In contrast, experienced-3xTg-AD-h mice retained a training effect at this age. These mice showed no significant impairments at 1.5 h ($p = 0.37$ vs NonTg experienced) but were again significantly impaired at 24 h ($p = 0.018$ vs NonTg experienced), although significantly less so compared with naive 3xTg-AD-h mice ($p = 0.002$ experienced 3xTg-AD-h vs NonTg experienced; $p < 0.05$ naive vs experienced 3xTg-AD-h mice).

No probe trials were conducted at 18 months, because the 3xTg-AD-H mice failed to acquire the location of the platform during training, similar to their performance at 15 months. Notably, neither NonTg nor PS1-KI mice exhibited any deficits in memory during MWM probe trials at this advanced age (Fig. 4). Therefore, previous training not only improves acquisition but also retention for a spatial reference memory task.

Study 2: effects of previous training on AD-related neuropathology

Learning induces changes in Aβ localization and aggregation

Because previous training produced a clear and transient benefit on acquisition and retention, we next explored the effect it had on the Aβ and tau pathology in the 3xTg-AD mice. To determine the impact of longitudinal training on the levels of Aβ and tau pathology in the brain, additional groups of 3xTg-AD-H ($n = 8$) and NonTg ($n = 8$) mice were trained and tested on the MWM at 2, 6, 9, and 12 months longitudinally and were killed after the last training trial (Fig. 1). The brains from these experienced 3xTg-AD mice were compared with brains from the corresponding 12-month-old naive 3xTg-AD mice [from study 1, $n = 7$ (Fig. 1)] to determine whether the training effect could be attributed, in part, to training-induced alterations in Aβ and tau neuropathology. One-half of each hemisphere was used for immunohistochemistry, whereas the cortex and hippocampus were dissected from the other half and used for biochemical analysis. The mice in this longitudinal study (study 2) (Table 1) exhibited learning curves and memory retention virtually identical to the experienced mice in study 1 (data not shown).

Significantly, we found that both Aβ plaque load and insoluble $A\beta_{42}$ levels were markedly reduced in the experienced group compared with the naive animals (Fig. 5*A–D*), as measured by sandwich ELISA. No difference was seen in intraneuronal Aβ staining via immunofluorescence (data not shown), despite elevated soluble $A\beta_{42}$ in the experienced animals (Fig. 5*D*). We reported previously that intraneuronal accumulation of Aβ coincides with early cognitive impairments in 4-month-old 3xTg-AD mice and that spatial memory continues to worsen as extracellular Aβ levels increase (Billings et al., 2005). At ~9–12 months of age, extracellular Aβ plaques begin to develop, indicating an efflux of Aβ from inside the cell to outside in which plaque formation occurs (Oddo et al., 2006) as seen in the naive animals. This leads to an overall increase in total Aβ levels. From these initial observations, it seems that this normal redistribution of Aβ has been severely retarded in the experienced mice relative to the naive mice, resulting in Aβ pathology more similar to 4-month-old 3xTg-AD mice rather than the 12-month-old naive animals, namely intraneuronal Aβ accumulation and no detectable extracellular Aβ. Likewise, experienced animals present with deficits only at 24 h but not at the 1.5 h time point, consistent with the behavior in younger (4-month-old) mice (Billings et al., 2005). In contrast, naive mice display marked deficits at both 1.5 and 24 h probe trials. In agreement, we also observed reduced CD45 staining in the experienced animals, indicating fewer plaque-associated activated microglia, consistent with lower inflammation in younger animals (Kitazawa et al., 2005) (Fig. 5*A*). Inflammation has been associated previously with cognitive impairments (McGeer and McGeer, 1995, 1998); therefore, this decrease in activated microglia may account, in part, for the preserved cognitive phenotype observed in the experienced group.

To investigate changes that could explain the differences in the localization of Aβ in the experienced versus the naive animals, we analyzed the mechanisms that lead to both the generation and degradation of the Aβ peptide. Western blot analysis of APP and the Aβ precursor fragment C99 showed no differences between experienced and naive animals (Fig. 5*E*,*F*). Therefore, decreased production of Aβ is unlikely to account for the differences in Aβ

distribution between experienced and naive mice. To explore the possibility of increased Aβ degradation in the experienced mice, we evaluated levels of the two primary Aβ degrading enzymes, IDE and neprilysin. Surprisingly, steady-state levels of both degrading enzymes were substantially increased in the naive group relative to the experienced animals (Fig. 5*G*,*H*). Therefore, in this training paradigm, the reduction in extracellular Aβ in the experienced mice cannot be explained by increased enzymatic degradation. Indeed, it has been shown previously that neprilysin steady-state levels are dynamic, such that levels increase when Aβ levels are elevated (Mohajeri et al., 2002). This finding is consistent with the present results in which the levels of Aβ degrading enzymes are elevated in the naive animals that display a larger plaque load. Notably, this finding differentiates our study from environmental enrichment studies, in which increases in neprilysin mediates reductions in Aβ pathology (Lazarov et al., 2005).

The mechanism underlying the alteration in the distribution of Aβ from intracellular to the extracellular space that occurs ~12 months of age, as apparent in the naive animals, remains unknown. Possibilities include changes in plasma membrane composition that allow Aβ to pass through more readily, increased formation of Aβ oligomers, which can insert into the membrane as channels and then be extruded, or active transport of Aβ via a transporter or receptor such as the LRP receptor (Deane et al., 2004). Dot blot analysis of Aβ oligomers levels using conformation-specific antibody A11 revealed a trend for lowered Aβ oligomers in the experienced group ($p = 0.051$) (Fig. 6*A*,*E*). Immunoblotting of brain homogenates with 6E10 antibody revealed a 56 kDa band, which was significantly decreased in the experienced animals and to a similar amount as the A11 dot blot suggested, whereas APP levels remained constant (Fig. 6*B*,*E*). This band corresponds in size with Aβ 12-mer oligomers recently shown to correlate with cognitive deficits in Tg2576 mice, dubbed Aβ*56 (Lesné et al., 2006). Furthermore, injection of these Aβ oligomers into rat brain resulted in impaired spatial memory, similar to the deficits described here. To further confirm that this band was indeed Aβ oligomers, we repeated the immunoblot with monoclonal antibodies 4G8 and 20.1, antibodies against different regions of Aβ to 6E10, and found the same band (data not shown). More significantly, treatment of brain homogenates with 10% hexafluoroisopropanol (HFIP), a solvent known to break up Aβ oligomers by disrupting β-sheets, caused this band to dissipate, whereas APP levels remained intact (Fig. 6*C*).

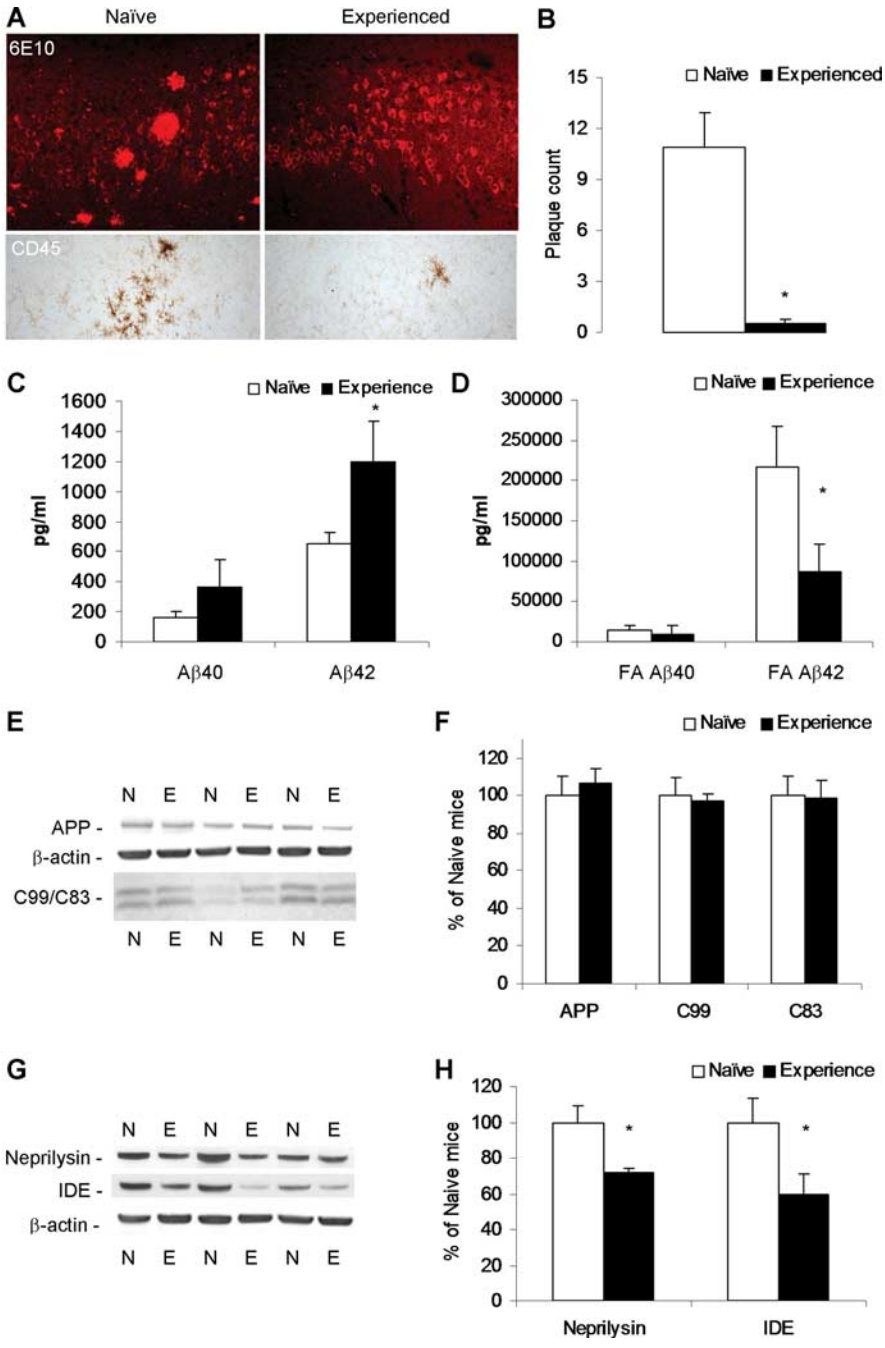

Figure 5. Experience decreases extracellular Aβ plaque formation. Aβ pathology was investigated in the 12 month experienced (denoted as E) 3xTg-AD mice and naive (denoted as N) 3xTg-AD-H mice to determine whether changes in pathology underlie cognitive improvements. 6E10 immunofluorescence illustrated increased Aβ plaque load and associated activated microglia (CD45) in the naive compared with the experienced mice (***A***). Quantification of plaque numbers revealed a significant decrease in the experienced animals (***B***). Correspondingly, sandwich ELISA $A\beta_{40}$ and $A\beta_{42}$ measurements showed increased soluble Aβ (***C***) but decreased insoluble Aβ in the experienced group (***D***). Steady-state levels of APP and the APP fragments C83 and C99 were unchanged (***E***) between groups as quantified and normalized to β-actin levels (***F***). Marked reductions in the Aβ degrading enzymes neprilysin and IDE were seen in the experienced animals (***G***) as quantified and normalized to β-actin levels (***H***), suggesting that Aβ plaque load did not stimulate increased Aβ degradation. *$p < 0.05$ significant differences with respect to naive mice.

In addition to oligomer formation, we looked at the putative Aβ transporter LRP and found small decreases in expression levels ($p < 0.05$) (Fig. 6*D*,*E*). Levels of ApoE, another reported Aβ

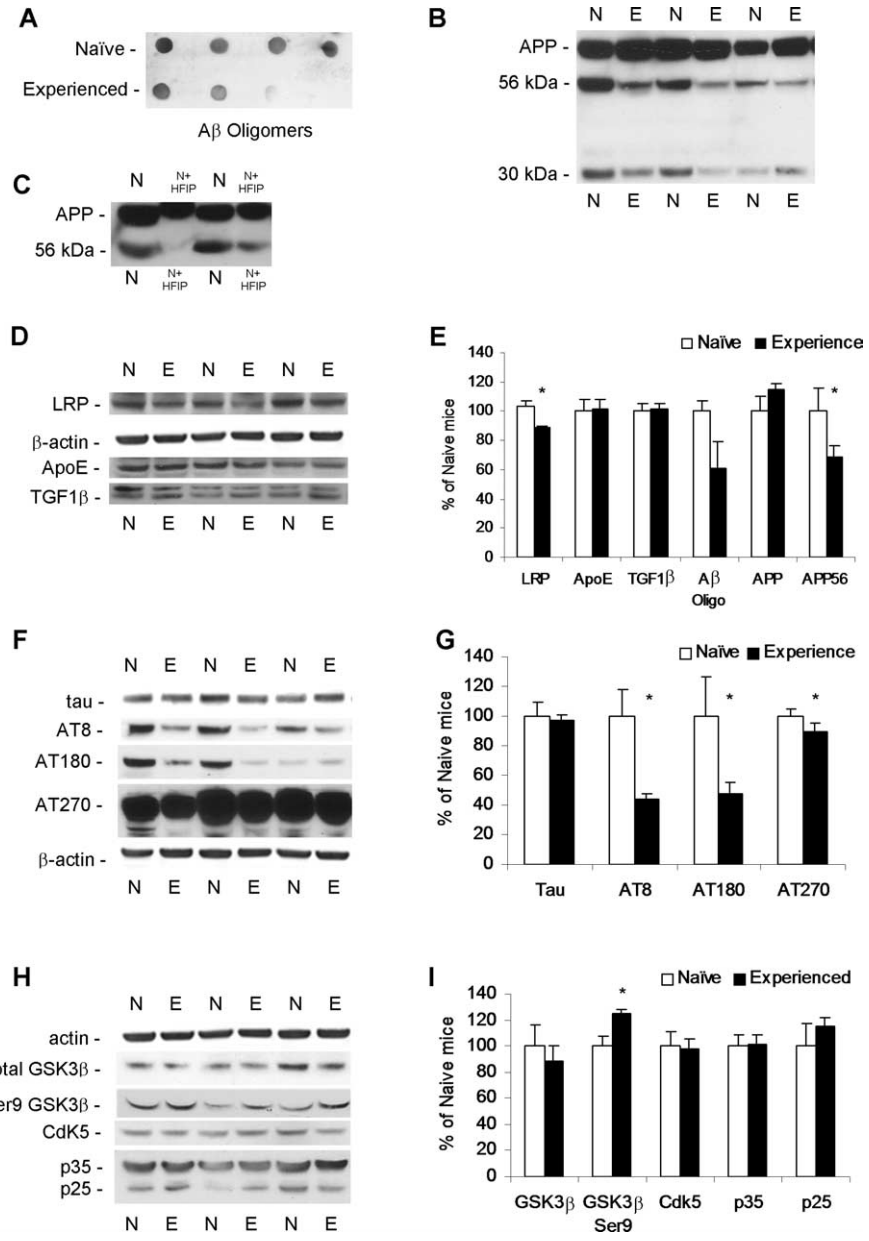

Figure 6. Experience diminishes Aβ oligomers formation and phospho-tau through GSK-3β. Additional characterization of Aβ pathology in the experienced (denoted as E) 12 month 3xTg-AD mice against the naive (denoted as N) animals revealed a decrease in Aβ oligomers levels, as shown by A11 dot blot (***A***, quantified in ***E***; $p = 0.051$). Analysis of 6E10 blots revealed differences in a band picked up at 56 kDa, which has been linked previously to cognitive decline, which significantly decreased in the experienced animals (***B***). In support of this band being, at least in part, made up of Aβ oligomers (12-mers), 10% HFIP treatment, which is known to break up oligomers, was found to decrease this band (***C***). To explore further why Aβ pathology was not relocalized in the experienced animals, as in the naive, putative Aβ transporter steady-state levels were investigated (***D***), and significant reduction was seen in LRP light chain but no differences in ApoE or TGF1β, as quantified and normalized to β-actin levels (***E***). Given the apparent retardation of Aβ pathology in the experienced group and the sequential relationship between Aβ and tau pathology, phosphorylated tau steady-state levels were studied. Whereas steady-state levels of total tau were unaltered, phosphorylation at AT8, AT180, and AT270 sites were significantly reduced in the experienced animals (***F***) as quantified and normalized to β-actin (***G***). Steady-state levels of GSK3β or Cdk5 were unaffected, as were levels of p25 and p35, which can underlie tau phosphorylation (***H***). However, phosphorylated GSK3β at Ser9, an inhibitory phosphorylation site, was significantly increased in the experienced group, indicating decreased GSK3β activity as quantified and normalized to β-actin (***I***). $^*p < 0.05$ with respect to naive mice.

transporter (Koistinaho et al., 2004), were unchanged between the groups, as were levels of TGF1β, which has been shown to increase intracellular Aβ levels by reducing secreted Aβ (Mazur-Kolecka et al., 2003) (Fig. 6*D*,*E*). Thus, it appears that repeated learning delays the age-related shift in Aβ from the intracellular to the plaque-forming extracellular compartment, perhaps mediated by lowered aggregation of Aβ, and that these changes underlie the preserved cognitive phenotype seen in the experienced animals. In other words, all of these changes that occur between 9 and 12 months of age in the 3xTg-AD mice, such as increased insoluble Aβ and plaque load, increased activated microglia, and increased formation of Aβ oligomers, in particular Aβ*56, contribute to cognitive decline but are delayed by repeated learning and thereby mitigate the cognitive decline in the 3xTg-AD mice.

Learning leads to decreased GSK3β activity and tau phosphorylation

In the 3xTg-AD mice, the appearance of phosphorylated tau within the somatodendritic compartments of hippocampal and cortical neurons coincides with an additional deterioration in cognition and memory. We found that phosphorylation of human tau was increased at the AT8, AT180, and AT270 sites in the naive animals, whereas overall steady-state levels of human tau were unaltered (Fig. 6*F*,*G*). Phosphorylation of tau is regulated by various protein kinases and phosphatases. Two major kinases, Cdk5 and GSK3β, have been shown to participate in the pathological hyperphosphorylation of tau (Kobayashi et al., 1993; Flaherty et al., 2000). Steady-state levels of Cdk5 and its activator p25 (Lew et al., 1994) were similar between experienced and naive mice, as were steady-state levels of GSK3β (Fig. 6*H*,*I*). However, levels of inactive GSK3β, which is phosphorylated at Ser9 (Dajani et al., 2001), were higher in the experienced group, suggesting reduced GSK3β activity in these mice. Therefore, longitudinal training appears to have established a state that favors inactive GSK3β, resulting in less tau phosphorylation.

Study 3: role of established pathology and effects of yoked swim on training effect

Training effect is influenced by established pathology

We next determined whether it was necessary for training to occur before the onset of overt Aβ and tau pathology to have a beneficial effect on learning and memory. Recall that, in our original experimental paradigm, the first training round for the experienced mice occurred at 2 months of age, which is before the development of overt Aβ and tau pathology in the 3xTg-AD mice (Oddo et al., 2003a,b). Consequently, we tested 3xTg-AD-H ($n = 10$), 3xTg-AD-h ($n = 7$), and NonTg ($n = 9$) mice beginning at 6 months of age, which corresponds to a time point after which Aβ has accumulated within neurons of the hippocampus and cortex (Oddo et

al., 2003a,b; Billings et al., 2005). Notably, 3xTg-AD-H mice trained and tested at 6, 9, and 12 months of age ("delayed training") showed reduced cognitive performance during MWM acquisition at 9 and 12 months compared with the experienced 3xTg-AD-H mice from study 1, in which the first training began at 2 months, before the Aβ pathology (Fig. 7*C*). Specifically, during acquisition, 3xTg-AD-H mice in the delayed training group were significantly worse on day 4 at the 9 month training and on days 2–4 at the 12 month training ($p < 0.02$ at 9 months, $p < 0.002$ for 12 month comparisons) (Fig. 7*C*). Hemizygous mice showed a significantly less robust difference between mice trained from 2 months of age (experienced, study 1) and animals trained beginning at 6 months (Fig. 7*B*). This effect is likely attributable to low levels of pathology in 6-month-old hemizygous mice.

The importance of training before the emergence of overt pathology was more striking during the probe trials, in which delayed-training 3xTg-AD-H mice were significantly worse than experienced 3xTg-AD-H mice on both probe measures at 9 and 12 months ($p < 0.05$ for all planned comparisons) (Fig. 7*F*). This pattern was not evident in hemizygous or NonTg mice. Thus, training before Aβ and somatodendritic tau accumulation in 3xTg-AD-H mice appears to be necessary to establish the full benefit of training on subsequent learning and memory.

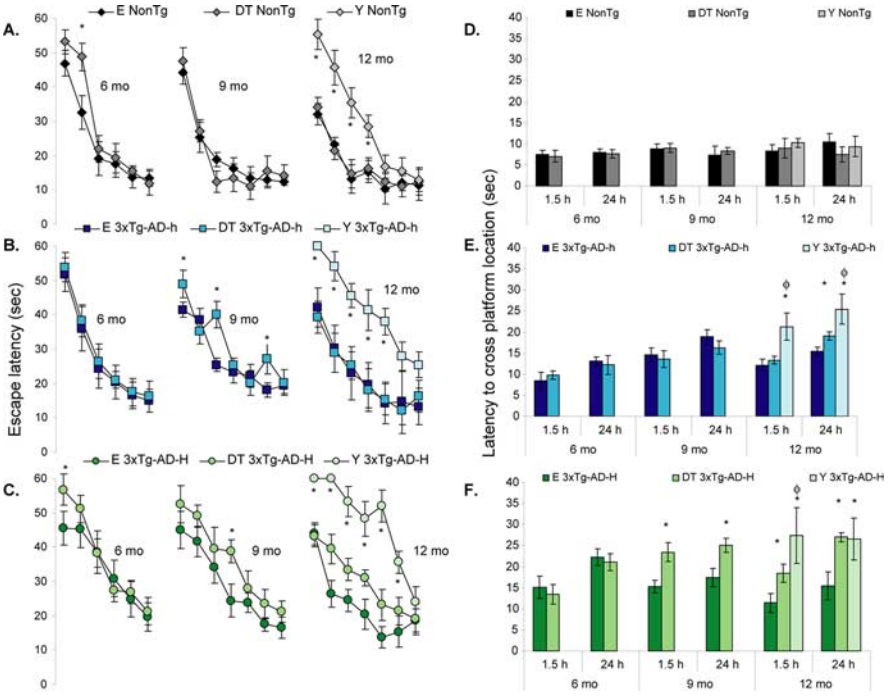

Figure 7. Training before the development of overt neuropathology is required for full benefit of longitudinal training. In the initial longitudinal study (see Fig. 1) (experienced mice, denoted as E), mice were trained and tested before the development of overt neuropathology. To assess the importance of training before the emergence of overt Aβ and tau neuropathology, a separate group of mice were trained and tested at 6, 9, and 12 months after they had developed early Aβ and tau deposits (delayed training mice, denoted as DT). In addition, a separate group of mice were made to swim the daily mean only at 2, 6, and 9 months but were trained and tested as all other groups at 12 months of age. This yoked (denoted as Y) group was included to control for exposure to the handling and the learning environment in the absence of a learning contingency. Although the 3xTg-AD DT mice learned similarly to experienced mice (***B***, ***C***), homozygous 3xTg-AD mice trained after the emergence of overt neuropathology were significantly impaired during probe trials relative to experienced mice at 9 and 12 months of age (***F***). Yoked mice were substantially impaired during acquisition, across all genotypes (***A–C***), perhaps attributable to a learned helplessness in response to the water maze. However, the NonTg yoked mice eventually acquired the task and exhibited no impairment during probe trials (***D***). In contrast, although the yoked 3xTg-AD mice eventually reached MWM criterion during training (***B***, ***C***), they were significantly impaired during probe trials (***E***, ***F***). $^{*}p < 0.05$ with respect to NonTg mice; $^{\varphi}p < 0.05$ with respect to the corresponding experienced group.

We also examined whether the effect of training on memory was attributable to an actual learning process or whether it was simply attributable to an effect of stress and/or enrichment from being exposed to the testing environment, being handled, and undergoing the motor assessments at each training stage. To examine the role of learning in the training effect, we analyzed a separate group of 3xTg-AD-H ($n = 8$), 3xTg-AD-h ($n = 8$), and NonTg ($n = 7$) mice that were handled similarly to the post-pathology and experienced groups but that were placed into the MWM without a learning contingency (i.e., only made to swim without a platform for the daily group means for their experienced counterparts) at 2, 6, and 9 months. At 12 months of age, these yoked mice were then trained and tested on the hidden platform MWM task to determine whether they would also show a training effect from having simply been exposed to the same learning environment. Remarkably, all three genotypes showed extensive impairments during acquisition of the hidden platform version of the MWM at 12 months, and, although all groups eventually reached a <30 s escape latency criterion (Fig. 7*A–C*), it is clear that they were relearning the contingencies of the task (presence of platform vs nothing in the tank). Although all groups of yoked mice eventually learned the task, both hemizygous and homozygous yoked 3xTg-AD mice were significantly impaired relative to experienced 3xTg-AD mice during both probe measures at 12 months (for all yoked vs experienced comparisons, $p \leq 0.002$) (Fig. 7*E*,*F*). In contrast, yoked NonTg mice showed no differences during probe trials compared with their experienced or delayed-training counterparts (Fig. 7*D*). These findings highlight the importance of cognitive stimulation before the onset of overt neuropathology, as well as the potential importance of engaging brain systems that are relevant to memory, in the protective effects of behavioral activity.

Discussion

Our findings indicate that early and repeated spatial water-maze training can profoundly affect the subsequent cognitive performance and neuropathology of the 3xTg-AD mice. Mice of all genotypes given repeated training (longitudinal paradigm) on the MWM subsequently exhibited improved acquisition and also demonstrated improved memory for newly acquired platform locations. Notably, particularly robust improvements were observed in the 3xTg-AD-H mice that brought their performance to NonTg levels at 12 months of age. In contrast, 3xTg-AD mice of different ages trained only once (i.e., in a cross-sectional paradigm) exhibited a progressive decline in learning and memory from 2 to 18 months of age. Remarkably, the training benefit exhibited by the longitudinally trained mice up to the age of 12 months was accompanied by a significantly reduced plaque burden, a decrease in insoluble and oligomeric Aβ, as well as a reduction in somatodendritic phospho-tau. These findings fit well with reports from studies in humans that suggest that life-long

learning as well as mental and physical activity may promote successful aging (for review, see Fillit et al., 2002). Indeed, the findings presented here suggest that such mental activity not only promotes successful aging as indicated by cognitive performance but can also delay emerging pathology associated with a neurological disease state.

The truly remarkable aspect of these findings is the vast global protective effect that repeated cognitive stimulation has on AD pathology, although the repeated stimulation is only once every 3 months. Our observations indicate that learning has helped to delay the normal age-related progression of both Aβ and tau pathology, and the associated cognitive decline, in the 3xT-AD mice. Therefore, at the testing time point of 12 months of age, insoluble and oligomeric Aβ levels are reduced as is phosphorylation of tau. Previously, the field has seen hints that the age of the brain allows certain AD pathologies to develop, most notably in humans in which the biggest risk factor for AD is aging. In other words, a younger brain is likely to exhibit more protective mechanisms to prevent advanced AD pathologies from developing. This is clearly seen in the 3xTg-AD mice and other mouse models of AD in which pathologies develop in an age-dependent manner. For example, in the brains of young mice, we observe intraneuronal Aβ and early tau accumulation, whereas at later stages, we observe extracellular plaque formation and tau phosphorylation, despite constant and stable expression levels of the transgenes from birth. One explanation could be that it takes a long time for these pathologies to develop, so that one has to wait 12–15 months for Aβ levels sufficient to form plaques. However immunotherapy studies have dispelled this: total clearance of both extracellular Aβ plaques and intraneuronal Aβ via 6E10 immunotherapy occurs within 7 d in the aged 3xTg-AD mice (Oddo et al., 2004; Billings et al., 2005). Subsequently, Aβ plaques return to their preimmunotherapy levels within just 30 d, despite taking 15 months to appear in the first place. Hence, the aged brain appears to lose a protective mechanism that, in young mice, prevents the formation of such advanced AD pathologies. From the present results, it appears that repeated cognitive stimulation somehow increases the protective mechanisms, and thus the development of more advanced AD pathologies becomes delayed. Of particular interest was our finding that both insoluble and oligomeric Aβ are reduced in the experienced mice relative to the naive animals and the relationship of this change to cognition, given that the experienced mice were far less cognitively impaired than were the naive animals. Of particular relevance is the recent discovery that the Aβ 12-mer, dubbed Aβ*56, is highly linked to spatial memory deficits in the Tg2576 mouse model of AD (Lesné et al., 2006). This Aβ oligomer was found to occur predominantly in the extracellular space and found to impair spatial retention in the absence of neuronal loss, similar to what we described in the 3xTg-AD animals. We find that levels of this Aβ*56 are elevated at 12 months of age in the naive animals, at a time when Aβ pools shift to a primarily extracellular localization, suggesting that this shift and consequent elevated extracellular Aβ levels allow this 12-mer to form at a time when we see considerable cognitive decline. Therefore, if this Aβ 12-mer is, as reported, a key determinant of spatial memory impairment, then the reduction we see could explain the improved spatial memory we see in these experienced mice versus the naive animals. In support of Aβ oligomerization playing a role in the shift in Aβ pools to an extracellular location in which plaques form, the "arctic" mutation in APP, which causes increased Aβ oligomerization, when overexpressed in mice leads to Aβ plaque formation much younger than overexpression of un-mutated APP (Lord et al., 2006). Speculatively, Aβ oligomers could either form intracellularly and pass through the membrane or form within the extracellular space and act as seeds for plaques drawing more Aβ from the intracellular compartments. Of course, it is likely that the improved cognition we see in the experienced animals is attributable to a sum of decreased insoluble and oligomeric Aβ, decreased inflammation, and decreased tau phosphorylation, and we cannot say for certain the relative individual contributions of each of these pathologies to the improved cognition under the current paradigm or that, for example, reduced phospho-tau levels were a consequence of reduced Aβ oligomers.

The robust improvement in subsequent learning and memory in this training paradigm speaks to the importance of the activation of cellular consolidation processes and the role of downstream brain regions in maintaining active synaptic connections and plasticity. The magnitude of this training effect in 3xTg-AD mice in light of our experimental design is not altogether surprising given that (1) a training effect has been described previously in nontransgenic animals, and (2) consolidation processes have been shown to last for several hours after training (for review, see Alvarez and Squire, 1994; McGaugh, 2005). It is important to note that, under the conditions described here, the learning-induced reductions in Aβ and tau pathology were transient, although we cannot exclude the possibility that more frequent or rigorous cognitive stimulation might have produced longer effects. By 18 months of age, Aβ and tau levels of the repeatedly trained 3xTg-AD mice were elevated above their 12 month levels and comparable with those of naive mice. The 3xTg-AD mice also performed poorly on the MWM at 15 months, despite exhibiting a significant training benefit only 3 months earlier.

The findings of several studies indicate that extensive mental activity can provide some protection against AD. For example, clinical studies suggest that the incidence of AD is inversely correlated with educational level and that individuals with superior linguistic abilities are less likely to develop AD later in life (Katzman, 1993; Snowdon et al., 1996). Importantly, findings of animal studies indicate that increasing the complexity of an environment can protect against neurodegeneration. Both environmental enrichment and extensive exercise, distinct methodologically from the paradigm used here, have been shown to increase dendritic branching, neurogenesis, and the number of synapses in brain regions involved in learning and memory (Greenough et al., 1972, 1979; Greer et al., 1982; Comery et al., 1996; Biernaskie and Corbett, 2001; van Praag et al., 2005). The precise mechanisms underlying this increased plasticity may include a wide variety of neuroprotective factors, including increases in neurotrophic factors such as BDNF (Falkenberg et al., 1992; Zhao et al., 2001; Alonso et al., 2002; Pham et al., 2002). In AD mouse models, environmental enrichment has been shown to decrease pathology through mechanisms that are distinct from those described here (Lazarov et al., 2005), suggesting various routes by which different types of neural stimulation can be protective against AD-like pathology. Regardless, the present study demonstrates that a specific protocol of prolonged cognitive stimulation offers transient benefits for learning and memory and attenuates the neuropathology in a mouse model of AD. These findings add to a growing body of evidence that highlight the plasticity of the brain, including the diseased brain. More significantly, it is important to note that, although this beneficial effect was transient, it produced disease-modifying effects, attenuating the neuropathology through nonpharmacological means. It is our belief that this type of behavioral modification with pharmacological- or

biological-based interventions may provide the greatest chance for successfully treating this insidious disorder.

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