

A TgCRND8 Mouse Model of Alzheimer's Disease Exhibits Sexual Dimorphisms in Behavioral Indices of Cognitive Reserve

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Abstract.

Cognitive decline is sexually dimorphic in Alzheimer's disease (AD). Men show higher incidences of amnesic mild cognitive impairment yet women disproportionately phenocopy to AD. It is hypothesized that men maintain greater cognitive reserve than women under comparable amyloid- β (A β) challenge. One behavioral aspect of cognitive reserve in mice is the capacity to cope with A β -associated stereotypies by switching to increasingly effective navigational search strategies in the Morris water maze. To explore inherent sex differences in this paradigm, however, we require an A β PP mouse model wherein behavioral flexibility is impaired earlier in females than males despite equivalent A β load. Here, we show that when F1 C57Bl/6 \times C3H/HeJ TgCRND8 mice are placed on C57Bl/6 background, N5 Tg males and females exhibit equivalent A β pathologies at 2, 4, 6, and 8 months of age yet females display learning and memory deficits earlier than males. We further show that this N5 line does not carry the autosomal recessive *pde6b^{rd1}* mutation that impairs visual acuity and that the estrous cycle is not disrupted on this genetic background. At 5.3 months of age, Tg males, but not females, compensate for A β -associated stereotypic behaviors (i.e., hyperactive tight circling) by alternating navigational search strategies and adopting increasingly productive spatial search strategies. Females fail to overcome A β -associated stereotypies and do not efficiently switch from systematic to spatial learning strategies. Together, these data identify a novel A β PP mouse model that can be used for preclinical testing of interventions targeting sexual dimorphisms in behavioral indices of cognitive reserve.

Keywords: Alzheimer's disease, amyloid- β cognitive reserve, learning, memory, Morris water maze, search strategy, stereotypy, transgenic mouse, visual acuity

INTRODUCTION

Aberrant cleavage of the amyloid- β precursor protein (A β PP) into toxic oligomeric A β peptides and intraneuronal accumulation of neurofibrillary tangles composed of hyperphosphorylated tau are defining Alzheimer's disease (AD) pathologies [1–3]. The

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amyloid cascade has been modeled in transgenic mice by ectopically expressing human familial mutant A β PP and/or presenilin 1 (PS1) genes linked to early onset AD [4, 5]. AD-associated tauopathies have been further engineered using triple transgenics expressing mutant human tau [5–7]. These models have clearly demonstrated that higher A β loads are associated with greater learning and memory impairments in mice. For example, interventions that accelerate A β deposition and tau aggregation in 3xTg-AD mice exacerbate learning and memory deficits [8]. Similarly, memory impairments in A β PP_{Swe}/PS1 mice increase as soluble A β levels and plaque burden rise [9–11].

In humans, cognitive decline in AD is sexually dimorphic [12–17]. Two-thirds of AD patients are women [12, 18, 19]. Men show a higher incidence of amnesic mild cognitive impairment, often with a greater A β load than women, yet women disproportionately transition to AD [12–15]. It is hypothesized that men maintain greater brain and cognitive reserves in the face of A β challenge [20, 21]. Here, brain reserve refers to differences in brain structure or synaptic densities that enable individuals to compensate for declining neural function resulting from A β toxicity [22, 23]. Cognitive reserve is defined as the capacity to switch between cognitive strategies, using alternative brain networks and cognitive paradigms to cope with progressive A β pathologies [22, 23]. To our knowledge, no A β PP mouse model robustly recapitulates sexual dimorphisms in cognitive reserve given pre-existing sex differences in A β biogenesis. While female A β PP transgenics often display more severe learning and memory deficits [24, 25], they also consistently exhibit higher A β ₄₀ and A β ₄₂ levels and greater A β plaque loads than males [26–29]. Accelerated A β accumulation in female mice is attributed, in part, to sex-dependent increases in BACE-1 expression [25], decreases in insulin-degrading enzyme expression [25], and enhanced γ -secretase activity [30]. Interestingly, mild learning and memory impairments have been reported in 3xTg-AD females at ages when A β and tau pathologies begin to diverge [24, 31]. While these data hint at sex differences in A β vulnerability, robust sex-specific impairments are only reported once A β load in females exceeds that of males [26, 32]. Identifying an A β PP transgenic model in which females and males exhibit comparable A β burden at all ages yet females phenoconvert earlier than males would provide an ideal preclinical model for testing strategies designed to enhance cognitive reserve

and would further our understanding of AD sexual dimorphisms.

The TgCRND8 line is an aggressive A β PP mouse model. TgCRND8 mice express the human A β PP gene with double KM670/671NL+V717F Swedish and Indiana familial AD mutations [33]. Extracellular A β ₄₀ and A β ₄₂ are detected by one month of age [33]. Soluble A β ₄₂/A β ₄₀ ratios are elevated by two months of age [33]. Learning and memory deficits manifest by three months of age [33]. Since this line exhibits a high mortality rate, males and females are commonly pooled for behavioral testing [33–35]. Sex differences have yet to be evaluated. We have previously shown that backcrossing C57Bl/6 \times C3H/HeJ TgCRND8 mice onto a C57Bl/6 lineage for 5 generations (N5) delays the onset of learning and memory impairment in females [36]. We have yet to characterize males on this genetic background. Here, we asked whether N4 and N5 C57Bl/6 \times C3H/HeJ TgCRND8 (Tg) mice display sexual dimorphisms in behavioral indices of cognitive reserve. We show that A β load is equivalent between Tg sexes yet Tg males exhibit fewer learning and memory deficits in the Morris water maze (MWM). We analyzed the search strategies used by NonTg and Tg mice to navigate the MWM to assess behavioral flexibility associated with cognitive reserve. We find that Tg males can overcome A β -associated non-productive behaviors (hyperactive repetitive tight circling) by adopting increasingly productive spatial search strategies whereas Tg females cannot, ultimately affecting behavioral measures of learning and memory. Together, these data identify the N4/N5 Tg line as a mouse model capable of recapitulating clinically relevant sexually dimorphic differences associated with risk of AD phenoconversion.

MATERIALS AND METHODS

Animals

A total of 78 Tg males, 194 Tg females, 61 NonTg males, and 96 NonTg females were used in this study. All mice were derived from F1 TgCRND8 C57Bl/6 \times C3H/HeJ hybrid mice [33] generously provided by Dr. Paul Fraser (University of Toronto). Mice were backcrossed for 4 and 5 generations (N4/N5) to wild-type C57Bl/6 mice in our laboratory [36] and then maintained by filial breeding of N4 Tg with N4 NonTg females. All mice were genotyped for the human A β PP transgene [36] and the autosomal

recessive retinal degeneration phosphodiesterase 6B (*Pde6b^{rd1}*) mutation [37] (Supplementary Table 1). At 50 days of age, males and females were single-housed on a 12:12 light:dark cycle with zeitgeber time (ZT) 0 set to 6:00 AM. Mice were fed a normal chow diet (Harlan Laboratories, 2018 Teklad Global 18% Protein Rodent Diet) *ad libitum*. Natural survival rates were calculated between 50–181 days of age. All experiments were approved by the Animal Care Committee of the University of Ottawa and performed in strict accordance with the ethical guidelines for experimentation of the Canadian Council for Animal Care.

Western blotting

Soluble protein was extracted from the cortex of NonTg and Tg mice at 2, 4, and 6 months of age using RadioImmunoprecipitation Assay (RIPA) buffer (1% Nonidet P40 substitute, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 300 μ g/mL aprotinin and 100 μ g/mL phenylmethylsulfonyl fluoride in 10 mM phosphate buffered saline (PBS: 10 mM sodium phosphate, 154 mM sodium chloride)). Protein samples of 15 μ g were processed as previously described [36]. Briefly, proteins were incubated at 70°C in NuPAGE lithium dodecyl sulfate sample buffer, resolved on a NuPAGE 4–12% SDS-PAGE gel (Invitrogen), transferred to nitrocellulose membrane (Pall Life Sciences #66485), and blocked in 5% non-fat milk in TBS-T (50 mM Tris base, 150 mM NaCl, 0.1% Tween 20). Immunoblotting was performed with overnight probe at 4°C using an A β antibody that recognizes human A β PP, C-terminal fragment β (CTF β) but not CTF α , and A β peptides (6E10 Cedarlane SIG-39320 at 1:2000 dilution). Secondary antibodies were horseradish peroxidase-conjugated anti-mouse IgG (Rockland 610-1319-0500 at 1:10 000 dilution) visualized by chemiluminescence using Immobilon Western substrate (Millipore WBKLS0500).

A β plaque counts

Tg males and females were euthanized at 2, 4, 6, and 8 months of age ($n=3-6$ per genotype/age/sex). Animals were injected with euthanyl (1EUS001, Bimeda-MTC Animal Health Ins) prepared in sterile water to a final concentration of 65 mg/ml and transcardially perfused with 10 mM PBS followed by either 3.7% paraformaldehyde in 10 mM PBS

for serial coronal sectioning or by Lana's fixative (4% paraformaldehyde, 0.2% picric acid in 0.16 M sodium phosphate buffer, pH 7.1) for sagittal sectioning to assess A β plaque distribution. Brains were removed, post-fixed for 24 h in either 3.7% paraformaldehyde in 10 mM PBS or for 1 h in Lana's fixative, and cryoprotected in 20% sucrose. Lana's fixed sagittal 30 μ m serial sections were collected between interaural 0.24 and 1.92 mm using the coordinates of Franklin and Paxinos [38] on a Leica CM1900 cryostat (Leica Microsystems). Sagittal sections were processed for immunohistochemistry using mouse anti-4G8 (1/250; SIG-39220, Covance) recognizing human and murine A β , biotin-labeled anti-mouse IgG (1/300; B9904, Sigma), extravidin-peroxidase (1/20; E2886, Sigma), and visualized by a reaction with Sigma FAST 3,3'-diaminobenzidine (D4418-50SET, Sigma). Sections were dehydrated in an increasing series of ethanol washes (50–100%) and coverslipped in DPX Mountant (44581, Fluka). All antibodies were diluted in antibody buffer (3% bovine serum albumin (A6003, Sigma), 0.3% Triton X-100 (T9284, Sigma), in 10 mM PBS, pH 7.0). For plaque quantification, 10 mM PBS-buffered 3.7% paraformaldehyde-fixed 10 μ m serial coronal cryosections between bregma -1.0 to -2.6 using the coordinates of Hof et al. [39] were collected. Total plaque number in the dorsal hippocampus and cortex as we have previously described [40] and defined by Fanselow and Dong [41], restricted to the dorsal retrosplenial, parietal association, and primary sensory cortices as identified according to Hof et al. [39], were quantified on a DMXRA2 epifluorescent microscope (Leica Microsystems) using the Advanced Measurement Module of OpenLab 5.0.2 (Improvision) software. Counting methodologies were as described [40], adapted to assess A β plaques. Briefly, a series of coronal sections (10 μ m) were incubated overnight at 4°C with mouse anti-4G8 (1:500). Sections were washed with 10 mM PBS, incubated with FITC-labeled anti-mouse IgG (1:1000, 715-095-150, Jackson ImmunoResearch) for 1 h at room temperature, and washed in 10 mM PBS prior to being coverslipped in 0.05% p-phenylenediamine (w/v, P6001, Sigma) in glycerol (BP229-1, Fisher) adjusted to pH 8.0 with 0.5 M sodium carbonate/sodium bicarbonate buffer. Plaques were defined as immunoreactive round or annular aggregates with diameters of 5 μ m or greater representing the minimum diameter of deposits meeting this morphological criteria detected. The maximum plaque size detected in these animals was 50 μ m. Average plaque

size was $27.2 \pm 12 \mu\text{m}$. An average of five 10- μm sections, serially sampled along the entire anterior-posterior axis of the hippocampus between bregma -1.0 and -2.6 , were analyzed per animal. Sections were a minimum of 30 μm apart to ensure the same plaque was not counted twice in adjacent sections. A single investigator blinded to the identity of the sections assessed plaque numbers. Total number of plaques per animal was calculated as the sum of the plaque counts per region in both hemispheres multiplied by the total number of serial sections collected, divided by the number of sections sampled and further divided by three to account for plaque size and thus overlap given assessment in 16 series of 10 μm sections.

Quantification of A β ₄₀ and A β ₄₂ peptides

Tg males and females were euthanized at two or six months of age ($n=4-5$ per sex) using euthanyl (1EUS001, Bimeda-MTC Animal Health Ins) prepared in sterile water to a final concentration of 65 mg/ml. Brains were extracted and the cerebrum dissected, weighed, and flash-frozen in liquid nitrogen. Tissue was prepared as per ELISA kit instructions (human A β ₄₂: Invitrogen #KHB3442; human A β ₄₀: Invitrogen #KHB3482). Briefly, tissue was homogenized in a 5 M guanidine hydrochloride, 50 mM Tris hydrochloride solution and then diluted 1:25 in Dulbecco's PBS (0.2 g/L KCl, 0.2 g/L KH₂PO₄, 8 g/L NaCl, 1.15 g/L Na₂HPO₄) with 5% BSA (Fisher, CAS 9048-46-8) and 0.03% Tween (Sigma-Aldrich, P1379) augmented with 1x protease inhibitor cocktail (Calbiochem #539131). Six-month samples were further diluted 1:1000 and two-month samples 1:2 to fall within range of commercial standard curves using age-matched NonTg mice as a negative control. Each sample was run in duplicate. Data are reported as ng A β peptide per gram of tissue wet weight.

MWM behavioral testing

Behavioral indices of spatial learning and memory were assessed in the MWM paradigm. Testing began when mice were 158 ± 10 days (5.3 months) of age and ended when mice were 167 ± 10 days (5.6 months) of age. An additional cohort of male mice was tested when mice were 338 ± 50 days (11.27 months) of age and ended when mice were 347 ± 50 days (11.57 months) of age. The apparatus, a circular plastic pool (ENV-594M-B, Med Associates

Inc.), measured 134.5 cm in diameter and 53.3 cm deep. The pool was filled with water maintained at 21°C and made opaque by the addition of white, non-toxic, water-soluble paint (506-BT12801O, Scholar's Choice). The water level was maintained at a depth of 1 cm above the 10 cm diameter circular escape platform. Visual cues included a black "X" and square on the front and left walls respectively. Mice were acclimated to MWM facilities using a white noise generator (70 dB; 2325-0144, San Diego Instruments) with overhead white light of 100 lux for one hour before being placed in the pool. Mice underwent four trials with 20-min intervals per day for eight test days. Each test day, mice were placed in four equidistant locations around the pool in a random order and navigation was tracked for 60 s. If mice did not find the escape platform independently, the experimenter guided the mouse to the platform ensuring the mouse remained on the platform for 5 s before being removed. On day nine, mice were subjected to a probe test whereby the platform was removed and mice were placed in the pool for 60 s to evaluate reference memory. Separate cohorts of Tg and NonTg mice were tested in the cued MWM at 5.3 months of age using the same experimental paradigm except that the platform location was made clearly visible with a flag emerging from the center of the hidden escape platform. In all experiments, mice were tracked using a video camera (Bosch, LTC0355/20; Pentax 3.5–8 mm Ins, TS2V314BED) and data collected using either Ethovision XT7 or XT8 (Noldus).

Navigational search strategy analysis

As defined by Janus [34] and Brody and Holtzman, [42], swim patterns were used to assess the cognitive strategies used by mice to acquire the MWM. Swim patterns were classified as one of three overarching search strategies: (1) spatial, (2) non-spatial systematic, or (3) repetitive looping. Spatial strategies were defined by focally specific searches in which there is a clear emphasis on the quadrant where the escape platform is located [34, 42, 43]. These included swim trajectories in which the mouse navigates directly to the target or concentrates its search in the correct quadrant. Systematic strategies represented a non-spatial navigational approach [34, 42, 43]. Searches were comprehensive but are not selective for the escape quadrant in that they included random or scanning behaviors in multiple quadrants as well as focused searches of an incorrect quadrant.

Looping referred to a primarily repetitive circling approach where mice swim in either small, tight circles or wider, concentric loops [34, 42, 43]. Floating was further assessed as a possible confounding phenotype as this behavior lacks a deliberate trajectory or an intentional navigational search strategy. Floating was defined by a velocity under 6 cm/s [34] and an escape latency of over 50 s for a given trial. A mouse with an average velocity of under 6 cm/s and average escape latency of over 50 s for more than four of the eight test days was considered a floater and was excluded from analysis. Only one N5 NonTg female was identified as a floater and excluded from this study. Search strategies were analyzed by (a) two investigators blinded to the subjects' genotypes and (b) using a fully automated algorithm, MWM Visual, developed in-house. Both the inter-rater agreements and the consensus agreement of the two raters with the software were comparable at 85–90% ($\kappa=0.825$ and $\kappa=0.804$ respectively). Data reported in this study represent classifications assigned exclusively using the MWM Visual algorithm.

Slow angled descent forepaw grasping test (SLAG)

Two days before cued MWM testing, visual acuity was assessed using the SLAG test [44]. The performances of 5-month-old N4 NonTg and Tg mice were compared to those of an in-house colony of N15 C57BL/6 as well as to C3H/HeNcrI mice (Charles River). C57BL/6 mice have no visual deficits; C3H/HeNcrI are homozygous for *Pde6b^{rd1}* mutation and exhibit retinal degeneration [37]. Mice heterozygous for *Pde6b^{rd1}* have normal retinas but increased photosensitivity [45]. The F1 TgCRND8 parental line is *Pde6b^{rd1/+}*. Our N4 NonTg and Tg either retained one copy of the mutant allele or were wildtype (*Pde6b^{+/+}*). N5 mice were all *Pde6b^{+/+}*. Briefly, the behavior of mice descending to a wire mesh cage lid in either ventral or dorsal orientations was recorded using a Sony High Definition Camcorder (HDR-CX210/R) in the presence of a desk lamp (Illuminada Gooseneck Desk Lamp, 17341–000) that emitted 600–700 lux located on the opposite side of the cage. Mice were subjected to six trials (three in each orientation) separated by five-minute intervals over two test days. Trial videos were scored for the presence (score = 1) or absence (score = 0) of the SLAG reflex. A SLAG reflex was defined by the following behaviors: (1) persistent

extension of the forepaws toward the wire lid, (2) multiple reaches of the forepaws towards the wire lid, (3) forepaw extension with head elevation or hind limb extension in attempt to reach the wire lid, or (4) rotating the body to reach the wire lid. Absence of a SLAG reflex was defined by: (1) no extension of forepaws toward the wire lid, (2) rotation of the body to reach the hind limb or tail and not the wire lid, (3), or extension of the forepaws in the correct direction only when the mouse was less than 4 cm away from the wire lid thus guided by whisker proprioception [44]. The percentage of total trials in which an animal exhibited a SLAG reflex was reported. Animals with an incidence of 30% or less were considered severely visually impaired, between 30% and 90% moderately to mildly photosensitive, and greater than 90% were defined as without visual impairment.

Estrous staging

Vaginal smears were collected from a separate cohort of N4 NonTg ($n=7$) and N4 Tg ($n=7$) mice for at least two consecutive cycles and estrous cycle length determined for each mouse and each genotype as we have described [46].

Statistical analyses

GraphPad Prism 6.0 software (GraphPad, San Diego, USA) or IBM SPSS Statistics v22 (IBM, Armonk, USA) was used for all statistical measures. Alpha values of $p < 0.05$ were deemed significant for assessment of main and interaction effects. For amyloid load analyses, we performed two group power analyses to estimate effect sizes afforded by the number of subjects assessed at 6 months of age with α set to 0.05 and β set to 0.2. Subject size in this study was sufficient to detect a 1.7 fold-change in $A\beta_{42}$, a 2.1 fold-change in $A\beta_{40}$, and a 2.1 fold-change in plaque number. Groups of two were analyzed by unpaired Student's *t*-tests. Changes in groups of three or more were assessed by one-way or two-way ANOVA or *t*-test followed by Holm-Sidak, Tukey, or Dunnett's *post-hoc* tests adjusting the alpha levels for pairwise and multiple comparisons as indicated. Paired or repeated measures analyses were performed where warranted. Reference memory was analyzed using a one-sample *t*-test. Natural survival of different sexes and genotypes was compared using Mantel-Cox log-rank tests.

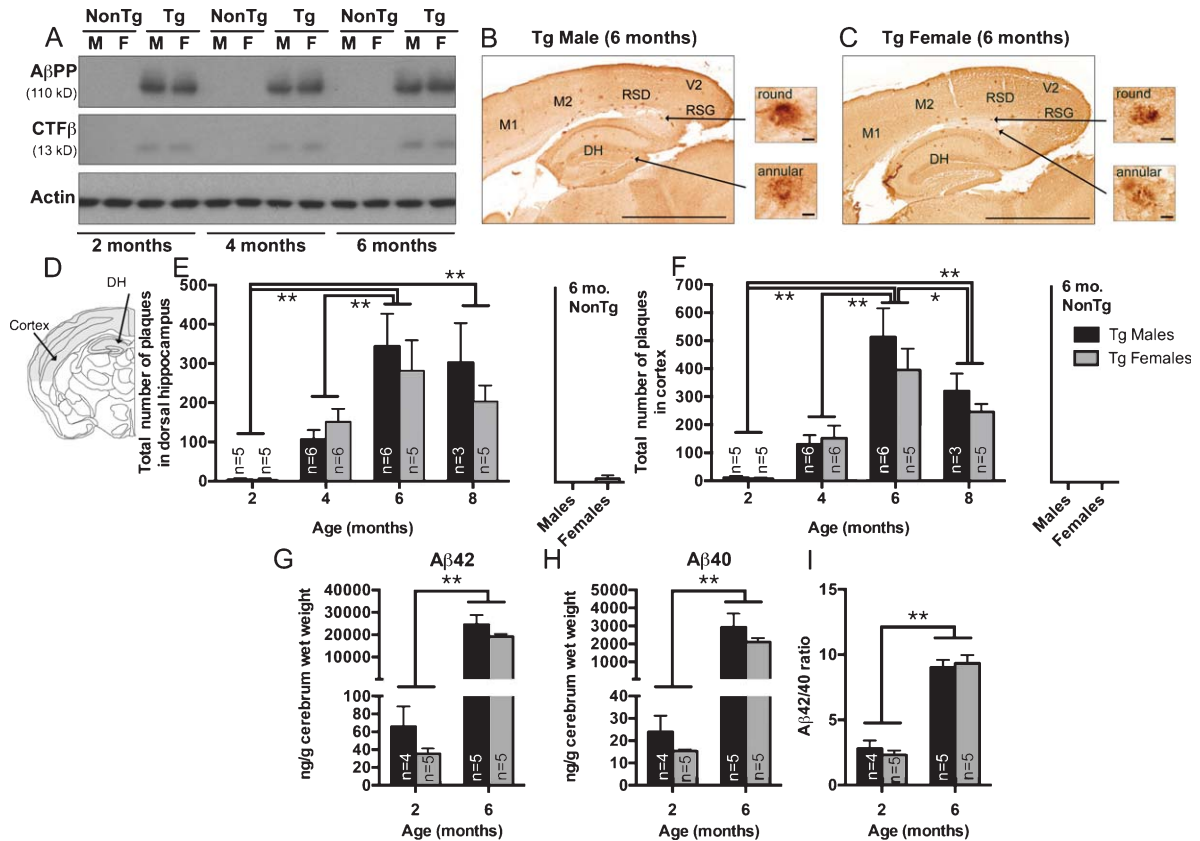


Fig. 1. A β burden is comparable in 2-8-month-old Tg male and female mice. A) No sex-differences in cortical A β PP and CTF β protein levels are detected at 2, 4, or 6 months of age ($n = 2$ animals/sex/genotype). M, males; F, females. Representative immunoblots are depicted. An increase in CTF β levels is apparent in both sexes at 6 months of age. Note that the CTF β panel is presented at a higher exposure than the A β PP panel (see Supplementary Figure 1 for the full blot at both exposures). B,C) Dense-core plaques, with either round or annular morphologies (insets), were enriched in dorsal hippocampus and parietal/temporal cortex of both male and female Tg mice at 6 months of age. Scale bars: 2000 μ m; insets, 20 μ m. The total number of plaques $> 5 \mu$ m diameter were quantified stereologically from bregma -1.0 to -2.6 in the (D,E) dorsal hippocampus and (D,F) cortex of male and female Tg mice and in 6 month-old NonTg littermates. No sex differences in plaque number were detected at any age. In both males and females, a significant increase in plaque number was evident at 6 and 8 months of age compared to 2 and 4 month old animals. In cortex but not hippocampus, plaque number was lower in 8 month-old compared to 6 month-old animals. Levels of A β_{42} (G) and A β_{40} (H) peptides and relative A β_{42} /A β_{40} ratios (I) were quantified at 2 and 6 months of age by ELISA. Data represent mean \pm standard error of measurement (SEM) of $n = 3-6$ mice per group. No sex-differences in A β peptide levels were detected. Statistics were two-way ANOVA followed by *post hoc* Tukey's tests. * $p < 0.05$, ** $p < 0.01$. DH, dorsal hippocampus; M1, primary motor cortex; M2, secondary motor cortex; RSD, retrosplenial dysgranular cortex; RSG, retrosplenial granular cortex; V2, secondary visual cortex.

RESULTS

A β PP, CTF β , A β peptide, and A β plaque load are comparable in N4 and N5 Tg males and females at 2, 4, 6, and 8 months of age

We first assessed protein levels of soluble 6E10-reactive A β PP and CTF β protein levels in the cortex of male and female Tg mice at 2, 4, and 6 months of age by western blotting (Fig. 1A). No sex differences in protein levels were detected (Supplementary

Figure 1). We next determined the distribution and morphology of 4G8-immunoreactive deposits at 6 months in sagittal sections of Tg males and females (Fig. 1B,C). A β deposits with dense-core (both round and annular) morphologies were enriched in the cortex (specifically retrosplenial, parietal association, and primary sensory cortices) and hippocampus (Fig. 1B,C). We quantified total plaque numbers in serial coronal sections at 2, 4, 6, and 8 months of age in dorsal hippocampus and these cortical regions by stereology (Fig. 1D-F). Data were analyzed by

two-way ANOVA identifying main effects of age (Hippocampus: $F_{(3,33)}=13.15$, $p<0.001$; Cortex: $F_{(3,33)}=22.14$, $p<0.001$) but not sex (Hippocampus: $F_{(1,33)}=0.590$, $p>0.05$; Cortex: $F_{(1,33)}=1.092$, $p>0.05$). Rare A β deposits were detected at 2 months of age in both Tg males and females (Fig. 1E,F). Statistically significant increases were evident at 6 and 8 months compared to 2 or 4 months (*post hoc* Tukey's tests Fig. 1E,F). Plaque load in hippocampus and cortex was equivalent in males and females at all time points. Few to no 4G8-reactive deposits were detected in NonTg mice (Fig. 1E,F). Soluble and insoluble A β_{40} and A β_{42} peptide levels were quantified in the cerebrum of male and female Tg mice at 2 and 6 months (Fig. 1G,H). A significant increase in A β_{40} and A β_{42} peptide levels was evident between 2 and 6 months of age in both males and females (Fig. 1G,H; A β_{42} : $F_{(1,15)}=85.6$, $p<0.0001$; A β_{40} : $F_{(1,15)}=33.65$, $p<0.0001$) with a significant increase in the A β_{42} /A β_{40} ratio evident by 6 months of age (Fig. 1I; $F_{(1,15)}=139.1$, $p<0.0001$). No sex differences in peptide levels were detected (A β_{42} : $F_{(1,15)}=1.319$, $p>0.05$; A β_{40} : $F_{(1,15)}=0.9201$, $p>0.05$; Ratio: $F_{(1,15)}=0.0241$, $p>0.05$).

Tg males undergo a higher A β -associated mortality rate than females

In cortex but not hippocampus, a statistically significant decline in plaque number was observed at 8 compared to 6 months of age in both sexes (Fig. 1D–F). To test whether this decline was due

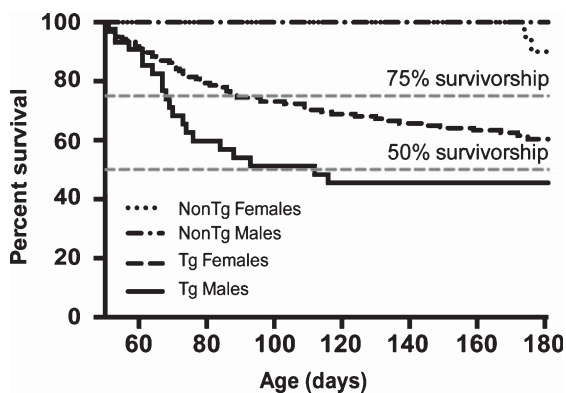


Fig. 2. Tg females modulate lethality associated with A β PP overexpression better than Tg males. Cumulative survival as a function of sex was tracked between 50–181 days of age ($n=21$ NonTg males, $n=44$ Tg males, $n=54$ NonTg females, $n=161$ Tg females). Survival of female Tg mice was significantly better than males. Statistics were Mantel-Cox log-rank tests.

to mortality, we compared the natural survival rates of N4 and N5 Tg mice with the parental F1 C57Bl/6 \times C3H/HeJ TgCRND8 line over a 6-month observation period. The 75% and 50% survivorship of the F1 TgCRND8 line is 85 days (2.8 months) and 260 days (8.7 months) of age, respectively, when males and females are considered collectively [33]. Our N4 and N5 Tg mice exhibited a comparable collective 75% survivorship (80 days, 2.6 months). Fifty-six percent of N4/N5 Tg mice survived to the end of our observation period (6 months of age) [33]. When males and females were analyzed separately (Fig. 2), we found that survival was significantly compromised in both sexes (Mantel-Cox: Males $\chi^2=8.8$, $df=1$, $p<0.01$; Females $\chi^2=12.5$, $df=1$, $p<0.01$). Mutant A β PP overexpression, however, was less lethal to Tg females than males (Mantel-Cox: $\chi^2=4.3$, $df=1$, $p<0.05$). The 50% survivorship for males was 112 days (3.7 months); 60% of females survived to 6 months of age. Thus, while we cannot rule out definitively that the decline in plaque load between 6 and 8 months is not due to mortality of animals with highest A β load, these data do suggest that males are more susceptible to A β PP-associated lethality than females despite equivalent A β and plaque load.

We further asked whether hormonal status was disrupted in female Tgs by comparing estrous cycles with NonTg littermates. Acyclicity indicates the onset of reproductive senescence, the human equivalent of menopause and is associated with increased risk of AD phenoconversion. Tg females exhibited regular estrous cycles (Supplementary Figure 2). There were no differences in the length of cycles between Tg and NonTg females (Supplementary Figure 2).

NonTg males adopt spatial navigational strategies faster than females in the MWM; NonTg females exhibit higher indices of anxiety than males when first exposed to the MWM

To determine whether adult NonTg males and females exhibit inherent sex differences in learning and memory in the absence of A β pathology, we compared their acquisition of the MWM (Fig. 3A). Data were analyzed by two-way repeated measures ANOVA followed by Tukey's *post hoc* for multiple comparisons. As expected, time to find the escape platform progressively decreased with repeated testing in both sexes indicative of learning and memory (main effect of time: $F_{(7,322)}=24.64$,

$p < 0.001$, Fig. 3A). Performance was comparable between sexes (main effect of sex: $F_{(1,46)} = 3.25$, $p > 0.05$, Fig. 3A). After eight days of testing, the escape platform was removed and spatial bias for the correct quadrant was quantified in a probe trial (Supplementary Figure 3A). Both males and females exhibited significant (and comparable) spatial biases for the correct quadrant, indicative of *bona fide* spatial learning and memory (One sample t -test: Males, $t = 8.147$; $df = 20$, $p < 0.0001$; Females $t = 5.069$; $df = 26$, $p < 0.0001$, Supplementary Figure 3A).

Analysis of simple effects of test days suggested that platform acquisition was, however, mildly accelerated in males. Escape latencies differed significantly over the first three days in males compared to their maximal MWM performance (i.e., test day eight, simple effect of days, *post hoc* Tukey's pairwise comparisons, Fig. 3A). In females, escape latencies were significantly elevated on test day 4 (Fig. 3A). To assess whether this subtle difference reflected sexual dimorphisms in behavioral flexibility, we asked whether spatial learning was triggered in males and females at different rates. The murine MWM learning process has been well characterized [34, 42, 43]. Typically, on first exposure to the pool, mice swim in a looping or chaining fashion in close proximity to pool wall [43] (Fig. 3B, left panel). Mice must exhibit behavioral flexibility to overcome this tendency and randomly deviate into the pool interior to make first contact with the escape platform. This independent discovery of the platform initiates the spatial learning process triggering a systematic maze exploration wherein mice deliberately explore the entire pool in search of the escape platform [43] (Fig. 3B, middle panel). With repeated testing, cognitively intact animals will then transition from predominantly systematic to predominantly spatial search strategies [34, 42, 43] (Fig. 3B, right panel). Spatial searches require that animals use distal intra- and extra-maze cues to navigate directly to the escape platform [34, 42, 43]. Once spatial navigation strategies are adopted, escape latencies plateau and animals are considered to have acquired the MWM to their maximal performance [43]. We found that NonTg males adopted spatial search strategies faster than NonTg females (Fig. 3C,D). Males exhibited a higher incidence of spatial navigation as early as test day 1 ($t = 2.569$, $df = 40$, $p < 0.05$, Fig. 3C). Males also transitioned from a predominantly systematic to spatial strategy one day earlier than females (Two-way

repeated measures ANOVA, test day \times strategy interaction: Males $F_{(14,420)} = 17.05$, $p < 0.0001$; Females, $F_{(14,546)} = 16.20$, $p < 0.0001$, *post hoc* Tukey's pairwise comparison, $p < 0.05$, Fig. 3D). As expected, once both sexes adopted primarily spatial strategies, escape latencies plateaued (test day 3–8 in males, test day 4–8 in females). No further navigational improvements were attained (compare Fig. 3A,D).

We next asked why spatial learning is triggered moderately faster in NonTg males compared to females. If female mice do not independently find the platform as fast as males, they will not initiate the spatial learning process as rapidly [43]. To test this hypothesis, we calculated the number of trials needed for both sexes to independently discover the MWM platform (Fig. 3E). Males randomly found the escape platform significantly earlier than females ($t = 2.130$, $df = 46$, $p < 0.05$). We next assessed known impediments to behavioral flexibility. Anxiety manifested as thigmotaxis has been shown to delay random exploration of the interior of the MWM [43]. NonTg females exhibited more thigmotactic swim patterns when first introduced to the MWM on test day 1 than males (Test Day 1, $t = 2.242$, $df = 46$, $p < 0.05$, Fig. 3F) yet without significant overall differences in anxiety with repeated MWM exposure when averaged over the entire test period (ANOVA, $F_{(3,76)} = 1.823$, $p > 0.05$, Supplementary Figure 3B). Elevated thigmotaxis on test day 1 in females was independent of motor impairment. Average distance moved was comparable regardless of sex both on test day 1 (ANOVA, $F_{(3,76)} = 21.88$, $p < 0.0001$, *post hoc* Holm-Sidak's multiple comparisons NonTg males versus NonTg females, $p > 0.05$, Supplementary Figure 3C) and across the entire test period (ANOVA $F_{(3,76)} = 27.67$, $p < 0.0001$, *post hoc* Holm-Sidak's multiple comparisons NonTg males versus NonTg females, $p > 0.05$, Supplementary Figure 3D). Moreover, both males and females swam at the same speed on day 1 (ANOVA, $F_{(3,76)} = 17.68$, $p < 0.0001$, *post hoc* Holm-Sidak's multiple comparisons NonTg males versus NonTg females, $p > 0.05$, Supplementary Figure 3E) and across the entire test period (ANOVA, $F_{(3,76)} = 5.197$, $p < 0.01$, *post hoc* Holm-Sidak's multiple comparisons NonTg male versus NonTg female, $p > 0.05$, Supplementary Figure 3F). Taken together, these data suggested that learning and memory is comparable regardless of sex on this genetic background but that NonTg females do exhibit an initial anxiety-based delay in the independent discovery of the escape platform.

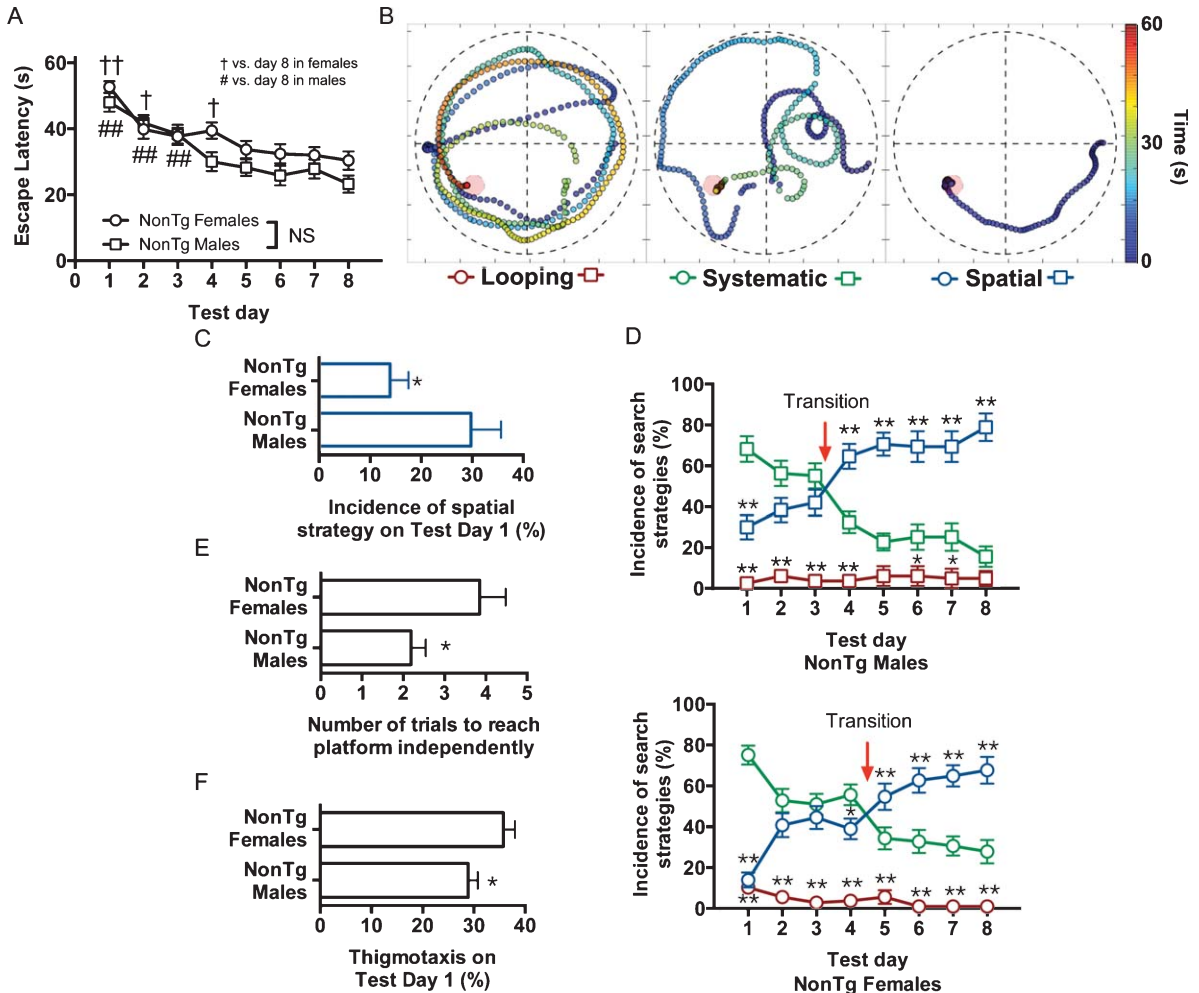


Fig. 3. NonTg mice do not exhibit sexual dimorphisms in learning and memory in the MWM. A) Escape latencies of 5.5-month-old male and female NonTg mice are comparable across all test days. Data represent average escape latency \pm SEM. Statistics were two-way repeated measures ANOVA. *Post-hoc* Tukey's multiple comparisons testing simple effects of the main effect of test day indicated learning was mildly accelerated in males, $\dagger p < 0.01$, $\ddagger p < 0.05$ in NonTg females versus test day eight. $\#\# p < 0.01$ in NonTg males versus test day eight. B) MWM search strategy classifications. Time in maze is color-coded as indicated in the time bar. C) The percentage of spatial strategies \pm SEM used by NonTg males was higher on test day one compared females. Statistics were unpaired Student's *t*-test, $*p < 0.05$. D) NonTg male mice (top panel) transitioned from primarily systematic to primarily spatial search strategies one day earlier than females (bottom panel). Symbols are defined in (B). Data represent the percentage of search strategies used in the four trials per day over eight test days \pm SEM. Statistics were two-way repeated measures ANOVA followed by *post hoc* Tukey's test examining the significant test day \times strategy interaction, $*p < 0.05$, $**p < 0.01$ looping or spatial versus systematic. E) NonTg males independently find the escape platform faster than females. Data represent mean number of trials \pm SEM to obtain the escape platform. Statistics were unpaired Student's *t*-test, $*p < 0.05$. F) NonTg females display more thigmotactic behaviors indicative of anxiety on test day one than males. Data represent the percent thigmotaxis \pm SEM in $n = 21$ NonTg males and $n = 27$ NonTg females. Statistics were unpaired Student's *t*-test, $*p < 0.05$.

Learning and memory deficits in Tg mice are sexually dimorphic by 5.5 months of age

Previous studies have tested learning and memory in the MWM in TgCRND8 males and females either collectively [33–35, 48] or restricting analyses to a single sex [49, 50]. To our knowledge, the

performances of males and females have yet to be directly compared in the MWM. When we included equal numbers of 5.5 month-old N4 and N5 male and female Tg mice, we found significant deficits in escape latency (Two-way repeated measures ANOVA: Main effect of genotype, $F_{(1,62)} = 32.26$, $p < 0.0001$) and average time spent in the platform

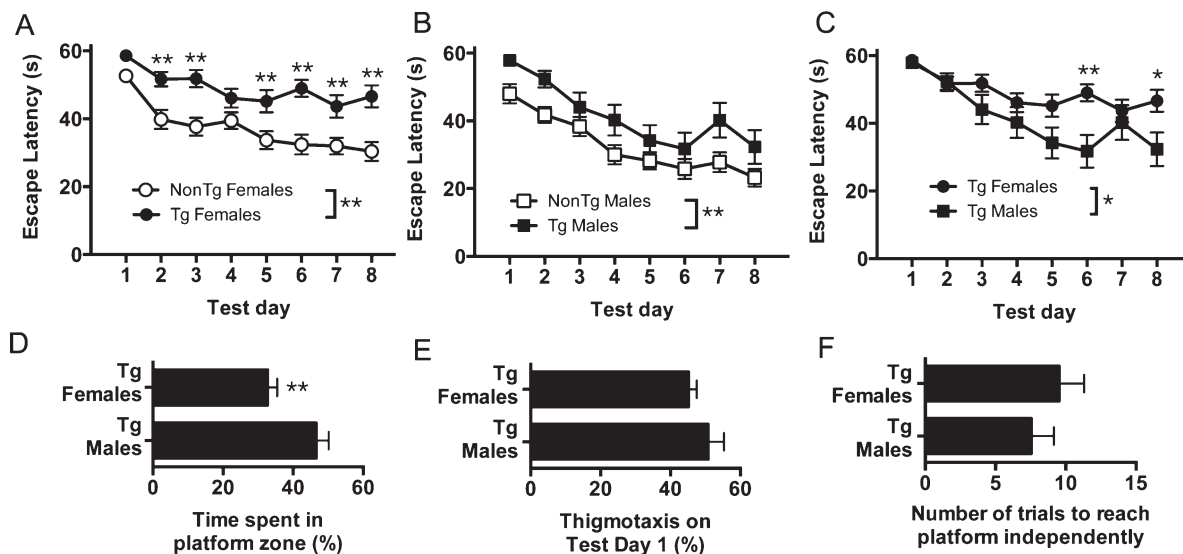


Fig. 4. Tg males and females exhibit different behavioral impairments in the MWM independent of anxiety or motor behaviors. A) Tg females are significantly impaired compared to their NonTg female littermates at 5.5 months of age. B) Tg males are only mildly impaired at 5.5 months of age with little to no difference in their rate of MWM acquisition compared to NonTg male littermates. C) Male Tg mice outperform female Tg mice. In A–C, data represent average escape latencies \pm SEM. Statistics were a two-way repeated measures ANOVA. Main effects of genotype are indicated in the legends, * $p < 0.05$, ** $p < 0.01$. Significant genotype \times test day interactions were analyzed by *post-hoc* Holm-Sidak multiple comparison tests comparing mice on each test day as indicated, * $p < 0.05$ and ** $p < 0.01$. D) Tg males spend significantly more time exploring the escape quadrant than Tg females. E) Tg males and females exhibit comparable thigmotaxis on test day one. F) Tg males and females independently obtain the escape platform within comparable numbers of trials. In D–F, statistics were unpaired Student's *t*-tests, ** $p < 0.01$. Data represent $n = 11$ Tg males and $n = 21$ Tg females.

zone ($t = 3.992$, $df = 62$, $p < 0.001$) (Supplementary Figure 4A,B). When compared separately, females exhibited markedly different learning and memory deficits than males (Fig. 4A–F, Supplementary Figure 3A–F). MWM acquisition was severely impaired in Tg females (Two-way repeated measures ANOVA: Main effect of genotype, $F_{(1,46)} = 23.32$, $p < 0.0001$, Fig. 4A). Tg females did not show the progressive improvement in escape latencies over the 8 test days seen in NonTg females (Fig. 4A). Moreover, Tg females failed to exhibit any spatial bias greater than chance for the escape quadrant during the probe trial (One sample *t*-test: $t = 0.552$, $df = 20$, $p > 0.05$ Supplementary Figure 3A). These data were not due to an irregular estrus cycle in Tg females. Tg females exhibited comparable cycle lengths to NonTg females (Supplementary Figure 2). By contrast, Tg males exhibited only minor deficits in learning and memory (Fig. 4B). Despite higher overall average escape latencies (Two-way repeated measures ANOVA: Main effect of genotype $F_{(1,30)} = 8.088$, $p < 0.01$), Tg male learning curves were comparable to that of NonTg males (Fig. 4B). Tg males were clearly capable of spatial learning as

they exhibited significant spatial bias for the escape quadrant during the probe trial ($t = 4.174$, $df = 10$, $p < 0.01$, Supplementary Figure 3A). Furthermore, Tg males consistently outperformed Tg females (Two-way repeated measures ANOVA: Main effect of sex $F_{(1,30)} = 5.675$, $p < 0.05$; Fig. 4C). Tg males spent significantly more time searching the correct quadrant over the 8 test days ($t = 3.03$, $df = 30$, $p < 0.01$; Fig. 4D) and exhibited significantly higher spatial bias during the probe trial than Tg females (ANOVA, $F_{(3,76)} = 5.05$, $p < 0.01$, *post hoc* Holm-Sidak's multiple comparisons, $p < 0.01$; Supplementary Figure 3A)

Sexually dimorphic learning and memory deficits in N4 and N5 Tg mice are independent of anxiety, motor impairment, and visual deficits

These sex differences were not due to higher test-related anxiety in Tg females (Fig. 4E). Both Tg males and females exhibited comparable thigmotactic behaviors on the first test day ($t = 1.221$, $df = 30$, $p > 0.05$; Fig. 4E) and overall anxiety indices did not differ between male or female Tg or NonTg mice over the entire test period (ANOVA, main effect of

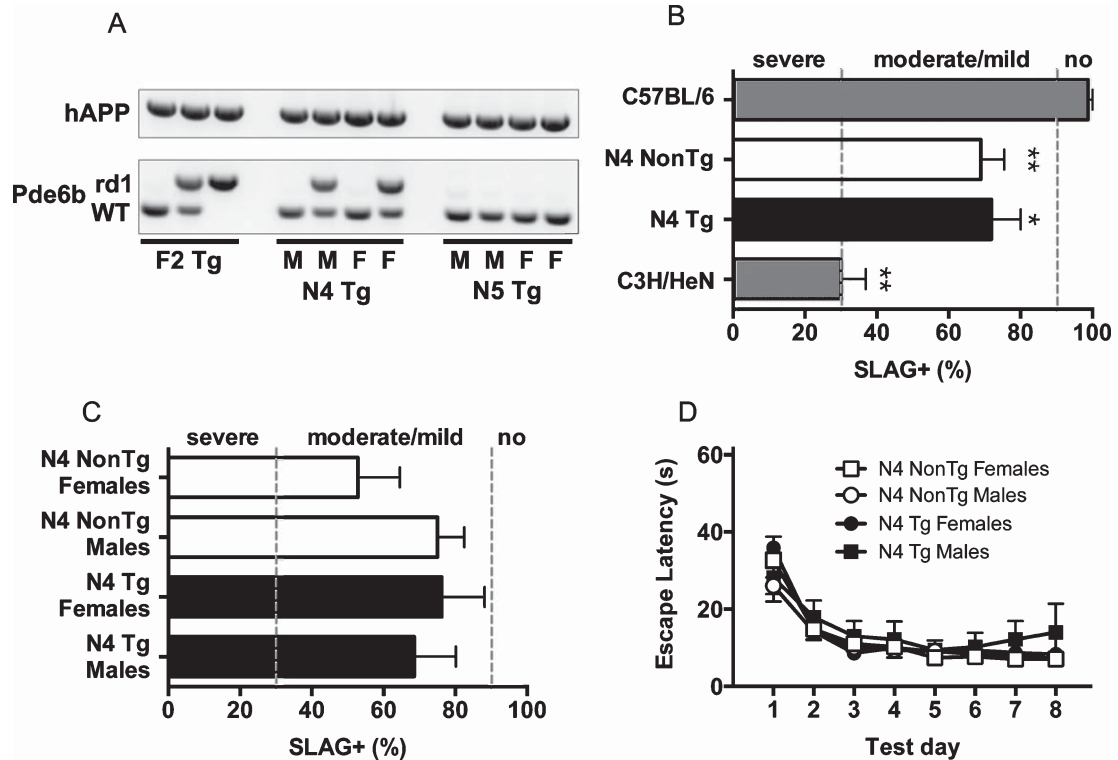


Fig. 5. N4 Tg mice are photosensitive yet without significant impairment of visual acuity in the MWM. A) Genotypes of representative progeny derived from sibling mating of the C57BL/6 \times C3H/HeJ TgCRND8 parental line (F2 Tg, left panel), N4 Tg mice (center panel), and N5 Tg mice (right panel) for the A β PP transgene (top panel) and the *pde6b^{rd1}* retinal degeneration mutation (bottom panel). B) SLAG testing of sighted *pde6b^{+/+}* C57BL/6 mice, functionally blind *pde6b^{rd1/rd1}* C3H/HeNCl mice, and N4 *pde6b^{rd1/+}* and *pde6b^{+/+}* NonTg and Tg males and females. (C) No sex difference in photosensitivity was detected in N4 NonTg or Tg mice. In (B,C), data represent average percentage of SLAG+ responses \pm SEM. Statistics were ANOVA with *post hoc* Dunnett's *t*-tests comparing each group to the C57BL/6 control, * $p < 0.05$, ** $p < 0.01$. (C) SLAG analysis of sex differences within NonTg and Tg genotypes. There are no sex differences in photosensitivity. (D) NonTg and Tg males and females rapidly acquire the MWM when the escape platform is made visible. Data represent average escape latency \pm SEM. Data represent $n = 16$ NonTg males, $n = 9$ Tg males, $n = 6$ NonTg females and $n = 7$ Tg females.

sex: $F_{(1,76)} = 3.355$, $p > 0.05$; Main effect of genotype: $F_{(1,76)} = 0.038$, $p > 0.05$, Supplementary Figure 3B). The frequency of thigmotaxic behaviors in Tg males and females on Test Day 1 was, however, higher than that observed in NonTg females although this comparison did not reach statistical significance (compare Fig. 3F with Fig. 4E). Both Tg males and Tg females required more trials than NonTg mice to independently find the escape platform for the first time (compare Fig. 3E with Fig. 4F); however unlike cognitively intact NonTg mice, Tg males and Tg females were equally impaired ($t = 0.7238$, $df = 30$, $p > 0.05$; Fig. 4F). Tg males did not independently obtain the platform faster than Tg females thus had no temporal advantage in their initiation of spatial learning ($t = 0.7238$, $df = 30$, $p > 0.05$; Fig. 4F). Motor performance was also comparable between Tg males and females (Supplementary Figure 3C–F). Both sexes

swam significantly further (ANOVA, $F_{(3,76)} = 27.67$, $p < 0.0001$, *post hoc* Holm-Sidak's multiple comparisons, $p < 0.01$) and faster (i.e., more hyperactive) (ANOVA, $F_{(3,76)} = 5.197$, $p < 0.01$, *post hoc* Holm-Sidak's multiple comparison, $p < 0.01$) than their NonTg littermates.

A potential confound in the assessment of learning and memory in A β PP Tg mouse models is the impact of their genetics on vision. Spatial navigation in the MWM requires adequate vision under defined illumination conditions to process intra and extra-maze cues. C57BL/6 mice exhibit no visual impairments [44]. C3H/HeJ mice are homozygous for the autosomal recessive retinal degeneration *pde6b^{rd1}* mutation and thus functionally blind [51]. The F1 TgCRND8 parental line is *pde6b^{rd1/+}* and expected to show increased photosensitivity [45]. In our colony, sibling matings produced sighted N4

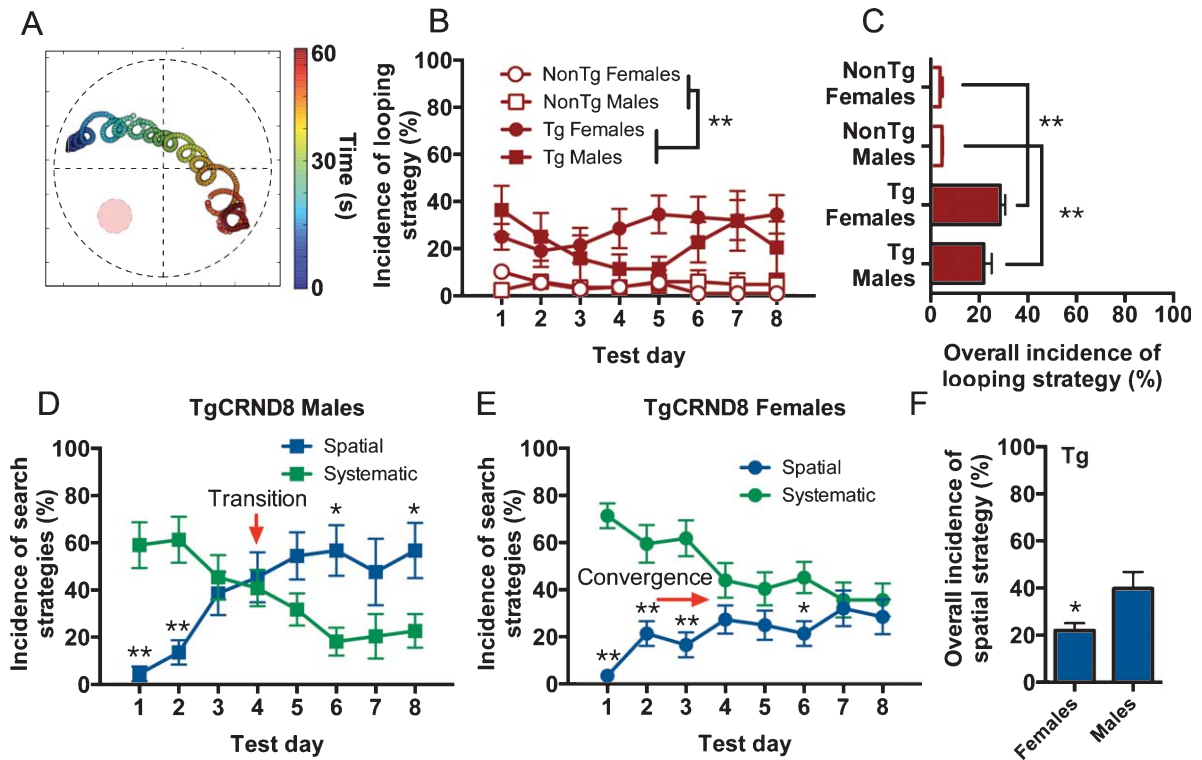


Fig. 6. Tg male but not female mice exhibit behavioral indices of cognitive reserve. A) Representative example of the non-productive repetitive circling behavior exhibited by both Tg male and female mice in the MWM. B) Tg male and female mice exhibit increased incidences of looping search strategies in the MWM compared to NonTg mice. Data represent the percentage of looping search strategies used per day over eight test days (32 trials) \pm SEM. Statistics were a three-way repeated measure ANOVA. The significant main effect of genotype is indicated in the legend, $**p < 0.01$. C) Phenotypic impairments (looping) were comparable between Tg males and females and significantly elevated compared to NonTg sexes. Data represent the percentage of looping search strategies used over the entire test period \pm SEM. Statistics were a one-way ANOVA with *post-hoc* Holm-Sidak multiple comparisons, $**p < 0.01$. D) Tg males were capable of adopting alternative search strategies, transitioning from predominantly systematic to predominantly spatial navigation strategies on test day 4. E) Tg females were unable to adopt spatial learning strategies effectively. By the end of the test period, their use of systematic and spatial strategies converged with incidences equivalent to that of non-productive pathological looping. In (D,E), data represent average daily search strategy incidence \pm SEM. Statistics were two-way repeated measures ANOVA followed by *post hoc* Holm-Sidak tests examining the significant test day \times strategy interactions, $*p < 0.05$, $**p < 0.01$ looping or spatial versus systematic. F) Overall incidence of spatial strategies was significantly higher in Tg males than females. Data represent average \pm SEM. Statistics were unpaired Student *t*-test, $*p < 0.05$. Data represent $n = 21$ NonTg males, $n = 11$ Tg males, $n = 27$ NonTg females and $n = 21$ Tg females.

pde6b^{+/+} wild-type mice, photosensitive *pde6b*^{rd1/+} heterozygotes, and blind *pde6b*^{rd1/rd1} homozygotes (Fig. 5A). The mice used in this study (both males and females) retained the mutant *pde6b*^{rd1} allele to the N4 generation and were a mix of heterozygotes and wild-type (Fig. 5A). This allele was bred out of breeding pairs by the N5 generation (Fig. 5A). We verified that our N4 Tg and NonTg *pde6b*^{rd1/+} mice were photosensitive using the SLAG paradigm [44]. As expected, C57BL/6 *pde6b*^{+/+} mice showed no visual impairments (Fig. 5B). C3H/HeNcr1 *pde6b*^{rd1/rd1} mice were severely impaired by 6–9 months of age (Fig. 5B). We detected a mild to moderate photosensitivity in N4 Tg and Non Tg mice

compared to C57BL/6 controls when tested under 600–700 lux illumination (ANOVA $F_{(3,58)} = 7.773$, $p < 0.0001$; Dunnett's *post hoc* test versus C57BL/6 mice; Fig. 5B). No sex differences were evident (ANOVA $F_{(3,34)} = 0.842$, $p > 0.05$, Fig. 5C). We next used the cued MWM to confirm that this photosensitivity did not impair visual acuity required for spatial learning under the 100 lux MWM illumination levels. N4 NonTg and Tg male and female mice rapidly acquired the cued MWM confirming visual status. Performance approached the limit of detection by Ethovision software (approximately 10 s). There were no significant main effects of genotype or sex (Two-way repeated measures ANOVA: Genotype:

$F_{(1,29)} = 0.855, p > 0.05$; sex: $F_{(1,29)} = 0.007, p > 0.05$, Fig. 5D). Taken together, these data indicate that Tg sex differences in MWM performance are not a consequence of sexual dimorphisms in thigmotaxis (anxiety), visual impairment (photosensitivity), or hyperactivity (swim speed and distance).

Tg mice exhibit sex differences in behavioral indices of cognitive reserve

We asked whether the sex differences observed in MWM performance in N4 and N5 Tg mice reflected sexual dimorphisms in behavioral indices of cognitive reserve, specifically compensation for A β -associated behavioral impairments by effectively alternating between navigational search strategies in the MWM. In humans, cognitive reserve refers to the extent to which an individual can switch between alternative cognitive paradigms to cope with progressive A β pathology [22, 23]. It has been previously established that (a) hyperactivity and (b) an elevated incidence of inefficient looping strategies in both MWM and open field tests are characteristic A β -associated behavioral impairments in F1 TgCRND8 mice [34, 52, 53]. Our N4/N5 Tg males and females at 5.5 months of age exhibited these same A β -associated impairments manifested as repetitive circular swimming patterns in the MWM (Three-way repeated measures ANOVA, time \times sex genotype interaction: $F_{(7,532)} = 3.142, p < 0.05$, main effect of genotype: $F_{(1,76)} = 24.576, p < 0.001$, Fig. 6A,B) with faster swim speeds and longer swim distances (Supplementary Figure 3C–F). While these swim trajectories fall under the overarching MWM looping search strategy (Fig. 3B), their repetition are not considered part of the typical MWM acquisition sequence but rather indicative of pathology-associated stereotypy that reduces behavioral flexibility and thereby impedes the initiation of the spatial learning process [43]. Both Tg males and females exhibited significantly higher (yet equivalent) overall incidences of non-productive looping strategies across the entire test period (ANOVA $F_{(3,28)} = 37.50, p < 0.0001$, *post hoc* Holm Sidak's multiple comparisons, $p < 0.01$; Fig. 6C). To assess whether Tg mice were able to overcome this stereotypy by adopting alternative learning strategies (a behavioral index of cognitive reserve), we evaluated how well they were able to adopt to more efficient strategies with repeated MWM exposure. We found that Tg males were capable of switching navigational search strategies more

efficiently than females as demonstrated by a progressive increase in the incidence of spatial search strategies (Fig. 6D). Tg males transitioned from predominantly systematic to predominantly spatial search strategies by test day 6 (Two-way repeated measures ANOVA, test day \times strategy interaction, $F_{(7,140)} = 9.156, p < 0.001$; *post hoc* Holm-Sidak multiple comparisons $p < 0.05$, Fig. 6D). Tg females were unable to effectively switch between search strategies and thus unable to initiate the spatial learning process (Fig. 6E). Tg females maintained a significantly higher incidence of systematic search strategies until test day 6 (two-way repeated measures ANOVA, test day \times strategy interaction, $F_{(7,280)} = 9.053, p < 0.0001$; *post hoc* Holm-Sidak multiple comparisons $p < 0.05$, Fig. 6E). They failed to transition to a predominantly spatial learning strategy, rather their use of systematic, spatial, and non-productive looping strategies converged, with females using all three strategies equally by the end of the test period (Fig. 6B,E). Males did not exhibit this impairment in behavioral flexibility until approximately 1 year of age thus modeling higher behavioral indices of cognitive reserve (Supplementary Figure 5). As a result, Tg females at 5.5 months of age used significantly fewer spatial strategies than males ($t = 2.287, df = 14, p < 0.05$; Fig. 6F).

DISCUSSION

Here, we report that, when TgCRND8 mice are placed on an N4/N5 C57BL/6 background, they display sex differences in spatial learning that recapitulate some of the sexual dimorphisms in cognitive reserve associated with the greater risk of earlier AD phenoconversion in women [12–15, 54, 55]. Despite equivalent A β burden as males, Tg females at 5.5 months fail to overcome A β -associated stereotypy (i.e., hyperactive repetitive tight circling behaviors [34, 52, 53, 56]) and disproportionally transition to an AD-like phenotype in the MWM. By contrast, Tg males exhibit robust behavioral indices of cognitive reserve, effectively adopting alternative, increasingly productive search strategies with repeated testing. Phenoconversion and impaired behavioral flexibility is not observed until Tg males are approximately 12 months of age. These sexual dimorphisms in learning and memory are only observed in Tg mice in response to A β pathology NonTg male and female littermates show no differences in spatial learning and few dimorphisms in behavioral flexibility beyond

a mildly higher level of anxiety when females are first exposed to the MWM. Taken together, these data describe a new phenotype in N4/N5 Tg mice that can be used to model sex differences in cognitive reserve associated with A β vulnerability.

To our knowledge, this is the first demonstration of sex differences in learning and memory in an A β PP mouse model wherein both sexes exhibit comparable A β load. In other A β PP models, the severity of learning and memory deficits corresponds with greater A β pathology in females [24–29]. Here, our N4/N5 Tg model behaviorally recapitulates clinical reports that men exhibit greater cognitive reserves in the face of equivalent (or higher) A β challenge than women [20, 21, 54]. It is important to note that we refer exclusively to the capacity to switch between cognitive strategies to cope with progressive A β pathology as the aspect of cognitive reserve experimentally modeled in this study. Interestingly, these mice also model sex differences in lethality associated with A β -overexpression [33]. In studies of mild cognitive impairment, mortality has been reported to be higher in men than women despite equivalent A β load [47, 57, 58]. We find that A β -associated lethality is also higher in N4/N5 Tg males than females between 2 and 6 months of age despite comparable A β load in cortex, hippocampus, and cerebrum. We have yet to assess whether this genetic background also recapitulates the sexual dimorphisms in age-associated memory impairment that occur independently of A β biogenesis. For example, in contrast to individuals with high A β load, learning and memory are reported to decline more rapidly in cognitively normal men than women in the absence of significant A β pathology [59]. This decline is hypothesized to contribute to risk of late-life AD phenoconversion rates in males [59] also modeled in N4/N5 Tg males by their later life phenoconversion at 12 months of age. However, further research into age-associated cognitive decline in NonTg males will need to be investigated to elicit the degree of phenoconversion found in Tg males at 12 months. These distinctions are critical to therapeutic interventions targeting cognitive reserve in men and women with high and low A β load over time and it will be important to follow N4/N5 NonTg and Tg males and females beyond 8 months of age to evaluate whether this model reflects all three phenotypes (i.e., sexually dimorphic vulnerability to A β -associated learning and memory impairment, A β -associated lethality, age-associated cognitive decline).

Our data indicate that the N4/N5 Tg model can be used to assess inherent sex differences in A β vulnerability in males and females raised under identical conditions, independent of reproductive senescence, alterations in estrous cycle/hormonal status, visual acuity deficits, or motor impairment. Thus, this model is uniquely suited to exploring molecular underpinnings of cognitive reserve and preclinical response to potential therapeutic intervention difficult to study in the clinic. Sociological differences in education level, occupational attainment, leisure activities, exercise, heart disease, and hypertension associated with gender, not sex, are purported to modulate both A β -associated mortality and cognitive reserve [57, 60]. Moreover, age-dependent hormonal status likely also contributes to enhanced risk of earlier AD phenoconversion in females than males [20], albeit with controversy [61]. As a result, it has been difficult to study sex differences in A β vulnerability directly without an optimal mouse model. The majority of studies examining cognitive reserve in A β PP transgenics have focused on assessing the effect of diet, environmental enrichment, or strategies designed to enhance neurogenesis on phenoconversion in a single sex to control for differences in A β biogenesis [62–66]. While invaluable, these studies have not been able to address directly why males and females respond differently to A β challenge and to these interventions in particular. For example, when females from our parental TgCRND8 line are placed in an enriched environment or given voluntary access to a running wheel, hippocampal neurogenesis and plasticity are enhanced [67], vascular dysfunction is reduced [68], and behavioral indices of learning and memory improve [49]. TgCRND8 males show a different response. Voluntary wheel running in males reduces A β -associated stereotypy in males but without significant impact of learning and memory [69]. Placed in context with our study, it is likely that amelioration of A β -associated behavioral impediments to spatial learning is more effective in females than males given that this stereotypy impairs behavior flexibility and reduces cognitive reserve to a greater extent in Tg females than males.

In summary, this study characterizes an A β PP transgenic model in which females and males exhibit comparable A β burden at all ages yet Tg females show enhanced vulnerability to A β pathology and A β -associated stereotypy, phenocover in the MWM maze earlier, and exhibit fewer behavioral indices of cognitive reserve than males. We suggest that this line represents a novel preclinical model useful for test-

ing strategies designed to enhance cognitive reserve by targeting the different A β vulnerabilities exhibited by males and females.

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SUPPLEMENTARY MATERIAL

The supplementary material is available in the electronic version of this article: <http://dx.doi.org/10.3233/JAD-150587>.

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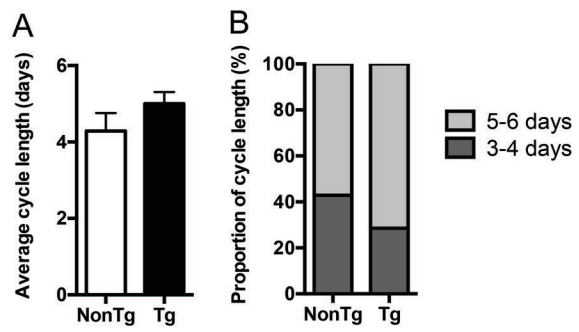
Supplemental Figure 1. No sex-differences in cortical A β PP and CTF β protein levels are detected at 2, 4, or 6 months of age. Full immunoblots at both exposures depicted in Figure 1 detecting human A β PP and CTF β are provided to confirm specificity of labeling.

Supplemental Figure 2. Tg and NonTg females have comparable estrous cycles. (A) Average estrous cycle length is comparable between NonTg and Tg females. Data represent mean \pm SEM. (B) In both genotypes, mice exhibited comparable numbers of 3-4 or 5-6 days cycles. Longer cycles in both genotypes were attributed to extensions the metestrus/diestrus stages. Data represent n=7 NonTg females and n=7 Tg females.

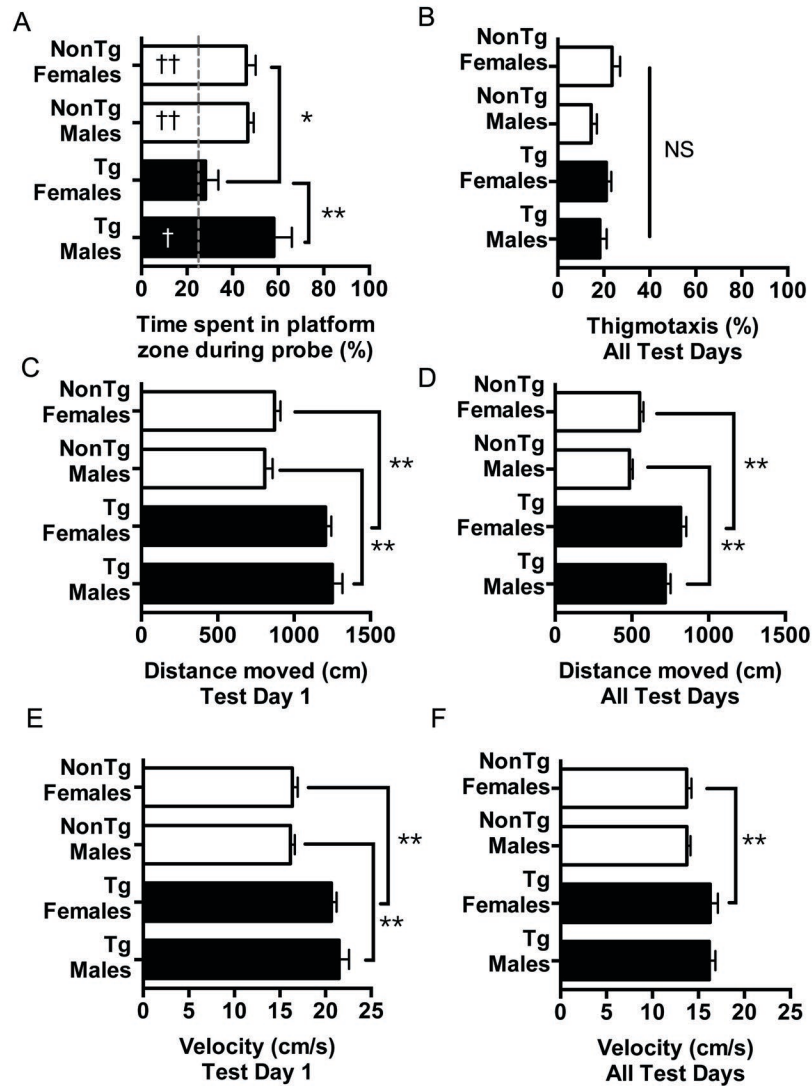
Supplemental Figure 3. Indices of learning and memory, anxiety, and motor capacity in NonTg and Tg males and females. (A) Spatial bias for the escape quadrant was assessed during the probe trial. Data represent the average amount of time mice spent exploring the correct escape quadrant \pm SEM. Statistics were a one-sample t-test comparing each group to a hypothetical value of 25% representing the amount of time a mouse would be expected in the platform quadrant by chance, †p<0.01 ††p<0.001. Between group comparisons were performed by ANOVA and post-hoc Holm-Sidak comparisons, **p<0.01, *p<0.05. (B) Overall percentage of thigmotaxic behaviors across the entire test period \pm SEM. (C) Average distance moved on test day one \pm SEM. (D) The Average distance moved across all test days \pm SEM. (E) Average velocity on test day one \pm SEM. (F) Average velocity across the entire test period \pm SEM. In (B-F), between group comparisons were performed by ANOVA and post-hoc Holm-Sidak comparisons, **p<0.01, *p<0.05. Data represent n=21 NonTg males, n=11 Tg males, n=27 NonTg females and n=21 Tg females.

Supplemental Figure 4. Tg mice exhibit learning and memory impairment in the MWM when both sexes are assessed collectively. (A) MWM acquisition in equal numbers of male and female Tg and NonTg mice. Data represent average escape latencies \pm SEM. Statistics were two-way repeated measures ANOVA. The main effect of genotype is indicated in the legend, ** $p < 0.01$. (B) Tg mice spend less time exploring the correct quadrant across all test days. Data represent mean \pm SEM. Statistics were an unpaired Student's t-test, ** $p < 0.01$. Data represent $n=21$ NonTg males, $n=21$ NonTg females, $n=11$ Tg males, and $n=11$ Tg females randomly selected from the entire cohort.

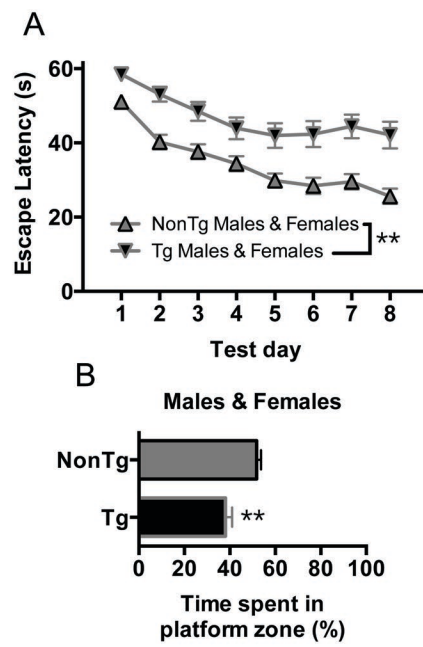
Supplemental Figure 5. Tg males are unable to achieve a predominant spatial strategy at approximately 12 months age. Impairment in strategy shifting is detected in males by 12 months of age. By the end of the test period, the employment of systematic and spatial strategies converged with Tg males failing to adopt primarily spatial strategies. Data represent average daily search strategy incidence \pm SEM. Statistics were two-way repeated measures ANOVA followed by *post hoc* Holm-Sidak tests examining the significant test day x strategy interactions, * $p < 0.05$, ** $p < 0.01$ spatial versus systematic.



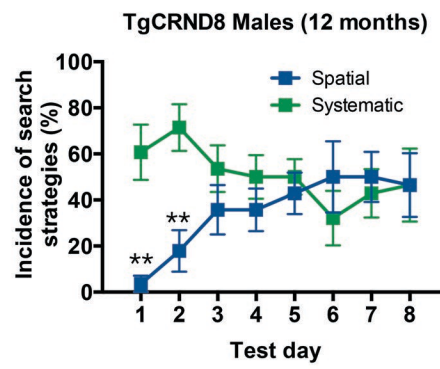
Supplemental Figure 2



Supplemental Figure 3



Supplemental Figure 4



Supplemental Figure 5

Supplementary Table 1. Genotyping Protocols

| Gene | Reaction Conditions and Amplicon Sizes | Cycling parameters |
|--------------|--|---|
| A β PP | 191: 5'-GGCCGCGGAGAAATGAAGAAACGCCAAG-CGCCGTGACT-3' 229: 5'-TGTCCAAGATGCAGCAGAACGGCTACGAAAA-3' Final primer concentrations: 0.8 pmol/ μ L each Volume of DNA: 2 μ L Final reaction volume: 12.5 μ L (Tg allele = 1000 bp) | 94°C 3 m 35 cycles: 94°C 20 s 68°C 20 s 72°C 90 s 72°C 7 min |
| Pde6b | RD3: 5'-TGACAATTACTCCTTTTCCCTCAGTCTG-3' RD4: 5'-GTAAACAGCAAGAGGCTTTATTGGGAAC-3' RD6: 5'-TACCCACCCTTCCTAATTTTCTCACGC-3' Final primer concentrations: 0.5 pmol/ μ L each Volume of DNA: 1 μ L Final reaction volume: 25 μ L (Wildtype allele = 550 bp) (Mutant allele = 400 bp) | 94°C 2 m 35 cycles: 94°C 30 s 68°C 30 s 72°C 120 s 72°C 10 min |