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Long-term prevention of Alzheimer's disease-like behavioral deficits in PDAPP mice carrying a mutation in Asp664

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Abstract

The deficits of Alzheimer's disease (AD) are believed to result, at least in part, from neurotoxicity of β -amyloid (A β), a set of 38–43 amino acid fragments derived from the β -amyloid precursor protein (APP). In addition, APP generates the APP-C31 and Jcasp toxic fragments intracellularly by cleavage at Asp664. We reported that mutation of Asp664 to A in a FAD-human APP transgene prevented AD-like deficits but did not affect A β production or deposition in PDAPP mice, arguing that D664A plays a crucial role in the generation of AD-like deficits. Whether D664A simply delays or completely prevents AD-like deficits, however, remained undefined. To address this question, we performed behavioral studies longitudinally on a pretrained mouse cohort at 9 and 13 months of age. While behavioral deficits were present in PDAPP mice, performance of Tg PDAPP(D664A) mice was not significantly different from non-Tg littermates' across all ages tested. Moreover, aberrant patterns in non-cognitive components of behavior in PDAPP mice were ameliorated in PDAPP(D664A) animals as well. A trend towards poorer retention at 9 mo and poorer learning at 13 mo that did not reach statistical significance was observed in PDAPP(D664A) mice. These results support and extend recent studies showing that cleavage of APP at Asp664 (or protein-protein interactions dependent on Asp664) is a crucial event in the generation of AD-like deficits in PDAPP mice. Our results thus further demonstrate that the D664A mutation either completely precludes, or markedly delays (beyond 13 mo) the appearance of AD-like deficits in this mouse model of AD.

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Keywords

Memory; transgenic mice; amyloid; Morris water maze; caspase; behavior

Alzheimer's disease (AD) is the most common form of dementia, and a serious healthcare issue. Deficits in AD are believed to result in large part from the toxic effects of amyloid-beta (A β), a peptide released after proteolysis of the amyloid precursor protein (APP). APP may also be cleaved intracytoplasmically at Asp664 by caspases [8,20], liberating a cytotoxic carboxy (C)-terminal peptide, APP-C31[6,20], which is thought to activate apoptosis through an N-terminal Smac/DIABLO-like motif[9]. The remaining membrane-associated fragment, Jcasp, was also shown to be toxic *in vitro*[23]. Various mechanisms mediating A β toxicity have been proposed on the basis of *in vitro* data, but genetic evidence for their relevance *in vivo* is still largely lacking. We recently showed[7,32] that a significant component of A β toxicity *in vivo* may be dependent on the cleavage of APP at Asp664, since transgenic mice otherwise identical to a well-characterized model of AD, PDAPP mice[13,27], but carrying a mutation that obliterates the Asp664 cleavage site in APP [PDAPP(D664A)mice] continue to produce and deposit A β , but do not develop AD-like deficits. One potential link between A β production and APP Asp664 cleavage has been described, with the demonstration that A β binds APP and induces APP multimerization[2,19,21,22,34,35], leading in turn to cleavage of the APP cytosolic tail at Asp664[21,22,34], followed by synaptic and neuronal damage[22,34].

We had previously shown[7,32] that AD-like deficits were abolished in two independent PDAPP(D664A) transgenic mouse lines, in the moderate-expressing PDAPP(D664A)B21 line up to 12 months of age (mo) and in the high-expressing PDAPP(D664A)B254 line until 7 mo. To determine whether mutation of Asp664 in high-expressing PDAPP(D664A)B254 mice delays or completely prevents AD-like pathology, we examined cognitive function in transgenic PDAPP and PDAPP(D664A)B254 animals at 9 and 13 mo and AD-like histopathology after completion of the study at 13 mo. Spatial learning and memory were assayed longitudinally on a mouse cohort that was trained and tested at 7[7], 9 and 13 mo with the Morris water maze (MWM) paradigm[26]. Our results indicate that obliteration of the Asp664 cleavage site ameliorates AD-like spatial learning and memory deficits in the high-expressor PDAPP(D664A)B254 transgenic line until late in life (13 mo), when A β -plaque deposition was increased by 50% and gliosis was similar to that in brains of the high-expressor PDAPP(J20) line. Moreover, aberrant patterns of behavior involving non-cognitive (emotional) processes in Tg PDAPP mice were also alleviated in Tg PDAPP(D664A) animals at all ages tested. These observations indicate that Asp664 cleavage site significantly contributes to the generation of cognitive and non-cognitive aspects of AD-like behavioural deficits in the PDAPP transgenic mouse model until late in life. It has been proposed that a significant component of A β toxicity is mediated by its interaction with APP and requires Asp664 *in vitro*[21,22,34] and *in vivo*[7,32]. The present study provides further support for the hypothesis that a major pathway mediating A β toxicity requires Asp664 and suggests that additional, non Asp664-dependent pathway(s) of A β toxicity may be operant that could underlie trends to poorer performance observed in aged Tg PDAPP(D664A) mice.

METHODS

Mice

A G-to-C mutation was introduced in the PDGF β -chain promoter-driven human APP minigene carrying the Swedish and Indiana mutations[13,27] that mutated Asp₆₆₄ (APP₆₉₅ numbering) to Ala (PDAPP(D664A)) to generate PDAPP(D664A) transgenic mice, as described[7]. Mice from the high-expressing PDAPP(D664A)B254 transgenic line were used in this study. Mice from the high-expressing PDAPP(J20) line were kindly provided by Dr. Lennart Mucke,

Gladstone Institutes for Neurological disease, San Francisco, CA. Non-Tg littermates were culled in equal proportions from both transgenic lines and used as controls. All transgenic lines were maintained by heterozygous crosses with C57BL/6J breeders (Jackson Laboratories, Bar Harbor, ME). All transgenic animals were heterozygous with respect to the transgene. After removal of overt ‘floaters’ (see **Methods, Behavioral Testing**, below), experimental groups at 9 mo were: non-Tg, n=15 (11 males, 4 females); Tg PDAPP(D664A), n=13 (10 males, 3 females); Tg PDAPP, n=10 (6 males, 4 females). All animals had been trained in the MWM previously. Experimental groups at 13 months were: non-Tg, n=10; Tg PDAPP(D664A), n=10; Tg PDAPP, n=8. All animals were males. Two Tg PDAPP animals showed no motivation to swim (“floaters”) at 13 months and were removed from the group. Two additional Tg PDAPP animals that had been trained at 9 mo in the MWM previously were added to the 13 mo group to preserve sample size.

Behavioral testing

The Morris water maze[26] (MWM) was used to test spatial memory. All animals had normal motor and visual skills as determined by sensorimotor tasks performed prior to testing. Swimming ability was assessed with a straight water alley (15 by 200 cm) containing a submerged (1 cm) 12 × 12 cm platform. No significant differences were observed in swimming abilities between groups. The procedure described by Morris et al. [26] was followed. All groups had been tested in the MWM at 7 mo previously[7]. Details are included as *Supporting Text*. Animals were monitored daily and weights recorded weekly. Mice showing no motivation to swim (“floaters”, spending more than 60% of trial time making no attempt to swim) were taken out of the study. All “floaters” were in the transgenic PDAPP(J20) group.

Quantitation of A β deposits and GFAP immunoreactivity

Standard immunohistochemical methods were used. Details are included as *Supporting Text*.

Statistical analyses

Statistical analyses were performed using GraphPad Prism (GraphPad, San Diego, CA). In two-variable experiments, as when scoring performance of different genotypes across several days of training, repeated measures two-way ANOVA followed by Bonferroni’s post hoc tests were used to evaluate significance of differences between group means. When analyzing one-variable experiments with more than 2 groups, as when scoring retention across genotypes in probe trials, significance of differences between means was evaluated using one-way ANOVA followed by Tukey’s post-hoc test. Significance of differences in means between two different groups or between the same group at two testing times were done with Student’s *t* test. Values of $P < 0.05$ were considered significant. Slopes (m) of curves were calculated using the LINEST function in MS EXCEL. Pearson correlation coefficients between variables were calculated with regression analyses performed using GraphPad Prism (GraphPad, San Diego, CA).

RESULTS

Reference memory in 9 mo TgPDAPP and TgPDAPP(D664A) mice

We previously showed that PDAPP(D664A) transgenic (Tg) mice carrying the D664A mutation do not develop learning and spatial memory deficits in the Morris water maze test [6,32], a widely used tool in the assessment of hippocampal-dependent learning and memory in rodents[26]. Whereas AD-like anatomical, histopathological and cognitive parameters had been examined in aged (12 mo) mice from the moderate-expressing PDAPP(D664A)B21 Tg line[7,32], AD-like deficits had been assessed in the high-expressing PDAPP(D664A)B254 Tg line only at the earliest age at which Tg PDAPP mice begin to show detectable impairments, which for spatial learning and memory deficits is ~7 mo. At this age, soluble A β levels in

PDAPP(D664A) mice from the high-expressor B254 line were increased by approximately 1.5-fold with respect to high-expressing PDAPP(J20) mice, in a manner proportional to their levels of expression of the FAD-hAPP transgene[7]. In this study, plaque deposition was not assayed in the 7 mo cohort examined in behavioural studies since at that age Tg PDAPP mice do not show overt plaque deposition. Saganich et al., however, described plaque deposits in Tg PDAPP(D664A)B254 mice as early as at 6 mo[32]. Whether plaque deposition, learning and memory would be affected by the D664A mutation in Tg animals from the high-expressing PDAPP(D664A)B254 line at later times in life remained to be determined. To answer this question, we used a longitudinal experimental design[3] to examine a mouse cohort that was trained and tested in the MWM at different times across their lifespan (7[7], 9 and 13 mo).

First, we examined groups of Tg PDAPP(J20), Tg PDAPP(D664A)B254 (PDAPP and PDAPP(D664A) mice henceforth) and non-Tg littermates at 9 mo. Nine mo Tg PDAPP, Tg PDAPP(D664A) and non-Tg littermates showed no deficiencies in swimming abilities, directional swimming or climbing onto a cued platform during pretraining and had no sensorimotor deficits as determined with a battery of neurobehavioral tasks. However, Tg PDAPP mice, but not the other experimental groups consistently showed a higher tendency to float (swimming at speeds lower than 0.025 m/s), which was exacerbated in some animals. Tg PDAPP mice showing no motivation to swim (“floaters”) were taken out of the study. Although we removed overt ‘floaters’ from the Tg PDAPP group, the tendency to spend a portion of trial time making no attempt to swim was still present in this group (Figure 5b). Tg PDAPP mice also showed an increased tendency to thigmotactic swim (with swim paths restricted to distances ~10 cm from the tank wall) (Figure 5a), which is frequent in animals that don’t learn well[1,3,40]. As shown in Figure 5, the percentage of time spent floating and engaged in thigmotactic swim was increased in the Tg PDAPP group at all ages tested. Floating artificially reduces distance travelled, resulting in short distances (hence better scores) even for animals that do not find the platform, and also decreases the measured average swim speeds in each trial. Thigmotactic swim artificially increases the time spent in the target quadrant in probe trials. Thus, to avoid potential confounds arising from these patterns of behavior in the Tg PDAPP group, we used latency to reach the escape platform as a measure of performance during both conventional and cued MWM training, and number of crosses over the former platform location as measure of performance during probe trials.

Consistent with our previous observations[7], Tg PDAPP mice showed significant deficits in performance both during training (Figure 1a) and in the probe trial (Figure 1b) at 9 mo. Performance of Tg PDAPP mice was significantly impaired with respect non-Tg littermates’ ($P<0.01$) and to Tg PDAPP(D664A) animals [$P<0.001$, as a result of Bonferroni’s post-hoc test applied to a significant effect of genotype ($F(2, 35)=10.71$; $P<0.0002$), repeated measures two-way ANOVA]. Performance of 9 month-old Tg PDAPP(D664A) mice during training was indistinguishable from non-Tg littermates’. No significant interaction was observed between day number and genotype, thus genotype had roughly the same effect at all times during training. Overall, session number did not significantly affect performance at 9 months ($F(3,105)=0.28$, $P=0.83$; repeated measures two-way ANOVA), suggesting that all groups might have reached their optimum attainable performance, albeit at different levels, after training at 7 mo. Even though percent time spent floating was higher for Tg PDAPP mice during the first 2 days of training, it decreased thereafter to levels similar to those of non-Tg and Tg PDAPP mice. Thus, no significant effect of genotype on percent time spent floating was observed ($P=0.27$, $F(3,105)=1.32$, repeated measures two-way ANOVA). This biphasic pattern was present, albeit at lower levels, in the latter groups as well (significant effect of swim number on percent time spent floating, $P<0.0001$, $F(3, 105)=10.21$, repeated measures two-way ANOVA). Percent time spent in thigmotactic swim was significantly higher in Tg PDAPP mice than in all other groups (Figure 5a) and this effect was uniform across days of training ($P<0.001$ and $P<0.01$ with respect to non-Tg and Tg PDAPP(D664A) groups

respectively, Bonferroni's post test applied to a significant effect of genotype on percent time spent in thigmotactic swim, $F(2,35)=13.34$, $P<0.0001$, repeated measures two-way ANOVA].

Spatial memory for location of the escape platform was then evaluated by counting the number of crosses over the former platform location for each group in a probe trial administered after the last training trial. Retention of the former platform site was not significantly different for the non-Tg and Tg PDAPP(D664A) groups (Figure 1b). In contrast, Tg PDAPP mice crossed the former platform site significantly fewer times than Non-Tg mice ($P<0.05$, Tukey's multiple comparisons test applied to a significant effect of genotype ($P<0.01$) in one-way ANOVA), suggesting impaired retention of the former platform site. Albeit retention in Tg PDAPP (D664A) mice was not significantly different from non-Tg littermates', in contrast to our observations at 7 mo [7], at this age retention in Tg PDAPP(D664A) mice was not significantly different from that of the Tg PDAPP group either. These results suggest that mild spatial memory impairments may have developed in Tg PDAPP(D664A) mice at 9 mo.

Reference memory in 13 mo TgPDAPP and TgPDAPP(D664A) mice

We next determined spatial learning and memory on Tg PDAPP, Tg PDAPP(D664A) and non-Tg littermates at 13 mo. Thirteen month-old Tg PDAPP, Tg PDAPP(D664A) and non-Tg littermates showed no sensorimotor deficits as determined with a battery of neurobehavioral tasks. Similar to our observations at 7 (Galvan et al. 2006) and at 9 mo (Figure 1), performance of Tg PDAPP animals during conventional training was significantly impaired with respect to that of non-Tg littermates' ($P<0.05$) as a result of Bonferroni's post-hoc test applied to a significant effect of genotype ($F(2,25) = 4.56$; $P<0.02$; repeated measures two-way ANOVA), Figure 2a]. Consistent with our observations at 9 mo, performance of Tg PDAPP(D664A) was indistinguishable from non-Tg littermates' ($P>0.05$). At 13 mo, however, performance of the PDAPP(D664A) group during training was not significantly different from that of Tg PDAPP mice either ($P>0.05$). Thus, and although performance of the Tg PDAPP(D664A) group was not significantly different from non-Tg littermates', the observed trend suggests that, in spite of an overall significant improvement in performance with respect to Tg PDAPP mice, deficits in spatial learning may develop in Tg PDAPP(D664A) with increasing age. Performance improved through session 2 for all experimental groups during training at 13 mo and mean latencies remained constant for the PDAPP(D664A) group or actually increased for the Tg PDAPP and non-Tg groups hereafter (Figure 2a). In contrast to our observations during training at 9 mo, session number significantly affected performance ($F(4,100)=3.77$; $P=0.007$; repeated measures two-way ANOVA), with improvements observed only for the PDAPP(D664A) and non-Tg groups, arguing that these groups had not attained optimal performance during training at 7 mo and that cumulative carry-over (savings) effects of experience on performance were not negated by aging. The interaction between genotype and session number was not significant ($F(8,100)=0.57$; $P=0.79$, repeated measures two-way ANOVA), thus genotype had the same effect at all sessions. The lack of interaction between genotype and session number during training both at 9 and 13 mo may have resulted from a tendency of Tg PDAPP mice to display a biphasic pattern of performance during training, with decreased average latencies in the first 4-6 trials followed by increased latencies in subsequent trials (Figures 1 and 2). This behavioral pattern was absent in younger Tg PDAPP(D664A) mice, which showed monotonically decreasing latencies in successive trials at 7 [7] and 13 mo. The same was true at 9 mo except for a trend to worse performance on the last session of training (Figure 1a) which did not reach statistical significance. These observations suggest that mild deficits in learning and a trend to a biphasic pattern of performance at 9 mo may develop with increasing age in Tg PDAPP (D664A) mice, but that these deficits in learning may be modified by savings (carry-over) effects of experience. On the other hand, while average latencies of the non-transgenic group monotonically decreased across sessions both at 7 and 9 mo, they also showed a trend to a biphasic pattern at 13 mo. In contrast to our observations at 7 [7] and 9 mo, percent time spent

floating increased for all groups at 13 mo (Figure 5b) and for all experimental groups across sessions [significant effect of session number on percent time spent floating, $P < 0.0001$, $F(4,100) = 6.35$, repeated measures two-way ANOVA]. Interestingly, and similar to our observations at 9 mo, percent time spent floating was higher in the Tg PDAPP group only during the first two sessions of training. Thigmotactic swim was uniformly higher for the Tg PDAPP group throughout training at 13 mo as well ($P < 0.01$ with respect to both non-Tg and Tg PDAPP(D664A) groups, Bonferroni's post hoc test applied to a significant effect of genotype on percent time spent in thigmotactic swim ($F(2,4) = 8.04$, $P = 0.002$, repeated measures two-way ANOVA)]. At 13 mo, however, percent time engaged in thigmotactic swim monotonically decreased across sessions for all groups [$F(2,100) = 3.5$, $P = 0.009$, repeated measures two-way ANOVA].

Percent time engaged in thigmotactic swim decreased with increased experience (Figure 5a), and percent time floating was not strongly correlated with latency ($R^2 = 0.13$). Thus, effects of anxiety (Figure 5a) or helplessness (Figure 5b) do not explain the observed pattern of performance of non-Tg mice at 13 mo. Therefore, our results suggest that aging *per se* may negatively affect learning in mice.

Spatial memory for location of the escape platform was then evaluated by counting the number of crosses over the former platform location for each group in a probe trial administered after the last session of spatial training at 13 mo. Surprisingly, retention of the former platform site was not significantly different for any experimental group at 13 mo (Figure 2b). In contrast to the memory impairments observed in the Tg PDAPP group at 7 and 9 mo, all experimental groups crossed the former platform site a similar number of times at 13 mo and this effect was due to improved retention in the Tg PDAPP group (Figures 1b and 2b), regardless of the observed deficits of this group during training (Figure 2a). These observations suggest that, in spite of showing significant deficits during acquisition, retention impairments in Tg PDAPP mice may be compensated or positively affected by carry-over (savings) effects of prior experience due to practice [24]. In cued conditions (Figure 3), all groups readily navigated to the visible platform and showed a significant difference in performance over trials (significant effect of trial number on performance, $F(5,125) = 5.72$; $P = 0.0001$; repeated measures two-way ANOVA). Both transgenic groups, however, showed a tendency to longer latencies in the first two sessions that were improved in later ones, although this differences reached significance only for the first swim when the non-Tg and the Tg PDAPP(D664A) groups were compared ($P < 0.05$ as a result of Bonferroni's post hoc test applied to a significant effect of genotype on performance, $F(2,25) = 6.34$; $P < 0.0059$, repeated measures two-way ANOVA). It is possible that the initial difference in performance observed for the transgenic groups, which has been documented for other hAPP transgenic models [16], could be caused by differences in their reactivity to novel testing conditions. Although latencies for the Tg PDAPP mice decreased monotonically across trials, this group performed significantly worse than non-Tg and Tg PDAPP(D664A) animals during the last trial of cued training ($P < 0.05$ as a result of Bonferroni's post hoc test applied to a significant effect of genotype on performance, $F(2,25) = 6.33$; $P < 0.0059$, repeated measures two-way ANOVA). This increase in average latency of Tg PDAPP mice in the last trial of cued training was not due to increased floating, since genotype did not have a significant effect on percent time spent floating during cued training, and percent time spent floating decreased significantly across trials for all groups [significant effect of trial number on percent time spent floating, $F(5,125) = 5.7$, $P = 0.001$, repeated measures two-way ANOVA]. The increase in average latency observed for the Tg PDAPP group in the last trial was not due to an increase in percent time engaged in thigmotactic swim at that trial either ($P > 0.05$, Bonferroni's post hoc test applied to a significant effect of genotype on percent time spent in thigmotactic swim, $F(2,25) = 5.91$, $P = 0.008$, repeated measures two-way ANOVA), although time spent in thigmotactic swim was significantly increased for the Tg PDAPP group during cued training with respect to non-Tg and Tg PDAPP(D664A) mice

($P<0.001$ and $P<0.05$ respectively). Thus, the increased latencies observed for the Tg PDAPP group during the last trial of cued training might be explained by learning deficits (Palop, Galvan, etc.) present in this group.

Effect of experience on performance of Tg PDAPP and Tg PDAPP(D664A) mice

Although Tg PDAPP mice showed significantly impaired learning of the platform location during conventional training with respect to non-Tg littermates ($P<0.001$) and with respect to Tg PDAPP(D664A) mice ($P<0.05$, Bonferroni's post hoc test applied to a significant effect of genotype, $F(2,2)=23.76$, $P<0.0001$, repeated measures two-way ANOVA) at all ages tested, all groups significantly reduced their average escape latencies across training sessions between 7 and 13 mo as evidenced by a significant effect of age at testing on performance [Figure 4a, $F(2,79)=6.57$, $P=0.002$, two-way ANOVA], suggesting that a positive effect of prior experience on learning can also be observed in Tg PDAPP mice across ages at testing. The interaction between genotype and session number was not significant ($F(4,79)=0.13$; $P=0.96$, two-way ANOVA), thus genotype had the same effect at all ages. Average latencies across training were not significantly different between non-Tg and Tg PDAPP(D664A) mice, indicating that impairments in spatial learning of Tg PDAPP mice are rescued in PDAPP (D664A) animals up to 13 mo.

Long-term retention in Tg PDAPP and Tg PDAPP(D664A) mice

To determine long-term retention of the platform location between different experiments, we compared average latencies of all groups at first and last trials during training at 7 [7], 9 and 13 mo. Consistent with the deficits observed in the Tg PDAPP group in a longitudinal analysis of performance (Figure 4a) Tg PDAPP mice showed significant differences in long-term retention of the platform location between experiments with respect to non-Tg and Tg PDAPP (D664A) animals across all ages tested ($P=0.0003$ as a result of Bonferroni's post hoc test applied to a significant effect of genotype [$F(2,5)=2.45$, two-way ANOVA, Figure 4b]). Although learning was significantly impaired in Tg PDAPP mice at 7 mo, the performance of this group improved during the last 3 sessions of training[7]. In spite of this improvement, which was consistent with prior studies showing that spatial learning is impaired but not absent in Tg PDAPP mice[29,31] average latencies of Tg PDAPP mice at the end of training at 7 mo were still higher than those at the beginning of training ([7], Figure 4b). Non-Tg and Tg PDAPP (D664A) animals showed long-term retention of the platform location between 7 and 9 mo. Average latencies of the Tg PDAPP group in the first trial at 9 mo were also similar to those at the end of training at 7 mo[7], suggesting long-term retention of the platform location at 9 mo (Figure 4b), albeit at significantly decreased levels than transgenic PDAPP(D664A) mice ($P<0.05$) and non-Tg animals [$P<0.05$, Bonferroni's post hoc test applied to a significant effect of genotype, $F(2,1)=5.85$, two-way ANOVA, $P<0.005$]. Interestingly, performance of transgenic PDAPP(D664A) and non-Tg mice at the beginning of training at 13 mo was worse than their performance at the end of training at 9 mo, being the difference significant for the non-Tg group ($P=0.013$) but not for the PDAPP(D664A) group ($P=0.3$, unpaired Student's *t* test), suggesting an age-related effect on long-term retention of the platform location for non-Tg and possibly for Tg PDAPP(D664A) animals, which may be associated with normal aging. No further decline was observed in performance of the Tg PDAPP group between the last trial at 9 months and the first one at 13 mo (Figure 4b). A detectable effect of age in long-term retention was therefore not observed in Tg PDAPP mice. Since learning is impaired in Tg PDAPP mice at all ages analyzed, it is possible that the lack of effect of age on retention is due to limitations of the experimental design (e.g., scores for latency cannot be higher than 60 s).

Non-cognitive components of behavior in Tg PDAPP and Tg PDAPP(D664A) mice

Making no attempt to swim (floating) and swimming in close proximity to the pool wall ('wall hugging' or thigmotaxis) are frequent performance deficits to which certain strains of mice are prone that are frequently more pronounced in mice that have been genetically manipulated [38,41]. These patterned behaviours are believed to be manifestations of emotional responses, such as anxiety and helplessness in stressing situations. Thigmotaxis is a pattern of behavior frequently displayed by animals that do not learn well[3]. Immobility, defined as the absence of active, escape-oriented behaviors such as swimming in the water tank is considered indicative of depressive-like behavior in experimental paradigms such as the forced swim test [4,30]. Percent time spent engaged in thigmotactic swim decreased significantly for all groups as a function of age [Figure 5a, significant effect of age at testing on percent time spent engaged in thigmotactic swim ($F(2,99)=25.64$; $P<0.0001$; two-way ANOVA), suggesting long-term effects of increased experience that may result in decreased anxiety. This effect was more pronounced for Tg PDAPP than for all other groups (slopes $m=-6.9$, $m=-6.5$, $m=-15.5$ for non-Tg, Tg PDAPP(D664A) and Tg PDAPP respectively). Tg PDAPP mice, however, spent a significantly larger percent of time engaged in thigmotactic swim than all other groups at all ages tested [$P<0.001$ with respect to both non-Tg controls and Tg PDAPP(D664A) mice as a result of Bonferroni's post hoc test applied to a significant effect of genotype ($F(2,2)=31.82$; $P<0.0001$; two-way ANOVA), suggesting that, in contrast to non-Tg littermates and Tg PDAPP(D664A) mice, Tg PDAPP animals may experience increased anxiety when facing challenging or relatively stressful situations such as swimming in a brightly lit open pool. In contrast, percent time engaged in thigmotactic swim in Tg PDAPP(D664A) mice was indistinguishable from the non-Tg group at all ages tested, suggesting that obliteration of the Asp664 site at the C-terminus of FAD-hAPP prevents the emergence of this behavioral pattern. It is possible that compensatory mechanisms such as hyperactivity may be activated in Tg PDAPP(D664A) mice that 'mask' the anxiety response and produce a behavior that mimics that of non-Tg animals. This alternative hypothesis is, however, unlikely since increased spontaneous activity is present both in PDAPP and PDAPP(D664A) mice[7,31] and may represent a general effect of overexpression of the FAD-hAPP transgene.

Even though overt 'floaters' were taken out of the study, significantly increased floating was observed in the Tg PDAPP group across the ages tested [Figure 5b, $P<0.05$ with respect to both non-Tg controls and Tg PDAPP(D664A) mice, Bonferroni's post hoc test applied to a significant effect of genotype ($F(2,2)=8.49$; $P=0.0005$; two-way ANOVA), suggesting that Tg PDAPP mice experience increased helplessness as compared to non-Tg littermates' in mildly stressful conditions such as those present during water maze training. However, and in contrast to the effect of age at testing on thigmotaxis, floating significantly increased in all groups with increasing age ($F(2,75)=3.3$, $P=0.04$, two-way ANOVA). Percent time spent floating in Tg PDAPP(D664A) mice was not significantly different from that of the non-Tg group, suggesting that obliteration of the Asp664 site at the C-terminus of FAD-hAPP largely prevents the appearance of this pattern of behavior, possibly by decreasing reactivity to stress. Adequate learning may act to reduce reactivity to stress. Consistent with this notion and with their improved retention at 9 mo (Figure 1b), Tg PDAPP mice showed reduced floating at this age. This effect, however, was negated by increasing age (Figure 5b). A trend to increased floating, that nevertheless did not reach statistical significance in a pair-wise comparison within the group, was also observed in non-Tg animals, suggesting that aging per se may increase floating behavior. Albeit at lower levels than Tg PDAPP mice, Tg PDAPP(D664A) mice also showed an age-dependent increase in the proportion of time spent floating between 9 and 13 mo ($P<0.04$, paired Student's t test, with a mean of differences of -10.9). Rates of increase in floating time were, however, significantly different between Tg PDAPP and Tg PDAPP(D664A) mice, with a 2X higher rate of increase in floating time for Tg PDAPP with respect to the Tg PDAPP(D664A) group (slopes $m=21.6$ for the rate increase in Tg PDAPP, $m=11$ for

that in Tg PDAPP(D664A) mice). While the observed increase in floating time for all groups with increasing age suggests that augmented helplessness in the face of stress may be a feature of 'normal' aging processes in mice, our results indicate that Tg mice expressing FAD-hAPP may experience significantly higher levels of helplessness with respect to non-Tg littermates with increasing age. This behavioral pattern, however, was significantly attenuated in Tg PDAPP(D664A) animals.

Percent time spent engaged in thigmotactic swim and floating were not correlated across subjects at any age.

A β deposition in 13 month-old PDAPP and PDAPP(D664A) transgenic mice

Previous studies have shown that the D664A mutation does not affect overall A β 40 and A β 42 production in cultured cells[36,39], and similar results were obtained by us *in vivo*[7,32]. In those studies, plaque deposition was examined in older (12 mo) mice from the moderate-expressing PDAPP(D664A)B21 transgenic line and in younger (6 mo) mice from the high-expressing PDAPP(D664A)B254 transgenic line. While plaque deposition was documented in the high-expressing PDAPP(D664A)B254 transgenic line at ages that preceded overt plaque deposition in Tg PDAPP mice (6 mo), whether plaque deposition would be affected by the D664A mutation in transgenic animals from the PDAPP(D664A)B254 line at more advanced ages remained to be determined.

To ascertain whether cleavage of APP at Asp664 had an effect on plaque deposition in older animals, we examined brain sections from aged (13 mo) Tg PDAPP and PDAPP(D664A) animals after completion of behavioral studies by immunohistochemistry, using an antibody directed against the middle portion of A β (4G8). Quantitative determinations of 4G8 immunoreactivity in brain sections of 13 mo Tg PDAPP and PDAPP(D664A) mice showed multiple 4G8-immunoreactive A β deposits restricted to the hippocampus and cortex (PDAPP=78.3 \pm 8.3, PDAPP(D664A)=119.3 \pm 18.2 total; PDAPP=62 \pm 9.5, PDAPP(D664A)=97 \pm 15.3 hippocampal; PDAPP=16.3 \pm 1.2, PDAPP(D664A)=22.3 \pm 5.3 cortical plaques per section, Figure 6a). Plaque deposition in brains of Tg PDAPP(D664A) mice from the high-expressor PDAPP(D664A)B254 line was thus ~50% higher than in Tg PDAPP animals from the J20 line at 13 mo, consistent with the levels of expression of the hAPP transgene in each Tg line as well as with their A β production levels, which are ~20–25% higher and ~60% higher for A β 42 in PDAPP(D664A)B254 mice with respect to PDAPP(J20) mice respectively[7, 32]. Amyloid plaques in brain sections from both Tg PDAPP and Tg PDAPP(D664A) mice were fibrillar in nature, as evidence by thioflavin-S staining (data not shown). Taken together, these results confirm our previous observations in aged animals from the moderate-expressing PDAPP(D664A)B21 transgenic line [7,32], indicating that mutation of Asp664 in the intracytoplasmic domain of APP has neither an overt quantitative nor qualitative effect on the deposition of β -amyloid *in vivo*.

Astrogliosis in hippocampi of 13 month-old PDAPP and PDAPP(D664A) transgenic mice

Astrogliosis occurs in many neurodegenerative diseases, including AD[37]. This phenomenon is recapitulated in transgenic PDAPP mice as described[7]. In those studies, increased astrogliosis was detected in 12 mo Tg PDAPP mice from the J20 line but not in Tg PDAPP (D664A) mice from the moderate-expressing PDAPP(D664A)B21 line at the same age[7]. To determine whether astrogliosis would be present in 13 mo animals from the high-expressing PDAPP(D664A)B254 transgenic line, we stained brain sections from Tg PDAPP(J20) and Tg PDAPP(D664A)B254 mice with GFAP antibodies after completion of behavioral studies at 13 mo. Quantitative determinations of GFAP immunoreactivity revealed that the area of GFAP immunoreactive signal in hippocampi of Tg PDAPP mice was significantly increased with respect to non-transgenic littermates' ($P < 0.05$, as a result of Tukey's post hoc test applied to

a significant effect of genotype ($P < 0.0001$) in one-way ANOVA), as previously shown[7]. However, GFAP immunoreactivity in hippocampi from 13 mo Tg PDAPP(D664A)B254 mice was also significantly increased with respect to non-transgenic littermates' ($P < 0.001$, Tukey's post hoc test applied to a significant effect of genotype ($P < 0.0001$) in one-way ANOVA), but was not significantly different from PDAPP Tg mice (Figure 6b). Thus, and in contrast to our observations in Tg PDAPP(D664A) mice from the moderate-expressing B21 line[7], astrogliosis was present in Tg PDAPP(D664A)B254 mice in a manner roughly proportional to the levels of expression of the hAPP transgene and consistent with the observed 50% increase in plaque deposition in these brains (Figure 6a). These results suggest that astrocytic proliferation may constitute a response to the levels of expression of the hAPP transgene and/or to the levels of A β production and deposition in this mouse model of AD. Therefore, we conclude that the D664A mutation did not affect astrogliosis in the PDAPP mouse model of AD. Since astrogliosis was present in brains from Tg PDAPP(D664A) mice in the absence of detectable behavioral abnormalities, our results suggest that astrogliosis does not have a pathogenic role in this mouse model of AD.

DISCUSSION

We showed previously[7,32] that mutation of Asp664 in the C-terminal domain of a FAD-hAPP transgene abolishes AD-like deficits in the PDAPP mouse model. AD-like deficits were absent in PDAPP(D664A) transgenic mice from the moderate-expressor B21 line up to 12 mo (mo)[32] and in the high-expressor PDAPP(D664A) transgenic line B254 until 7 mo [7]. Tg PDAPP(D664A)B254 mice express ~1.5X higher levels of the FAD-hAPP transgene and produce proportionally higher levels of A β than Tg PDAPP(J20) mice [7]. To determine whether mutation of Asp664 completely abolishes or only delays the appearance of AD-like deficits in high-expressing PDAPP(D664A)B254 mice, we examined cognitive function longitudinally in transgenic PDAPP and PDAPP(D664A) at 9 and 13 mo and features of AD-like histopathology at 13 mo after completion of behavioral studies. Consistent with previous reports[7,29,31,32], transgenic PDAPP mice exhibited spatial learning and memory deficits with respect to non-Tg animals at all ages studied (Figures 1,2 and 4). Examination of these features of the AD-like phenotype in PDAPP(D664A)B254 mice revealed an overall normalization of learning and memory deficits in animals carrying a D664A mutation until 13 mo. These results are in agreement with our previous observations in younger Tg PDAPP (D664A)B254 mice (2.5 to 7 mo, [7,32]). Although overall performance of Tg PDAPP(D664A) mice across all ages examined in the present study was not significantly different from that of non-Tg littermates' (Figure 4), a trend towards poorer performance during retention at 9 mo and during spatial learning at 13 mo was present in Tg PDAPP(D664A)B254 mice (Figure 2a). None of these changes, however, resulted in significant differences with respect to non-Tg littermates' performance during training or at testing. Performance of Tg PDAPP(D664A) was therefore significantly improved with respect to Tg PDAPP animals at all ages examined. While retention in PDAPP(D664A) mice was not significantly different from the non-Tg group at 9 mo, it was not significantly different from the Tg PDAPP group either, suggesting that a trend to impairment in spatial memory may have developed in Tg PDAPP(D664A) mice at that age. This trend, however, was reversed by carry-over (savings) effects of experience (Figure 2b). Interestingly, experience had a positive impact on performance in all groups (Figure 4a), suggesting that long-term retention is operant in PDAPP mice even in the presence of significant learning and memory deficits. Retention of the platform location in Tg PDAPP mice was improved at 13 mo, in spite of the sustained deficits observed for this group during training (Figure 2). These observations are in agreement with the observed amelioration in cognitive deficits resulting from enriched experience in other Tg mouse models of AD[15], are consistent with the concept of "cognitive reserve" proposed for human subjects[33] and may provide support for cognitive training approaches to the prevention of AD.

PDAPP(D664A) mice from the high-expressing line used in these studies(B254), produce very high levels of soluble A β [7,32]. As would be expected from their higher levels of soluble A β production, we found that A β plaque deposition was increased by ~50% in 13 month-old Tg PDAPP(D664A) mice as compared to Tg PDAPP(J20) mice (Figure 6a). These observations are in agreement with those of Saganich et al. [32], who showed that A β deposition was present in Tg PDAPP(D664A)B524 mice at 6 mo, when it is still undetectable in Tg PDAPP(J20) mice. The distribution of A β deposits in hippocampus and in cortex was roughly equivalent in both transgenic lines (Figure 6a). Taken together, these results support and extend previous observations[7,32] and provide strong support for the hypothesis that Asp664 cleavage is required for the generation of AD-like deficits in the PDAPP mouse model of AD, possibly by mediating a significant component of A β toxicity. Considering the very high levels of A β production and accumulation in PDAPP(D664A), it is likely that Asp664-independent pathways of A β damage may be responsible for the emerging trends to poorer performance observed in aged Tg PDAPP(D664A) mice.

In addition to cognitive deficits, Tg PDAPP mice displayed aberrant patterns of behavior (increased floating and thigmotactic swim) during training (Figure 5). These may be associated with non-cognitive components of behavior such as anxiety and helplessness, which are thought to underlie thigmotactic swim and floating behaviors respectively. Supporting the notion that effective learning is associated with decreased anxiety, time spent engaged in thigmotactic swim decreased monotonically with increased experience in all groups (Figure 5a). Percent time spent swimming close to the tank wall was highest in the Tg PDAPP group at all ages, but their responsiveness to increased experience was also more pronounced than any other group, suggesting that, in spite of their deficits in learning and memory, long-term savings effects operant in Tg PDAPP mice may have an impact in their responses to stressful situations. Thus, our results suggest that effective learning may have an effect in decreasing anxiety that is not affected by aging processes in mice, and that this effect of learning is operant also in Tg PDAPP mice modelling AD. Moreover, a carry-over (savings) effect was present for this non-cognitive component of behavior across ages at training for all groups, with the lowest percent times spent engaged in thigmotactic swim at the end of the experiment in the last session at 13 mo.

Although increased floating and thigmotactic swim patterns were absent in non-Tg and PDAPP (D664A) mice at all ages tested, a trend to increased floating was observed both in non-Tg and Tg PDAPP(D664A) groups at 13 mo (Figure 5b). This trend may reflect processes associated with normal aging in mice.

We also observed a positive effect of prior experience on increased helplessness (floating) associated with testing conditions that was pronounced in Tg PDAPP mice between 7 and 9 mo; this decreasing effect on helplessness, however, and in contrast to our observations for anxiety, disappeared with increasing age (Figure 5b). A closer examination of percent spent floating in all groups at 9 and 13 mo, however, revealed that the overall increase in floating for the Tg PDAPP group arose from a stereotypical response during the first and second days of training that disappeared as training progressed at both ages. Thus, our results suggest that the observed increase in helplessness in mice modelling AD may be ascribed to a lack of familiarity with the conditions of training, arising from a deficit in learning [absent carry-over (savings) effect] between ages at training. Long-term effects (retention between days at training) are operant in Tg PDAPP mice, however, since helplessness subsided as training progressed both at 9 and 13 mo. Thus, Tg PDAPP mice, in contrast to non-Tg and Tg PDAPP (D664A) mice, did not show carry-over (savings) effect between 9 and 13 mo, arguing that this non-cognitive component of behavior is not significantly affected by prior experience in aged animals undergoing AD-like processes. Interestingly, and in contrast to our observations for anxiety (Figure 5a), floating also showed a trend to monotonic increase in both non-Tg and

Tg PDAPP(D664A) mice during training at 13 mo, possibly as a consequence of normal aging processes in mice.

Astrogliosis is commonly present in neurodegenerative conditions. Both beneficial and detrimental effects have been attributed to increased numbers of reactive astrocytes[28]. Astrogliosis was observed in Tg PDAPP mice[7], but its significance in this mouse model of AD remains unclear. In contrast to the improvements in spatial learning and memory observed in Tg PDAPP(D664A)B254 mice with respect to Tg PDAPP mice across all ages tested, astrogliosis was unchanged in brains from Tg PDAPP(D664A)B254 mice at 13 mo (Figure 6b). Although no causal relationships can be inferred from these observations, our results argue that increases in numbers of reactive astrocytes do not have a deleterious effect in spatial learning and memory in this mouse model of AD, and suggest that the astrocytic proliferative response may respond to increases in the levels of expression of the hAPP transgene and/or to proportional increases in A β production and deposition (Figure 6a). Moreover, our results can be interpreted as suggesting that an increase in the number of reactive astrocytes may have no role, or potentially a beneficial effect, in this mouse model of AD.

To summarize, the results of the present study confirm our previous findings[7,32] and indicate that the cleavage of hAPP at Asp664 by caspases (or possibly by a non-caspase protease or proteases) does not overtly affect A β production and deposition nor astrogliosis, but is a critical step in the development of behavioral abnormalities in FAD-hAPP transgenic mice. Therefore, A β -mediated learning and memory deficits in the PDAPP mouse model of AD may involve APP cleavage at Asp664. The results of the present study also lend support to a recently proposed model of AD, in which A β binds to and oligomerizes APP[2,19,21,22,34,35], leading to cleavage at Asp664 and cytotoxicity[21,22]. Our results do not exclude the possibility that the Asp664->Ala mutation affects the AD phenotype not by preventing cleavage at Asp664 but rather by destroying, or conversely stabilizing, an as-yet-uncharacterized protein-protein interaction at APP's C-terminus; however, in either case, the mediation of A β toxicity *in vivo* by APP via an intracytoplasmic mechanism (be it cleavage or modulation of protein-protein interaction, or both) is supported by the current observations.

The C-terminal cleavage of APP by caspases truncates APP aminoterminally to sequences that are required for its interaction with motor proteins, components of the stress response, and transcriptional transactivators[5,10–12,14,17,18,25]. Cleavage of APP by transiently activated caspases at neuronal terminals may therefore disrupt its interaction with several different protein complexes and thus alter the normal processing, turnover or function of the molecule. Thus, it is possible that protective mechanisms that partially compensate for A β toxicity may be upregulated when the C-terminal cleavage of APP is precluded. If upregulation of pathways that result in resistance to A β toxicity have a role in preventing the development of AD-like deficits in Tg PDAPP(D664A) mice, the appearance of a non-significant trend to poorer performance in older PDAPP(D664A) mice suggests that these pathways might be down-regulated with increasing age. Alternatively, it is conceivable that cognitive function in Tg PDAPP(D664A) mice can withstand ongoing low-levels of A β -mediated damage until late in life, when the accumulated amyloid or the consequences of prior A β -mediated damage may result in a trend to emergent cognitive abnormalities.

The results presented here point to a key role for the C-terminal cleavage of APP (or alternatively, a protein-protein interaction requiring Asp664) in the development of late functional AD-like deficits in a transgenic mouse model. Furthermore, our data indicate that the intracytoplasmic domain of APP may play an important role in the pathogenesis of AD, and suggest that the cleavage of APP at Asp664 may represent a therapeutic target.

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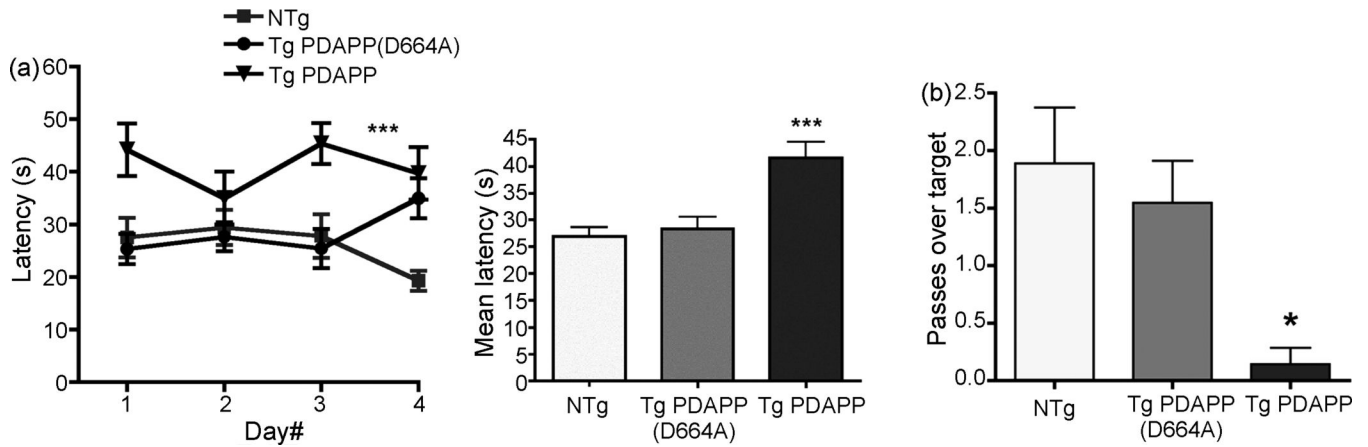


Figure 1. Performance of 9 mo Tg PDAPP, Tg PDAPP(D664A) and non-Tg littermates in the MWM
a. Spatial training *Left panel*, mean latencies to reach a hidden platform were significantly different for the Tg PDAPP mouse group with respect to non-Tg littermates ($P < 0.01$) and Tg PDAPP(D664A) animals [$P < 0.001$, as a result of Bonferroni's post-hoc test applied to a significant effect of genotype, $F(2,35) = 10.71$; *** $P < 0.0002$; repeated measures two-way ANOVA]. *Right panel*, mean latencies for all genotypes. **b. Probe trial.** Retention of the former platform site was impaired in Tg PDAPP mice with respect to the non-Tg group [$P < 0.05$, Tukey's multiple comparisons test applied to a significant effect of genotype ($P < 0.01$) in one-way ANOVA] but was not significantly different for the non-Tg and Tg PDAPP(D664A) groups nor between the Tg PDAPP and Tg PDAPP(D664A) groups. Data are mean \pm SEM.

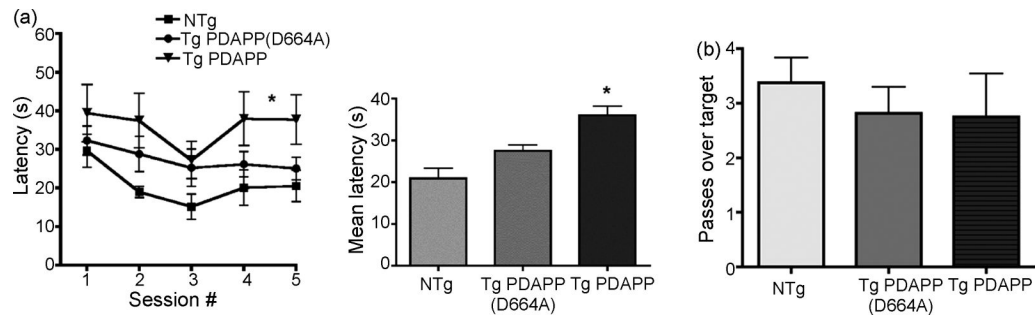


Figure 2. Performance of 13 mo Tg PDAPP, Tg PDAPP(D664A) and non-Tg littermates in the MWM

a. Spatial training *Left panel*, mean latencies to reach a hidden platform were significantly different in Tg PDAPP mice with respect to non-Tg littermates [$P < 0.05$ as a result of Bonferroni's post-hoc test applied to a significant effect of genotype ($F(2, 25) = 4.56$; $*P < 0.02$, repeated measures two-way ANOVA)]. Performance of 13 mo Tg PDAPP(D664A) was indistinguishable from non-Tg littermates' ($P > 0.05$) and from Tg PDAPP mice ($P > 0.05$). *Right panel*, mean latencies for all genotypes. Session number significantly affected performance of all groups ($F(4, 100) = 3.77$, $P = 0.007$, repeated measures two-way ANOVA). **b. Probe trial.** No significant difference in number of crosses over the target were observed between groups. Data are means \pm SEM.

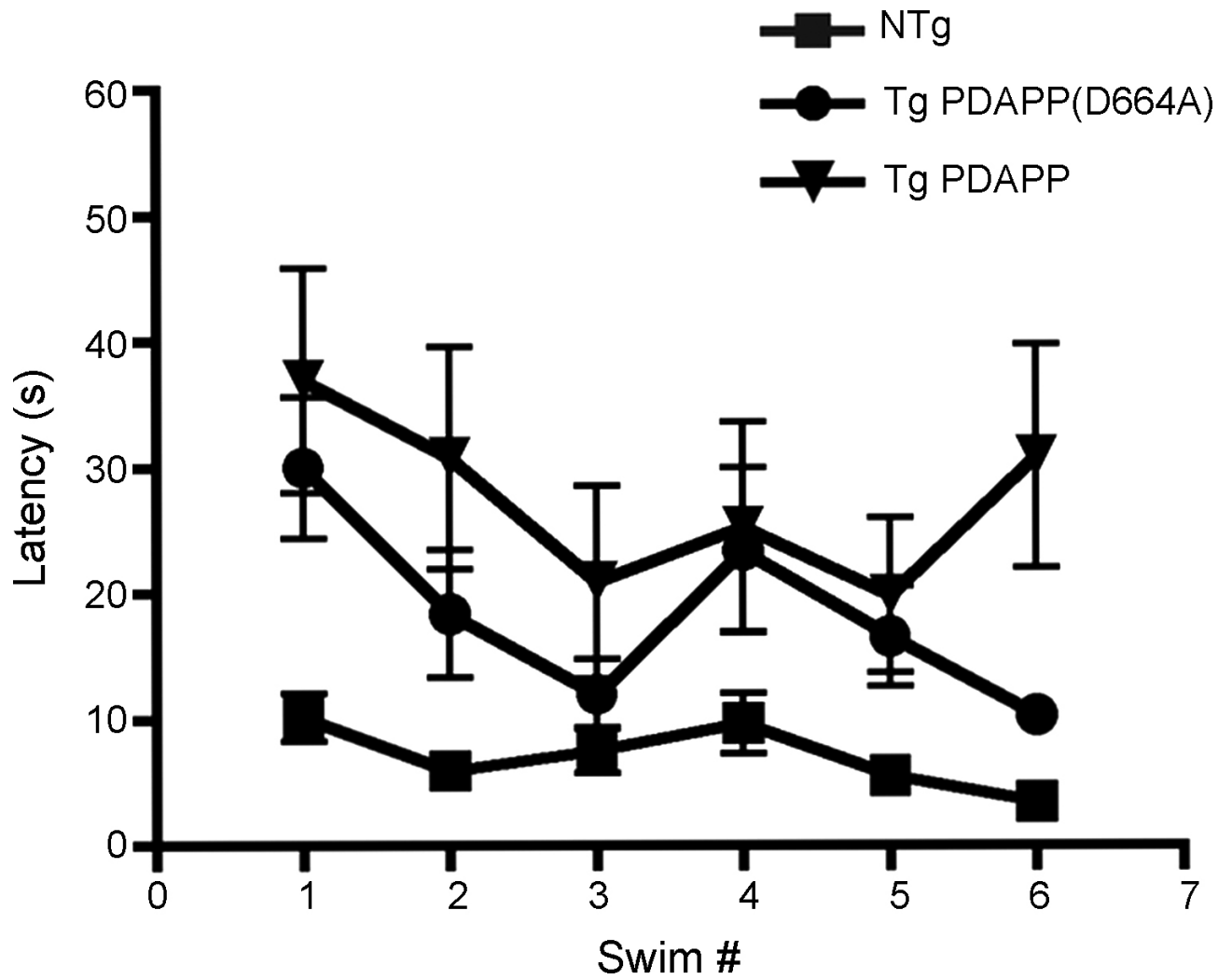


Figure 3. Performance of Tg PDAPP and Tg PDAPP(D664A) mice in the cued version of the MWM Tg PDAPP, Tg PDAPP(D664A) and non-Tg animals performed similarly during training in cued conditions, showing significantly reduced latencies over sessions [significant effect of session number on performance, $F(5,125)=5.72$; $P=0.0001$, repeated measures two-way ANOVA]. Data are means \pm SEM.

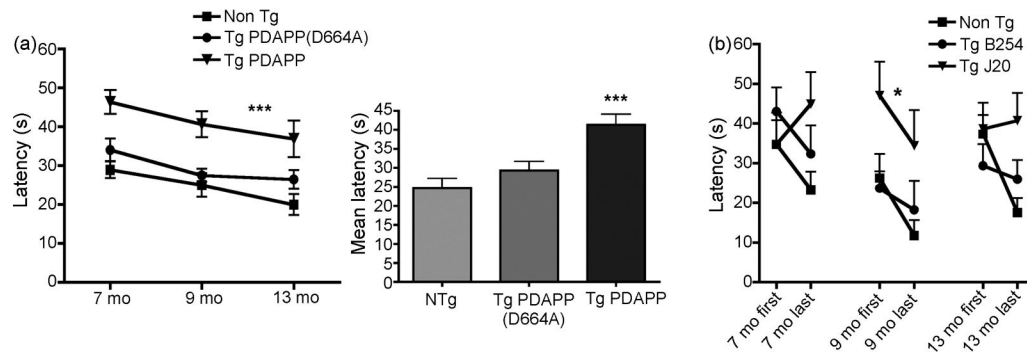


Figure 4. Effect of prior experience on performance of Tg PDAPP, Tg PDAPP(D664A) and non-Tg littermates in the MWM

a *Left panel*, performance of all experimental groups was significantly improved with increasing age and experience [significant effect of age at testing on performance, $F(2,79) = 6.57$, $P = 0.002$, repeated measures two-way ANOVA]. Tg PDAPP mice were significantly impaired with respect to non-Tg controls ($P < 0.001$) and with respect to Tg PDAPP(D664A) mice [$P < 0.05$, as a result of Bonferroni's post test applied to a significant effect of genotype, $F(2,2) = 23.76$; $***P < 0.0001$, repeated measures two-way ANOVA]. *Right panel*, mean latencies for all genotypes. **b. Performance at first and last swim during training at 7, 9 and 13 mo.** Latencies to find the escape platform were reduced at last swim with respect to the first swim of each training period for non-Tg and Tg PDAPP(D664A), but not for Tg PDAPP mice except at 9 mo, when performance of Tg PDAPP mice also improved as a result of training. *, significant effect of genotype on performance, $F(2,5) = 2.45$, $P = 0.0003$, two-way ANOVA. Retention was observed between 7 and 9 mo, but not between 9 and 13 mo for non-Tg and Tg PDAPP(D664A) groups. Data are means \pm SEM

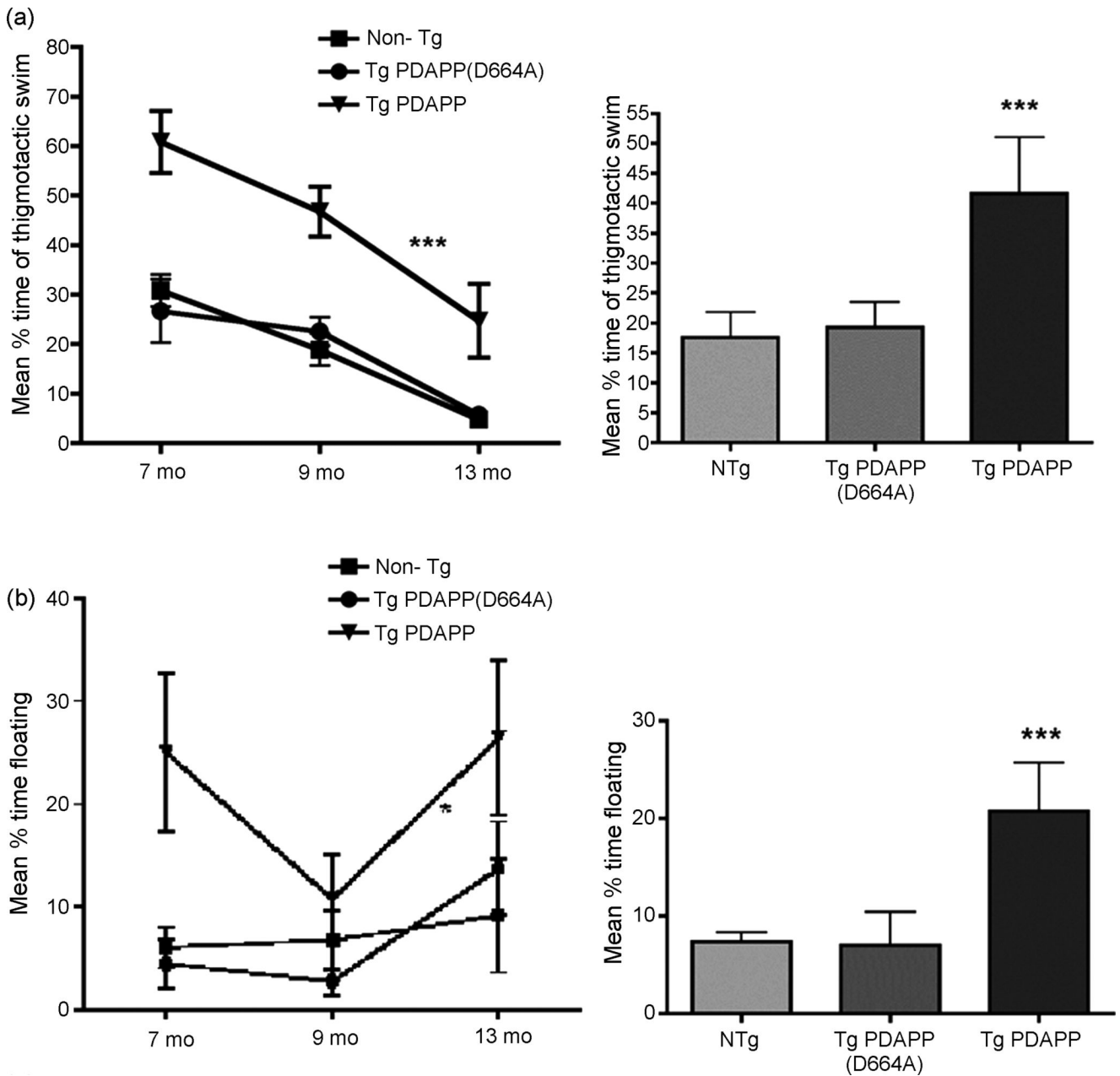


Figure 5. Non-cognitive components of behavior in Tg PDAPP and Tg PDAPP(D664A) mice

a. Thigmotaxis Percent time spent engaged in thigmotactic swim decreased significantly as a function of age at testing [$F(2,2)=25.64$, $P=0.0001$, two-way ANOVA]. Tg PDAPP mice spent a significantly larger percent of time during trials swimming close to the tank wall than all other groups across the ages tested [$P<0.001$ with respect to both non-Tg and Tg PDAPP (D664A) groups respectively as a result of Bonferroni's post hoc test applied to a significant effect of genotype ($F(2,2)=31.82$; $***P<0.0001$, two-way ANOVA)]. **b. Floating.** Percent time spent floating increased significantly as a function of age at testing [$F(2,75)=3.3$, $P=0.04$, two-way ANOVA]. Tg PDAPP mice spent a significantly larger percent of trial time floating than all other groups across the ages tested ($P<0.05$ with respect to both non-Tg and Tg PDAPP (D664A) mice respectively as a result of Bonferroni's post hoc test applied to a significant

effect of genotype ($F(2,2)=8.49$; $P=0.0005$; two-way ANOVA). “Floaters” were excluded from all analyses. Data are means \pm SEM.

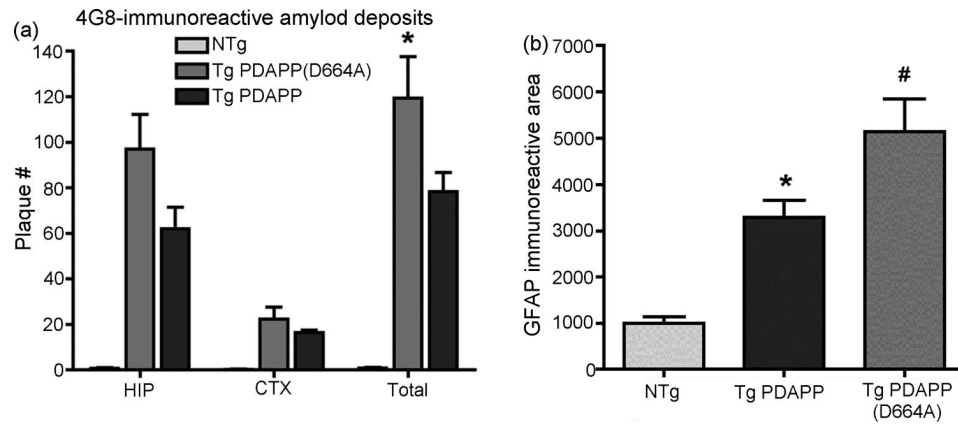


Figure 6. Plaque deposition in 13 mo Tg PDAPP and Tg PDAPP(D664A) mice
a Total hippocampal (HIP) and cortical (CTX) A β plaques (4G8-immunoreactive objects) were quantitated in 13 mo Tg PDAPP, Tg PDAPP(D664A) and non-Tg littermate control brain sections using ImageJ by investigators blinded to genotype. *, $P < 0.05$ as a result of Bonferroni's post-hoc test applied to a significant effect of genotype on number of plaques [$F(2,42) = 74.26$, $P < 0.0001$, two-way ANOVA]. **b. Astroglial markers in 13 mo Tg PDAPP and Tg PDAPP(D664A) mice.** Total GFAP-immunoreactive area was quantitated in 13 mo Tg PDAPP, Tg PDAPP(D664A) and non-Tg littermate control brain sections using Image J by investigators blinded to genotype. Total GFAP-immunoreactive area was higher both in Tg PDAPP and Tg PDAPP(D664A) animals as compared to non-Tg littermates (*, $P < 0.05$; #, $P < 0.01$ respectively as a result of Tukey's post-hoc test applied to a significant difference between genotypes in one-way ANOVA ($P < 0.0001$). GFAP-immunoreactive areas were not significantly different between Tg PDAPP(D664A) and Tg PDAPP groups. Data are means \pm SEM.