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Limited Impact of Social Isolation on Alzheimer-Like Symptoms in a Triple Transgenic Mouse Model

Susanna Pietropaolo
Swiss Federal Institute of Technology Zurich

Yan Sun and Ruixi Li
Fudan University

Corinne Brana, Joram Feldon, and Benjamin K. Yee
Swiss Federal Institute of Technology Zurich

Gene-environment interactions are known to play a major role in the ethiopathology of several neuropsychiatric disorders, including Alzheimer's disease (AD). The present study investigates whether environmental manipulations, that is, social isolation, may affect the genetic predisposition to develop AD-related traits in a triple transgenic mouse model (3 × Tg-AD), as suggested by our previous study employing physical exercise (Pietropaolo et al., 2008). Mutant and wild type mice of both sexes were housed singly or in groups from weaning, and evaluated behaviorally at 6 to 7 months of age. Independent of sex, the 3 × Tg-AD genotype was associated with enhanced acoustic startle response, improved performance in the cued version of the water maze and a clear impairment in the Y maze. Notably, the female (but not male) mutant mice showed increased anxiety. Although social isolation was effective in modifying several behaviors, it did not exacerbate any of the AD-like symptoms. Our findings demonstrated the differential susceptibility of the 3 × Tg-AD mouse line to environmental manipulations, showing that social isolation did not induce remarkable effects on the genetically determined AD-like symptoms, in contrast to what previously observed with physical exercise.

Keywords: acoustic startle response, gene-environment interactions, anxiety, spatial memory, sex differences

Converging lines of evidence from human research suggest that the lack of adequate social networks can promote the expression of neuropsychiatric disorders, including Alzheimer's disease (AD; Kessler, Price, & Wortman, 1985; Singer, Friedman, Seeman, Fava, & Ryff, 2005). These data provided impetus to animal studies devoted to investigate the mechanisms underlying the effects of insufficient social support. A relevant paradigm to investigate the impact of social deprivation on the pathogenesis of neuropsychiatric disorders in rodents consists of rearing animals in social isolation (Weiss & Feldon, 2001). The isolation housing condition is typically implemented at weaning because the early postweaning (juvenile) phase constitutes a critical period for the effects of social isolation in rats (Einon & Morgan, 1977; Einon, Morgan, & Kibbler, 1978) as well as in mice (Dyer & Southwick, 1974; Terranova, Laviola, & Alleva, 1993).

Postweaning social isolation induces a wide range of brain and behavioral changes in rats (Hatch et al., 1965) and in mice (Valzelli, 1973). This "isolation syndrome" may resemble the symptoms of several neuropsychiatric disorders, including alterations of spontaneous activity, sensorimotor response, emotionality, and cognition. Although most of the studies have been conducted only in male animals, the magnitude of the isolation effects can differ largely between sexes in both rats (Weiss, Domeney, Heidbreder, Moreau, & Feldon, 2001; Weiss, Pryce, Jongen-Relo, Nanz-Bahr, & Feldon, 2004) and mice (Abramov et al., 2004; Guo, Wu, Liu, Yang, & Chen, 2004; Pietropaolo, Singer, Feldon, & Yee, 2008).

One of the most robust effects of social isolation consists of increased spontaneous locomotor activity (mice: Abramov et al., 2004; Benton & Brain, 1981; Pietropaolo et al., 2008; rats: Domeney & Feldon, 1998; Einon et al., 1978; Gentsch, Lichtsteiner, Frischknecht, Feer, & Siegfried, 1988; Heidbreder et al., 2000), with few exceptions (Geyer, Wilkinson, Humby, & Robbins, 1993; Weiss, Di Iorio, Feldon, & Domeney, 2000; Weiss et al., 2001). Social isolation also reportedly enhanced acoustic startle response (mice: Dai et al., 2005; Sakaue, Ago, Baba, & Matsuda, 2003; rats: Varty, Braff, & Geyer, 1999; Weiss, Feldon, & Domeney, 1999;

Susanna Pietropaolo, Corinne Brana, Joram Feldon, and Benjamin K. Yee, Laboratory of Behavioural Neurobiology, Swiss Federal Institute of Technology Zurich, Schwerzenbach, Switzerland; Yan Sun and Ruixi Li, Department of Anatomy, Histology, and Embryology, Shanghai Medical College, Fudan University, Shanghai, China.

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Correspondence concerning this article should be addressed to Benjamin K. Yee, Laboratory of Behavioral Neurobiology, Swiss Federal Institute of Technology Zurich, Schorenstrasse 16, CH–8603 Schwerzenbach, Switzerland. E-mail: byee@ethz.ch

Wilkinson et al., 1994) and induced anxiogenesis (Guidotti et al., 2001; Wright, Upton, & Marsden, 1991), though the latter effect was not consistently detected across different studies (mice: Moragrega, Carrasco, Vicens, & Redolat, 2003; Pietropaolo et al., 2008; Voikar, Polus, Vasar, & Rauvala, 2005; rats: Hellemans, Benge, & Olmstead, 2004; Lapiz, Mateo, Durkin, Parker, & Marsden, 2001; Schrijver, Bahr, Weiss, & Wurbel, 2002).

Social isolation also impaired cognitive abilities in a variety of spatial and nonspatial memory tests in both rats (Hellemans et al., 2004; Jones, Marsden, & Robbins, 1991; Juraska, Henderson, & Muller, 1984; Lu et al., 2003; Weiss et al., 2004) and mice (Dai et al., 2005; Ibi et al., 2008; Voikar et al., 2005), although some exceptions have been reported (Lapiz et al., 2001; Moragrega et al., 2003; Schrijver et al., 2002). These effects have also been described in genetic mouse models of cognitive dysfunction: social isolation impaired passive avoidance in senescence-accelerated mice (Chida, Sudo, Mori, & Kubo, 2006) and contextual learning in the Tg2576 mouse model of AD (Dong et al., 2004). Furthermore, isolation from weaning until 6 months of age accelerated the deposition of β-amyloid plaques in Tg2576 mice, suggesting that isolation may affect the symptoms of AD by acting on the underlying brain processes (Dong et al., 2004).

The impact of isolation on genetically induced AD-like symptoms has been investigated so far only within the cognitive domain (Dong et al., 2004). Yet, AD is a heterogenous pathology characterized not only by memory impairments, but also by distractibility (Chiu et al., 2004; Helkala, Laulumaa, Soininen, & Riekkinen, 1989; Jessen et al., 2001; Loewenstein et al., 2004, 2003), increased irritability and altered emotionality (Aalten et al., 2003; Frisoni et al., 1999; Hope, Keene, Fairburn, McShane, & Jacoby, 1997; Lawlor & Bhriain, 2001). Most of these noncognitive symptoms can be mimicked by the triple-transgenic mouse model (3 × Tg-AD), harboring PS1M146V, APPSwe, and tauP301L transgenes (Oddo et al., 2003). At around 6 months of age, that is, at the early stage of the pathology when Aβ- immunoreactivity is first detected in the hippocampus and amygdala (Billings, Oddo, Green, McGaugh, & LaFerla, 2005; Oddo et al., 2003), 3 × Tg-AD mice of both sexes display signs of enhanced reactivity to aversive stimuli (Pietropaolo, Feldon, & Yee, 2008; Pietropaolo, Sun, et al., 2008), for example, enhanced acoustic startle response and improved performance in the cued version of the water maze. This noncognitive syndrome is associated with mild deficits in water maze and Y maze learning (Billings, Green, McGaugh, & LaFerla, 2007; Billings et al., 2005; Caccamo et al., 2006; Clinton et al., 2007; Gimenez-Llort et al., 2007; Oddo et al., 2003), although these behavioral alterations appear to be preferentially expressed in females (Pietropaolo, Feldon, et al., 2008; Pietropaolo, Sun, et al., 2008).

The aim of the present study was to evaluate the impact of social deprivation on the genetic predisposition to develop AD-like symptoms in the 3 × Tg-AD mouse model. We hypothesized that postweaning social isolation would exacerbate most of the noncognitive and cognitive symptoms displayed by 3 × Tg-AD mice at around 6 months of age. At weaning, wild type and mutant 3 × Tg-AD mice of both sexes were housed singly or in groups and at 6 to 7 months of age they were subjected to a battery of behavioral tests, including anxiety (elevated plus maze), locomotor activity (open field), sensorimotor response (the acoustic startle reflex), and spatial memory (water maze and Y maze). In addition, β-amyloid immunoreactivity was also assessed in the hippocampus to evaluate potential effects of isolation on the severity of AD-like brain pathology.

Method

Subjects and Housing Conditions

Subjects of both sexes were employed here. There were 33 transgenic 3 × Tg-AD and 38 wild type mice of matched genetic background. Both mouse lines were originally generated and maintained by Frank LaFerla and colleagues (University of California, Irvine), and the full descriptions of their generation have been reported previously (Oddo et al., 2003). Breeding pairs were obtained from LaFerla, and bred in the SPF (specific-pathogen-free) facility at the Laboratory of Behavioral Neurobiology (Swiss Federal Institute of Technology, Zurich). The genotype of each and every mouse was confirmed by standard PCR of DNA isolated from tail biopsies collected at the end of the experiments reported here.

The animals were weaned on postnatal day 21, and transferred to a separate temperature and humidity controlled (22 °C, 55%) animal room to begin the two experimental housing conditions: (a) grouped, in which mice of the same genotype and sex were housed in groups of 4 to 5 in Makrolon cages (Tecniplast, Milan, Italy), measuring 41 × 20 × 19 cm, and (b) isolated, in which mice were individually housed in Makrolon transparent cages of 26 × 21 × 14 cm. The grouped condition included 17 males (9 wild type and 8 mutant) and 18 females (10 wild type and 8 mutant), whereas the isolated group comprised 18 males (9 wild type and 9 mutant) and 18 females (10 wild type and 8 mutant). We ensured that members from a given litter were randomly allocated between the two housing conditions (i.e., either isolated or grouped), to minimize possible confounding due to litter effects (Zorrilla, 1997). We further ensured that each cage of the grouped condition comprised mice from at least two independent litters.

All cages were provided with sawdust bedding (Schill AG, Muttenz, Switzerland) that was renewed weekly and a stainless steel wired lid. Food chow (Provimi Kliba SA, Kaiseraugst, Switzerland) and water bottles were provided on the top of each cage. The animals were kept on an ad lib feeding condition and maintained under a 12:12hr reversed light–dark cycle (lights off: 0700–1900). The ambient light intensity in the animal vivarium during the light phase was 250 lux.

Animals were left undisturbed until the beginning of behavioral testing at the age of approximately 180 days. Here, we refrained from determining the oestrous cycle of the female mice (a) to avoid stressing the animals due to repeated vaginal smears, and (b) to prevent a confounding factor with sex because the male mice could not be equivalently treated.

Behavioral Procedures

Five female mutant animals (3 grouped and 2 isolated) died before the beginning of behavioral testing. Data were therefore collected from 38 (18 males, 20 females) wild type and 28 (17 males, 11 females) mutant mice.

Behavioral tests commenced when the animals reached the age of 180 days. Mice were maintained in the corresponding housing

conditions throughout the entire experimental period, which lasted for about a month. Five tests were included in this study: (a) elevated plus maze test of anxiety, (b) open field test of spontaneous locomotor activity and spatial exploration, (c) startle reactivity, (d) spatial reference memory in the water maze, and (e) spontaneous alternation in the Y maze. The animals were approximately 180, 183, 185, 190, and 208 days old at the beginning of the five tests, respectively. Whenever possible, tests that relied mainly on observations of spontaneous behavior were conducted first to minimize undesirable transfer effects.

All behavioral tests were always carried out during the dark phase of the cycle. All manipulations described here had been approved by the Cantonal Veterinary Authority of Zurich, and were in accordance to the European Union Directives (86/609/EEC).

Elevated Plus Maze

Apparatus. The elevated plus maze consisted of clear acrylglass made opaque by black self-adhesive foil ("d-c-fix"; Konrad Hornschuch, Weissbach, Germany) with a removable plastic gray floor. It was elevated 70 cm above floor level, and positioned in the middle of a testing room with diffuse dim lighting (25 lux in the center of the maze). The elevated plus maze consisted of four equally spaced arms radiating from a central square measuring 5 × 5 cm. Each arm was 30 cm long and 5 cm wide. One pair of opposing arms was enclosed with opaque walls, 14 cm high, except for the side adjoining the central square. The remaining two arms were exposed with a 3-mm high perimeter border along the outer edges. A digital camera was mounted above the maze. Images were captured at a rate of 5 Hz and transmitted to a PC running the Ethovision (Version 3.1, Noldus Technology, The Netherlands) tracking system.

Procedure. To begin a trial, the mouse was gently placed in the central square with its head facing one of the open arms. It was allowed to explore freely and undisturbed for 5 min.

Data analysis. Two anxiety-related measures were calculated: percentage time in open arms = time in open arms / time in all arms × 100%, and percentage entries into open arms = the number of entries into open arms / the number of entries into open and enclosed arms × 100%. In addition, the total distance traveled in the entire maze surface (i.e., arms and central platform) was recorded as a concomitant measure of locomotor activity.

Open Field Test

Apparatus. The apparatus consisted of four identical square arenas, each measured 40 × 40 cm in surface area and was surrounded from all sides by a 25 cm wall. The open field was made of wood with a white waterproof plastic surface. It was located in a testing room under diffused dim lighting (30 lux). A digital camera was mounted directly above the four arenas, capturing images from all four arenas at a rate of 5Hz. The images were transmitted to a PC running the Ethovision (Version 3.1, Noldus Technology, The Netherlands) tracking system.

Procedure. The mice were tested in squads of four (one in each arena). The mouse was gently placed in the center of the appropriate arena and allowed to explore undisturbed for 60 min. Afterward, they were returned to the home cage and the arenas cleansed with water and dried prior to the next squad.

Data analysis. Locomotor activity was indexed by distance traveled recorded in consecutive 5-min bins.

Assessment of the Acoustic Startle Reflex

Apparatus. The apparatus consisted of four acoustic startle chambers for mice (SR-LAB, San Diego Instruments, San Diego, CA) each comprised a nonrestrictive cylindrical enclosure made of clear Plexiglas attached horizontally on a mobile platform, which was in turn resting on a solid base inside a sound-attenuated isolation cubicle. A high-frequency loudspeaker mounted directly above the animal enclosure inside each cubicle produced a continuous background noise of 65 dB_A and the various acoustic stimuli in the form of white noise. Vibrations of the Plexiglas enclosure caused by the whole-body startle response of the animal were converted into analog signals by a piezoelectric unit attached to the platform. These signals were digitized and stored by a computer. A total of 130 readings were taken at 0.5-ms intervals (i.e., spanning across 65 ms), starting at the onset of the startle stimulus. The average amplitude over the 65-ms window was used to determine the subject's startle reactivity on a given trial. The sensitivity of the stabilimeter was routinely calibrated to ensure consistency between chambers and across sessions.

Procedure. Acoustic startle reflex was assessed during a session lasting for approximately 30 min, in which the subjects were presented with a series of discrete pulse-alone trials of different intensities and durations. Ten pulse intensities were used: 69, 73, 77, 81, 85, 90, 95, 100, 110, and 120dB_A lasting for either 20 or 40 ms. A session began when the animals were placed into the Plexiglas enclosure. They were acclimatized to the apparatus for 2 min before the first trial began. The first six trials consisted of six trials at 120 dB_A, with three trials at each of the two possible stimulus durations. These trials served to stabilize the animals' startle response and were analyzed separately. Subsequently, the animals were presented with five blocks of discrete test trials. Each block consisted of 20 pulse-alone trials, one for each intensity and duration, presented in a pseudorandom order. The intertrials interval was variable (10 to 20 s) with an average duration of 15 s.

Spatial Reference Memory in the Water Maze

Apparatus. The water maze consisted of a circular tank measuring 102 cm in diameter and 36 cm high that was positioned in the middle of a well-lit testing room enriched with distal visual cues. It was made of fiberglass and painted white. The bottom of the maze was raised 12 cm over the room floor. At the beginning of each day, the water maze was filled with a mixture of cold and hot tap water and the temperature maintained at 24 ± 1 °C. A stable solid Plexiglas cylinder measuring 7 cm in diameter and 18 cm high served as the escape platform. It had a rough surface that allowed the animal to climb onto it easily once its presence was detected. Four points, equally spaced along the circumference of the pool, were arbitrarily designated as: N, E, S, W. These points served as the starting positions at which the mouse was lowered gently into the water, with its head facing the wall of the water maze. The area of the pool was conceptually divided into four quadrants (NE, SE, SW, and NW) of equal size by two imaginary orthogonal lines running through the center of the pool. A digital camera was mounted above the water maze, capturing images at a

rate of 5 Hz and transmitting the data to a PC running the Ethovision (Version 3.1, Noldus Technology, The Netherlands) tracking system.

Procedure. On each experimental day, mice were habituated to the experimental room: They were individually housed in standard Makrolon cages (26 × 21 × 14 cm in size) and left undisturbed for about 10 min before testing began.

On Days 1 and 2, the animals were tested in the visually cued task across four consecutive trials with a 1-min ITI. The platform surface was 0.5 cm above the water level, and its presence was made visible by mounting a black disk of the same size (7 cm in diameter) directly above it. This cued task served to habituate the animals to the pool and to train them to escape from the water by climbing onto the platform. It further allowed the assessment of possible noncognitive alterations related to swimming, visual abilities, and general motivation to escape from the pool. The platform was positioned in different locations across the four trials: in the center of the pool and in the center of the three quadrants not used to locate the hidden platform in the subsequent reference memory task. The starting point for releasing the mouse in the pool was constant across trials within a day but changed from Day 1 to 2. Platform locations as well as the starting position were counterbalanced as much as possible with respect to all between-subjects factors. A trial ended when the animals escaped onto the platform or when 60 s had elapsed, at which time the animal was guided to the platform by the experimenter. The animal was allowed to spend 30 s on the platform; afterward it was placed into a waiting cage for a further 30 s prior to commencement of the next trial.

On Days 3 to 8, the animals were trained to locate the escape platform that was now hidden under the water surface and remained in a constant location (in the middle of one of the quadrants, 22 cm off the maze wall). Across the four trials in a day, the start positions varied among N, E, S, and W in a pseudorandom sequence. Otherwise, the testing procedures were identical to those described above.

On Day 9, in addition to the four trials of hidden platform training, two 30-s probe tests were conducted: 1.5 h before and 1.5 h after hidden platform training. The mice were returned to the home cage for the 1.5 h intervals. In the probe test, the platform was removed from the water maze, and the animal released into the quadrant opposite to the one in which the platform had been previously located (i.e., the "target" quadrant).

On Day 10, the animals underwent another 30-s probe test, as described above. A similar procedure with multiple-probe tests was employed by previous studies carried out in the 3 × Tg-AD model (Billings et al., 2007, 2005; Caccamo et al., 2006; Clinton et al., 2007; Gimenez-Llort et al., 2007; Oddo et al., 2003).

Data analysis. On each trial, the latency and distance swum to reach the platform (visible or hidden) were recorded. Performance on probe tests was evaluated by percentage time spent and distance swum in the target quadrant on its own, and in comparison to the other three (nontarget) quadrants. The number of annular crossings and the latency to the first annular crossing were also evaluated in each probe test. An annular crossing was scored whenever a swim path crossed into the area previously occupied by the platform.

The average swim speed in each trial at different stages of the experiment was also computed and analyzed.

Spontaneous Alternation in the Y Maze

Apparatus. Spontaneous alternation was assessed in a gray, wooden Y maze, elevated 80 cm from the ground and located in the middle of a room containing a variety of extra maze cues. The three arms of the Y maze were identical in appearance and spaced at 120° from each other. Each arm was 50 cm long and 10.5 cm wide. The entire maze was enclosed by a wall 10 cm high and 1 cm thick. The floor of the maze was covered with sawdust bedding. A digital camera was mounted above the water maze, capturing images at a rate of 5 Hz and transmitting the data to a PC running the Ethovision tracking system.

Procedure. Animals were habituated to the experimental room as described for the water maze experiment. Mice were assigned two arms (start and familiar arm) to which they were exposed during the first phase of the test (sample phase). The remaining third arm constituted the novel arm during the second phase (test phase). Allocation of arms (start, familiar, and novel) was counterbalanced within each experimental group.

During the sample phase, access to the novel arm was blocked by a gray wooden door, 12 cm high, 7.5 cm wide, and 0.5 cm thick. Mice were placed at the end of the start arm and allowed to freely explore both the start and the other unblocked arm for 5 min before being removed from the maze and returned to the waiting cage. Timing of the 5-min sample phase period began once the mouse had left the start arm. After 90 s in the waiting cage, the test phase began. During this phase, the door was removed and all three arms were unblocked. To begin the test phase, mice were placed at the end of the start arm and allowed to explore the entire maze for 2 min. Timing of the 2-min test phase period began once the mouse had left the start arm. In the interval between the exposure and the test phase the sawdust from each arm was mixed and randomly distributed to avoid olfactory cues.

Data analysis. Time spent in each arm of the maze was analyzed during both phases of the experiment as well as the total distance traveled. Performance on test phase was evaluated by time spent in the novel arm on its own as well as in comparison to the other two arms.

Assessment of β-amyloid Pathology

Immunohistochemistry. Animals were deeply anesthetized with an overdose of sodium pentobarbital (Nembutal; 40 mg/kg ip) and perfused transcardially with ice-cold saline. The brains were removed in toto, and then bisected into two hemispheres. The left hemisphere was immediately frozen and the right hemisphere was postfixed for 24 hr by immersion into a cold fixative (0.15M phosphate buffer with 4% paraformaldehyde and 15% saturated picric acid solution, pH 7.4). After fixation, the right hemisphere underwent microwave-assisted processing as previously described (Fritschy, Weinmann, Wenzel, & Benke, 1998). Following cryoprotection with sucrose solution, free-floating sections (40 μm thick) were cut on a freezing microtome and 8 series of about 20 coronal interleaved sections were sampled from bregma +2.58 to −4.16 mm (Paxinos & Franklin, 2001) and stored at −20 °C in cryoprotectant solution until further processing.

Immunohistochemistry was performed on one randomly sampled series of brain sections per animal, employing the mouse antihuman β-amyloid protein as the primary antibody (Chemicon, Temecula CA, 1:500). The free floating sections were rinsed in

PBS three times for 10 min and incubated for 1 hr in PBS containing 5% normal goat serum (NGS) and 0.3% Triton X-100 at room temperature (RT). Sections were incubated overnight at 4 °C in the primary antibody diluted in PBS containing 2% NGS and 0.3% Triton X-100 (antibody buffer). Sections were then washed with PBS and incubated with the biotinylated secondary antibody (Jackson ImmunoResearch Laboratories Inc., Pennsylvania, PA) diluted 1:500 in antibody buffer for 1 hr at RT. After three washes in PBS, sections were incubated in Vectastain ABC Kit (+++Vector Laboratories Inc., Burlingame, CA) diluted in PBS, for 1 hr at RT, and washed three times in 0.1 M Tris-HCl buffer, pH 7.4. Immunoreactivity was visualized by standardized DAB method: sections were incubated with 1.25% 3,3′-diaminobenzidine (DAB, Fluka, Buchs, Switzerland) and 0.08% H_2O_2 in 0.1 M Tris-HCl, pH 7.6 for 2 to 15 min and washed three times in PBS. Sections were then mounted on gelatin-coated slides, dried overnight, dehydrated and cover slipped with Eukitt (Kindler GmbH & Co, Freiburg, Germany).

Evaluation of β-amyloid immunoreactive cells in the hippocampus. β-amyloid immunoreactivity was mainly observed in pyramidal neurons, as suggested by the morphological evaluation of the β-amyloid-immunoreactive (ir) cells, and it was mainly localized in the cytoplasm. β-amyloid-ir cells were detected mainly in CA1 and CA3 of the dorsal and ventral hippocampus.

Stereological estimation of the number of β-amyloid-ir cells was performed in the hippocampus and the cell number was estimated in CA1 and CA3 hippocampal subfields using the optical fractionator method (Buckmaster & Jongen-Relo, 1999; Gundersen et al., 1988). Four nonadjacent sections for the dorsal (from bregma −1.34 to −2.30 mm) and two for the ventral hippocampus (from bregma −2.92 to −3.64 mm) were analyzed with the aid of the image analysis computer software Stereo Investigator (Version 6.50.1; Microbrightfield, Colchester, VT). The following sampling parameters were used: a fixed counting frame measuring 30 × 30 μm; and a sampling grid size of 99.7 × 55.1 μm. The counting frames were placed randomly at the intersections of the grid within the outlined structure of interest by the software. The cells were counted following the unbiased sampling rule (Howard & Reed, 2005) using the 20× objective lens (N.A. 0.75) and included in the measurement when they came into focus within the optical dissector (height, 10 μm). For this analysis, the total number of estimated β-amyloid-ir neurons per unit volume was evaluated.

Statistical Analysis

All data were analyzed by parametric analysis of variance (ANOVA) with genotype, sex, and housing condition as the between-subjects factors. Additional within-subjects factors (e.g., trials, days) were also included as determined by the nature of the dependent variables under consideration. Supplementary restricted analyses were also performed to assist data interpretation whenever appropriate. Post hoc comparisons were performed using Fisher's LSD test based on the overall error variance associated with the relevant factor. To better conform to the assumptions of parametric ANOVA, data from startle reactivity were transformed using the following logarithmic transformation: $y = ln(x + 1)$.

All statistical analyses were carried out using SPSS for Windows (release 13.0, SPSS Inc. Chicago) installed on a PC running the Microsoft Windows XP SP2 operating system.

Results

Elevated Plus Maze

Differences in anxiety-like behavior were observed between genotypes, but they were clearly dependent on sex and housing conditions (Figure 1A). In females, mutant mice displayed higher anxiety levels compared to wild type controls, but this effect was observed exclusively in the grouped condition. This was because isolation was anxiogenic in wild type female mice. In males, no differences between genotypes, sexes, or housing conditions were detectable. The 2 × 2 × 2 (Genotype × Sex × Housing) ANOVA of the percentage time spent in the open arms confirmed these conclusions yielding a significant Genotype × Sex × Housing interaction, $F(1, 58) = 4.31$, $p < .05$, although all main effects and

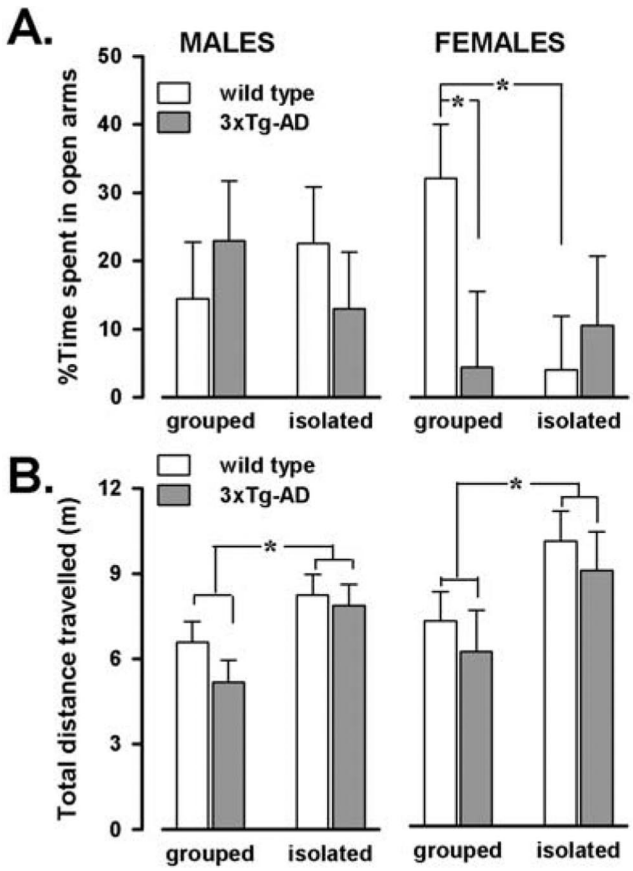

Figure 1. Anxiety and locomotion in the elevated plus maze. Differences between the genotypes were observed on anxiety-like behavior (A), and they were dependent on sex and housing conditions. Female mutants displayed higher levels of anxiety compared to wild type controls, but this phenomenon was detected only in grouped conditions. Social isolation induced an anxiogenic effect, which was detected exclusively in wild type females. The differences observed on anxiety levels could not be attributed to reduced locomotion (B). Activity levels did not differ between genotypes, but they were instead increased by social isolation in mice of both sexes. Data ($M \pm SEM$) were obtained from 18 wild type males (9 grouped and 9 isolated), 20 wild type females (10 grouped and 10 isolated), 17 mutant males (8 grouped and 9 isolated), and 11 mutant females (5 grouped and 6 isolated). $^{*}p < .05$.

two-way interactions were far from reaching statistical significance, all Fs < 1. Post hoc comparisons confirmed that in females, wild type grouped mice significantly differed from both mutant grouped and wild type isolated animals ($p < .05$).

A similar pattern of results was obtained from the analyses of the percent entries into the open arms, although the Genotype $\times$ Sex $\times$ Housing interaction only approached statistical significance, $F(1, 58) = 2.98$, $p = .09$ (data not shown).

The genotype effect on anxiety observed in the grouped females was not confounded by any changes in concomitant locomotor activity. Indeed, mutant and wild type female mice showed comparable levels of activity (Figure 1B). On the other hand, social isolation induced hyperactivity in mice of both genotypes and sexes. The ANOVA of the distance traveled in the maze provided support to these impressions, yielding a significant effect of housing, $F(1, 58) = 13.50$, $p = .001$.

Open Field Test

Locomotor habituation was evident in all groups (Figure 2A&B) with a gradual reduction in distance traveled across successive 5-min bins. This effect was equally observed in all experimental groups, although mutant mice were less active than wild type controls during the first 10 min of the test (Figure 2A). These conclusions were based on the 2 $\times$ 2 $\times$ 2 (Genotype $\times$ Sex $\times$ Housing) ANOVA of the distance traveled, which yielded a significant effect of bins, $F(11, 638) = 114.26$, $p < .0001$ and of the Genotype $\times$ Bins interaction, $F(11, 638) = 2.13$, $p < .05$.

Social isolation appeared to enhance locomotor activity, but the time course of this effect differed somewhat between sexes (Figure 2B). In the male this effect tended to be stronger as the session progressed, whereas the reverse was in the case of the females. The ANOVA of the distance traveled confirmed these impressions yielding a significant effect of housing, $F(1, 58) = 15.69$, $p < .0001$, Housing $\times$ Bins interaction, $F(11, 638) = 2.23$, $p < .05$, and Sex $\times$ Housing $\times$ Bins, $F(11, 638) = 4.21$, $p < .0001$. Restricted analyses further demonstrated the presence of a significant main effect of housing in males, $F(1, 31) = 9.80$, $p < .01$, whereas a significant effect of housing, $F(1, 27) = 6.75$, $p < .05$ and of the interaction Housing $\times$ Bins, $F(11, 297) = 5.38$, $p < .0001$ was both observed in females.

Assessment of the Acoustic Startle Reflex

Increasing intensity of the acoustic stimulus consistently led to stronger startle reaction. This dependency was equally observed in both sexes, although the initial levels of startle reactivity were lower in females in comparison to males regardless of genotype (Figure 3A). A 2 $\times$ 2 $\times$ 2 $\times$ 2 $\times$ 10 (Genotype $\times$ Sex $\times$ Housing $\times$ Stimulus Duration $\times$ Stimulus Intensity) ANOVA of the startle reactivity (following the logarithmic transformation: $y = ln(x+1)$) supported these conclusions, yielding a significant effect of stimulus intensity, $F(9, 522) = 249.63$, $p < .0001$ and its interaction with sex, $F(9, 522) = 2.03$, $p < .05$. Neither the main effect of stimulus duration, $F < 1$ nor any of its interaction terms attained statistical significance.

From the stimulus intensity of 85 dB_A upward, mutant mice of both sexes displayed a marked increase in startle response compared to wild type controls (Figure 3B), leading to the emergence of a significant effect of genotype, $F(1, 58) = 39.80$, $p < .0001$ and of its interaction with stimulus intensity, $F(9, 522) = 48.19$, $p < .0001$.

Social isolation slightly enhanced startle reactivity in mice of both genotypes and sexes, and this effect was observed from stimulus intensity of 81 dB_A onward (Figure 3C). These impressions were confirmed by the significant effect of housing, $F(1, 58) = 7.38$, $p < .01$ and of its interaction with stimulus intensity, $F(9, 522) = 4.32$, $p < .0001$.

Next, we performed an additional analysis to assess the stability of the above findings over the course of the test session. To this end, we explicitly compared the startle reactivity scores obtained in the first two blocks with the last two blocks of trials in the

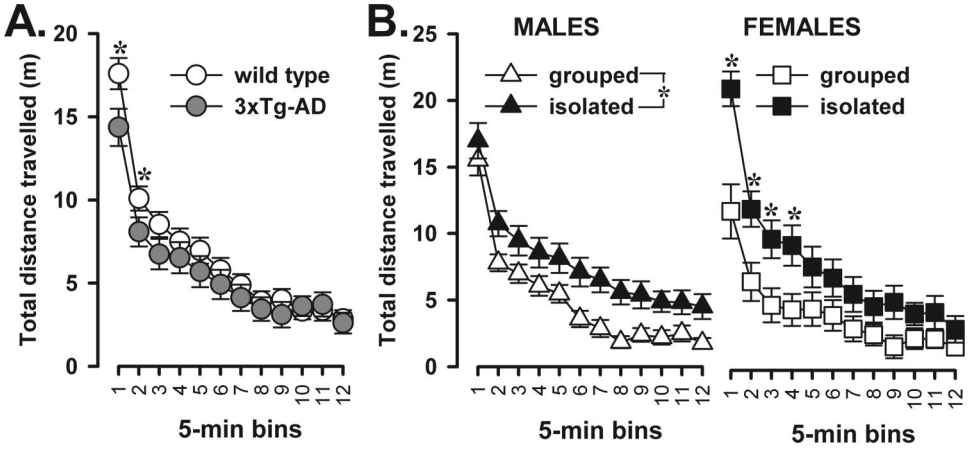

Figure 2. Locomotor activity in the open field. A reduction in locomotor activity was observed in mutant mice compared to wild type controls during the first 10 min of the test, and it was equally detected across sexes (A). Hyperactivity was induced by social isolation in male and female mice of both genotypes (B), although this effect had a different temporal profile in males and females. Data ($M \pm SEM$) were obtained from 18 wild type males (9 grouped and 9 isolated), 20 wild type females (10 grouped and 10 isolated), 17 mutant males (8 grouped and 9 isolated), and 11 mutant females (5 grouped and 6 isolated). $^{*}p < .05$.

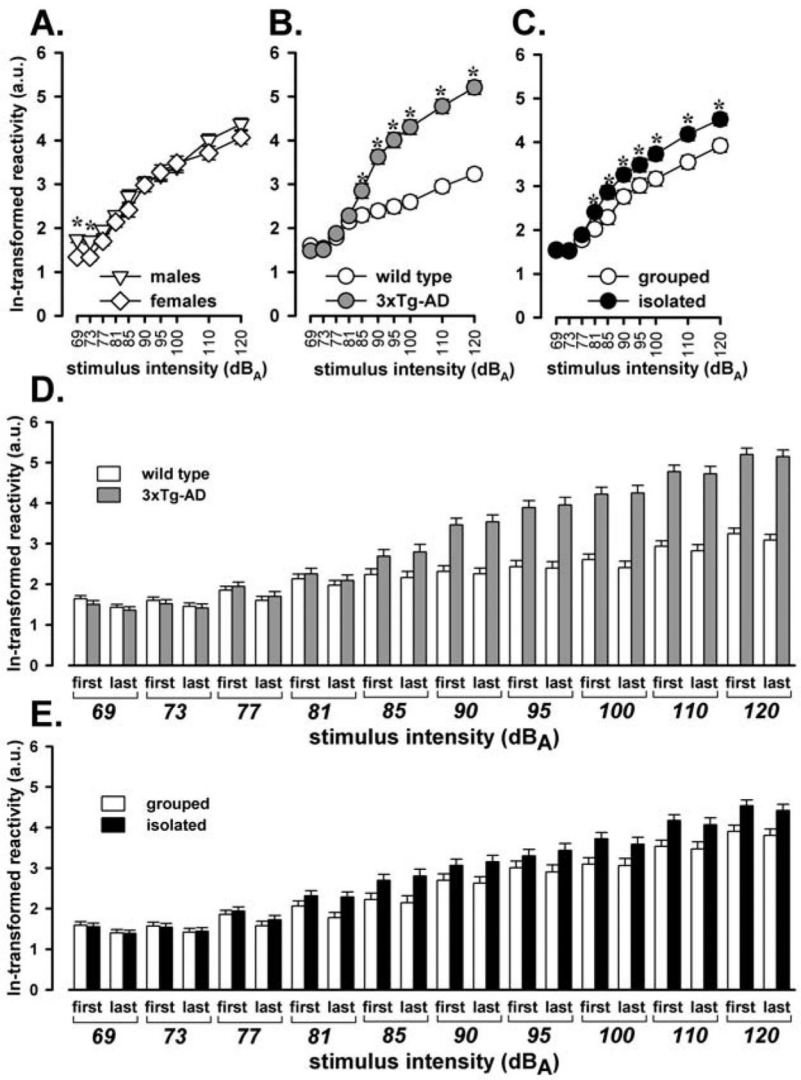

Figure 3. Acoustic startle response. Startle response gradually increased with the stimulus intensity, but the initial levels of reactivity were lower in females compared to males (A). Mutant genotype and social isolation both enhanced startle reactivity and these effects were equally observed across sexes. Mutant mice displayed a marked increase in startle response, starting from the stimulus intensity of 85 dBA (B), although social isolation slightly enhanced startle reactivity at the stimulus levels of 81 dBA and above (C). The comparison of the first and the last pair of blocks clearly demonstrated that the effects of genotype (D) and of isolation (E) were consistently expressed across blocks. Data ($M \pm SEM$) were obtained from 18 wild type males (9 grouped and 9 isolated), 20 wild type females (10 grouped and 10 isolated), 17 mutant males (8 grouped and 9 isolated), and 11 mutant females (5 grouped and 6 isolated). * $p < .05$ from the post hoc comparisons based on the overall error variance obtained from the relevant factor.

session (each block comprised one of each trial type: see Method). This comparison between the first and last two blocks is represented by the factor session time in a 2 × 2 × 2 × 2 × 10 × 2 (Genotype × Sex × Housing × Stimulus Duration × Stimulus Intensity × Session Time) ANOVA of the startle reactivity score followed by a logarithmic transformation: $y = ln(x + 1)$. In accordance to the analysis described in the previous paragraphs, the significant effects of genotype, $F(1, 58) = 38.64$, $p < .0001$; Genotype × Stimulus Intensity, $F(9, 522) = 48.43$, $p < .0001$; and of isolation housing, $F(1, 58) = 6.74$, $p < .05$; Housing × Stimulus Intensity, $F(9, 522) = 4.15$, $p < .0001$ was again observed. This supplementary analysis further revealed a significant effect of session time, $F(1, 58) = 5.95$, $p < .05$, because startle reactivity was in general lower in the last two blocks relative to the first two blocks of the session, regardless of genotype (Figure 3D) or housing (Figure 3E). The effect of session time appeared to be somewhat more clearly observed in trial types with lower stimulus intensities, leading to a significant Stimulus Intensity × Session Time interaction, $F(9, 522) = 2.2$, $p < .05$. Most important, the main findings regarding the effects genotype or housing on startle

reactivity did not significantly change over the course of the test session because there was no evidence of any significant interaction of session time with either of these factors. This suggested the stability of the genotype and isolation effect on startle reactivity over time (Figure 3D–E).

Water Maze

Cued task (Days 1–2). Escape performance showed a clear improvement over days (Figure 4A). Overall, wild type controls in the two housing conditions were performing similarly to each other. In contrast, although the mutant and wild type mice in the isolation condition were comparable, group-housed mutant performed better than group-housed wild type mice—in particular on Day 1. This complex pattern of results led to the emergence of a Genotype × Housing × Days interaction, $F(1, 58) = 6.88$, $p = .01$ in the 2 × 2 × 2 × 2 (Genotype × Housing × Sex × Days) ANOVA of escape latency. This interaction effect was accompanied by a significant main effect of days, $F(1, 58) = 113.93$, $p < .0001$, genotype, $F(1, 58) = 13.92$, $p < .0001$, and housing, $F(1, 58) = 8.82$, $p < .005$. The genotype by days interaction also attained statistical significance, $F(1, 58) = 4.07$, $p < .05$. Consistent with our interpretations above, separate analyses restricted to either housing condition yielded a significant effect of genotype, $F(1, 28) = 28.67$, $p < .0001$ and of its interaction with days, $F(1, 28) = 14.44$, $p = .001$ only in grouped animals. In the isolated mice, neither the main effect of genotype, $F(1, 30) = 2.27$, $p = .14$ nor the interaction with days, $F < 1$ approached statistical significance.

Parallel analyses performed on the dependent measure of path length essentially yielded an identical pattern of results and statistical outcomes. In addition, separate analysis of swim speed did not yield any significant effect.

Acquisition of Spatial Reference Memory (Days 3 to 9)

Days 3 to 8 (Before probe Test 1). Performance on the hidden platform task improved as training progressed as indicated by the overall reduction in escape latency over successive 2-day blocks (Figure 4B–C). Mutant genotype did not significantly affect the acquisition of the water maze task (Figure 4B). Isolation on the other hand significantly impaired performance relative to the group-housed controls (Figure 4C), regardless of sex and genotype. These conclusions were supported by a 2 × 2 × 2 × 3 (Genotype × Sex × Housing × Blocks) ANOVA of the escape latency, which yielded a significant effect of housing, $F(1, 58) = 9.58$, $p < .005$ and blocks, $F(2, 116) = 3.10$, $p < .05$. No other effect attained statistical significance.

Parallel analyses of path length yielded a highly similar pattern of results as described above. Separate analysis of swim speed data did not reveal any group difference.

Day 9. Analysis of performance on Day 9 was in full agreement with the results described above. Social isolation, but not mutant genotype, markedly impaired the performance in both sexes. A 2 × 2 × 2 × 4 (Genotype × Sex × Housing × Trials) ANOVA of the escape latency across the four trials on this day only revealed a significant effect of housing, $F(1, 58) = 7.47$, $p <$

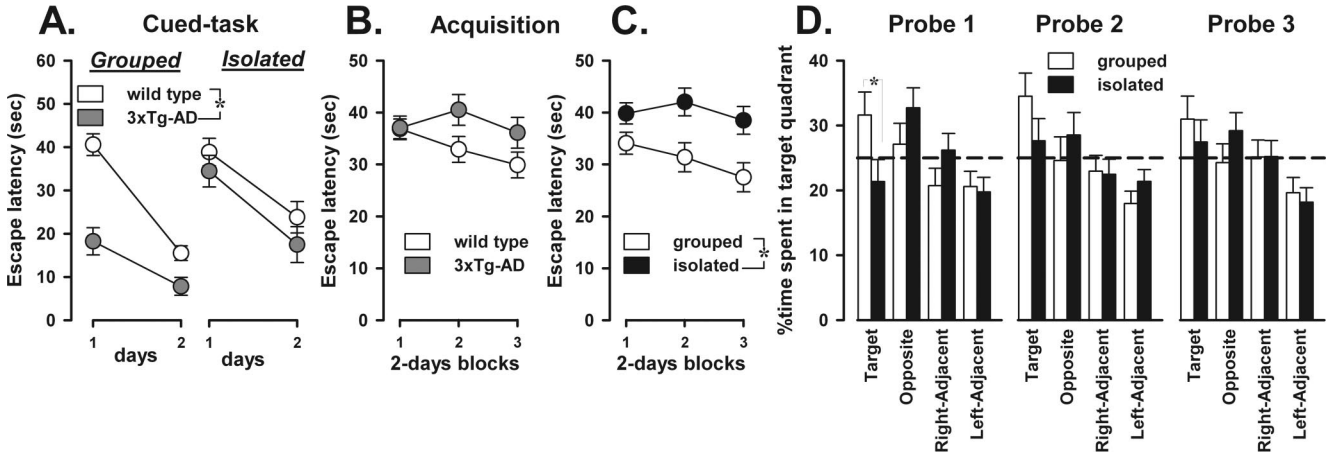

Figure 4. Spatial learning in the water maze test. Cued task (A): Mutant genotype reduced the escape latencies of animals of both sexes and this effect was attenuated by social isolation. Social isolation also induced an overall impairment in the performance of both male and female mice. Acquisition of a reference memory task (B–C): The first 6 days of training, that is, before the first probe test, were separately analyzed in 2-day blocks. Mutant genotype did not alter the acquisition of the water maze task, which was instead impaired by social isolation. Probe tests (D): Three separate 30-s probe tests were performed. The first two were conducted on Day 9, 1.5 hr before and after the last four trials of training. Probe 3 was performed 24 hr after the last training session. No differences between genotypes were detected on any of the three probes. The detrimental impact of isolated housing on the performance of female mice was confirmed on Probe 1, but disappeared on the last two probes. Dotted line indicates the chance level, that is, 25%. Data ($M \pm SEM$) were obtained from 18 wild type males (9 grouped and 9 isolated), 20 wild type females (10 grouped and 10 isolated), 17 mutant males (8 grouped and 9 isolated), and 11 mutant females (5 grouped and 6 isolated). $^{*}p < .05$.

.01. The mean (± *SEM*) escape latency (expressed in seconds) for each group were: 25.04 ± 3.18 (grouped), 37.05 ± 3.03 (isolated).

Probe tests. Altogether three separate probe tests were conducted (Figure 4D): (a) 1.5 hr prior to acquisition training on Day 9, (b) 1.5 hr after Day 9 acquisition training, and (c) 24 hr after Day 9 acquisition training. These were separately analyzed by comparing the percentage time spent in each of the four quadrants. Additional analyses restricted to the target quadrant (previously occupied by the escape platform) were conducted separately.

As depicted in Figure 4D, in the first probe test, mice preferentially displayed spatial search in the target quadrant. This behavior was comparably observed in both genotypes, but it was reduced by isolation. These impressions were suggested by the 2 × 2 × 2 × 4 (Genotype × Sex × Housing × Quadrants) ANOVA of percentage time per quadrant, which yielded a significant effect of quadrants, $F(3, 174) = 3.06$, $p < .05$ and an interaction Housing × Quadrants that approached statistical significance, $F(3, 174) = 2.46$, $p = .07$. Furthermore, the 2 × 2 × 2 (Genotype × Sex × Housing) ANOVA of percentage time spent in the target quadrant yielded a significant effect of housing, $F(1, 58) = 4.37$, $p < .05$.

No differences in the animals' performance were detected between genotypes, sexes or housing conditions in the last two probe tests (Figure 4D). A 2 × 2 × 2 × 4 (Genotype × Sex × Housing × Quadrants) ANOVA of percentage time per quadrant yielded only a significant effect of quadrants in Probe 2, $F(3, 174) = 4.27$, $p < .01$ and Probe 3, $F(3, 174) = 3.64$, $p = .01$. No other effects achieved or approached statistical significance also in the 2 × 2 × 2 (Sex × Genotype × Housing) ANOVA of the time spent in the target quadrant.

The above analyses were repeated by focusing on the first 15 s of each probe test, and the outcomes were essentially identical. Furthermore, parallel analyses were performed on the dependent measure of percentage distance swum per quadrant and these yielded highly similar outcomes as described above. The analysis of annular crossings and of the escape latency (data no shown) agreed with the pattern of results based on quadrant time, although no effect reached statistical significance. Finally, analyses of the swim speed revealed no difference among the experimental groups on any probe test.

Spontaneous Alternation in the Y Maze

Three mice (one wild type male isolated, one mutant male isolated, and one mutant female isolated) failed to leave the start arm within 10 min in the test phase and were therefore excluded from the data analysis.

Sample phase. During the sample phase social isolation enhanced locomotor activity and this effect was equally observed in mice of both sexes and genotypes (Figure 5A). This conclusion was supported by a 2 × 2 × 2 (Genotype × Sex × Housing) ANOVA of the total distance traveled in the maze, yielding only a significant effect of housing, $F(1, 55) = 33.35$, $p < .0001$.

A 2 × 2 × 2 × 2 (Genotype × Housing × Sex × Arms) ANOVA of the time spent yielded no evidence, all Fs < 1 for any difference between groups in terms of the relative time spent between the "start" and "familiar" arms of the Y maze in this phase of the test.

Test phase. During the test phase, social isolation again enhanced locomotor activity regardless of sex or genotype, housing effect: $F(1, 55) = 39.12$, $p < .0001$ (Figure 5B).

As expected, a general preference for the "novel" arm was observed in the test phase. However, this effect was considerably weaker in the mutant mice in comparison to the controls (Figure 5C), suggesting that the mutant mice might be impaired in spatial recognition memory. A 2 × 2 × 2 × 3 (Genotype × Housing × Sex × Arms) ANOVA of the time spent per arm revealed a significant effect of arms, $F(2, 110) = 13.60$, $p < .0001$ and its interaction with genotype, $F(2, 110) = 3.07$, $p = .05$. An additional analysis restricted to the novel arm also yielded a main effect of genotype, $F(1, 55) = 5.21$, $p < .05$.

Social isolation resulted in an effect similar to that of the triple mutation: Isolates displayed a weaker preference for the novel arm (Figure 5D). This impression was supported by the emergence of

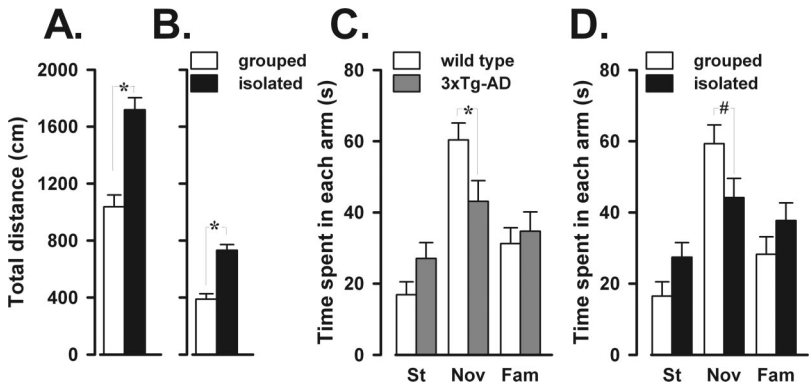

Figure 5. Spontaneous alternation in the Y maze. Social isolation enhanced locomotor activity in mice of both sexes and genotypes and this effect was observed during both the sample (A) and the test phase (B). The preference for the novel arm of the maze during the test phase was attenuated by mutant genotype (C) as well as by social isolation (D). These effects were comparably detected across sexes. Data (expressed as $M \pm SEM$) were obtained from 17 wild type males (9 grouped and 8 isolated), 20 wild type females (10 grouped and 10 isolated), 16 mutant males (8 grouped and 8 isolated), and 10 mutant females (5 grouped and 5 isolated). St = Start arm; Nov = novel arm; Fam = familiar arm. $^{*}p < .05$. #$p = .05$.

a significant Housing × Arms interaction, $F(2, 110) = 3.21$, $p < .05$. A near-significant effect of housing was also obtained in an analysis restricted to the novel arm, $F(1, 55) = 4.0$, $p = .05$.

Hippocampal β-amlyoid Immunoreactivity

Quantification of β-amyloid (Aβ) immunoreactivity was performed in CA1 and CA3 subfields of both dorsal and ventral hippocampus. Due to damage that occurred during brain processing, some sections could not be included in the final analysis. Quantification of Aβ-ir cells was performed on samples obtained from 15 males (7 grouped and 8 isolated), and 8 females (3 grouped and 5 isolated).

In mutant mice, hippocampal Aβ pathology was more severe in the female (see Figure 6). This sex difference was more pronounced in the dorsal hippocampus, where it reached statistical significance in CA1, $F(1, 19) = 18.81$, $p < .0001$ (Figure 6A) as well as CA3, $F(1, 19) = 10.66$, $p < .005$ (Figure 6B). The number of Aβ-ir cells in the ventral hippocampus of the mutant mice was also higher in the female, although this sex difference only achieved statistical significance in CA3, $F(1, 19) = 7.18$, $p < .05$ (Figure 6D) but not in CA1, $F(1, 19) = 3.04$, $p = .10$ (Figure 6C).

In contrast, social isolation failed to affect the severity of Aβ pathology regardless of sex and housing effect in all hippocampal areas: all Fs < 1.

Representative sections are presented in Figure 7 to illustrate the results obtained from the quantitative analysis.

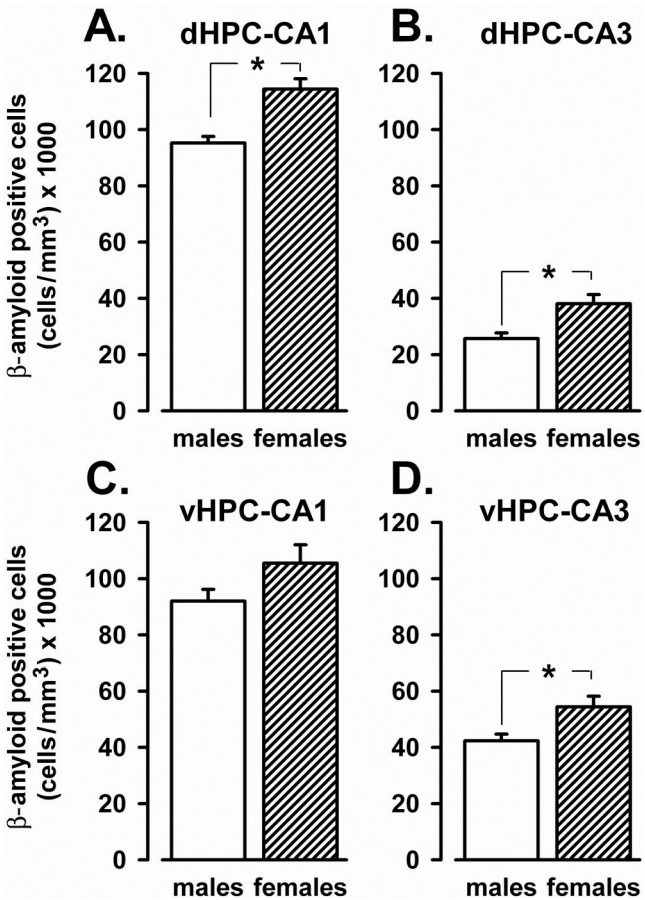

Figure 6. Quantification of hippocampal β-amlyoid pathology in 3 × Tg-AD mice**.** β-amyloid immunoreactivity was extensively observed in the hippocampus of mutant mice. In both grouped and isolated conditions, the β-amyloid pathology appeared to be more pronounced in female compared to male mutants. This sex difference was more apparent in the dorsal hippocampus (dHPC), where it was observed in both CA1 (A) and CA3 (B) subfields. In the ventral hippocampus (vHPC) a significant sex difference was detected in CA3 (D), but not in CA1 (C). The number of subjects included in each brain analysis was: 15 males (7 grouped and 8 isolated), and 8 females (3 grouped and 5 isolated). dHPC = dorsal hippocampus; vHPC = ventral hippocampus. Error bars represent ± *SEM*. * $p < .05$.

Discussion

In contrast to our prediction, isolation did not exacerbate any of the AD-like symptoms in the 3 × Tg-AD mouse line: It actually eliminated the effects of the triple mutations, although only on a limited number of behaviors. On the other hand, both 3 × Tg-AD genotype and social isolation affected several behaviors and these effects often differed in direction and magnitude, as illustrated by Table 1.

First, we partially confirmed the noncognitive and cognitive symptoms of the 3 × Tg-AD mouse line previously reported (Pietropaolo, Feldon, et al., 2008; Pietropaolo, Sun, et al., 2008). Signs of enhanced reactivity to aversive stimuli, for example, enhanced acoustic startle response and reduced escape latencies in the cued task of the water maze test were observed in mutant mice. These effects were consistently described by previous reports (Pietropaolo, Feldon, et al., 2008; Pietropaolo, Sun, et al., 2008) in mice of both sexes, suggesting that they constitute robust and reliable phenotypes of the 3 × Tg-AD mouse line. Furthermore, they can be interpreted as AD-like symptoms because they may resemble the enhanced irritability observed at the early stages of AD pathology (Aalten et al., 2003; Frisoni et al., 1999; Hope et al., 1997; Lawlor & Bhriain, 2001). On the other hand, other behavioral characteristics of the 3 × Tg-AD line appeared less robust. The cognitive impairment of mutant animals observed here was limited to the Y maze test, whereas AD mice of both sexes displayed normal acquisition and retention of the water maze task. Although the overall performance of wild type controls in the water maze here appeared to be relatively poor, it is unlikely that this could have completely masked the potential deficit in the mutant mice. Indeed, a null effect was also observed in a previous study of this mutant mouse line in the same water maze test when performance of the control group was considerably better—achieving a sub-25 s escape latency by the end of training (See Figure 6, Pietropaolo, Feldon, et al., 2008). On the other hand, it is possible that pretraining in the cued task might have an ameliorating effect on performance of mutant mice in the subsequent hidden-platform test, by minimizing the mouse tendency to search for alternative escape routes (Vorhees & Williams, 2006).

The present findings also demonstrated little sex differences on the behavioral phenotype of the 3 × Tg-AD mouse line. A reduction in locomotor activity was observed here in mutant mice of both sexes, but only in the open field and during the initial part of the test. Previous results reported the presence of hypoactivity in the open field in 3 × Tg-AD mice, but only in females (Pietropaolo, Feldon, et al., 2008; Pietropaolo, Sun, et al., 2008). Similarly, the results on Y maze and water maze learning obtained here did

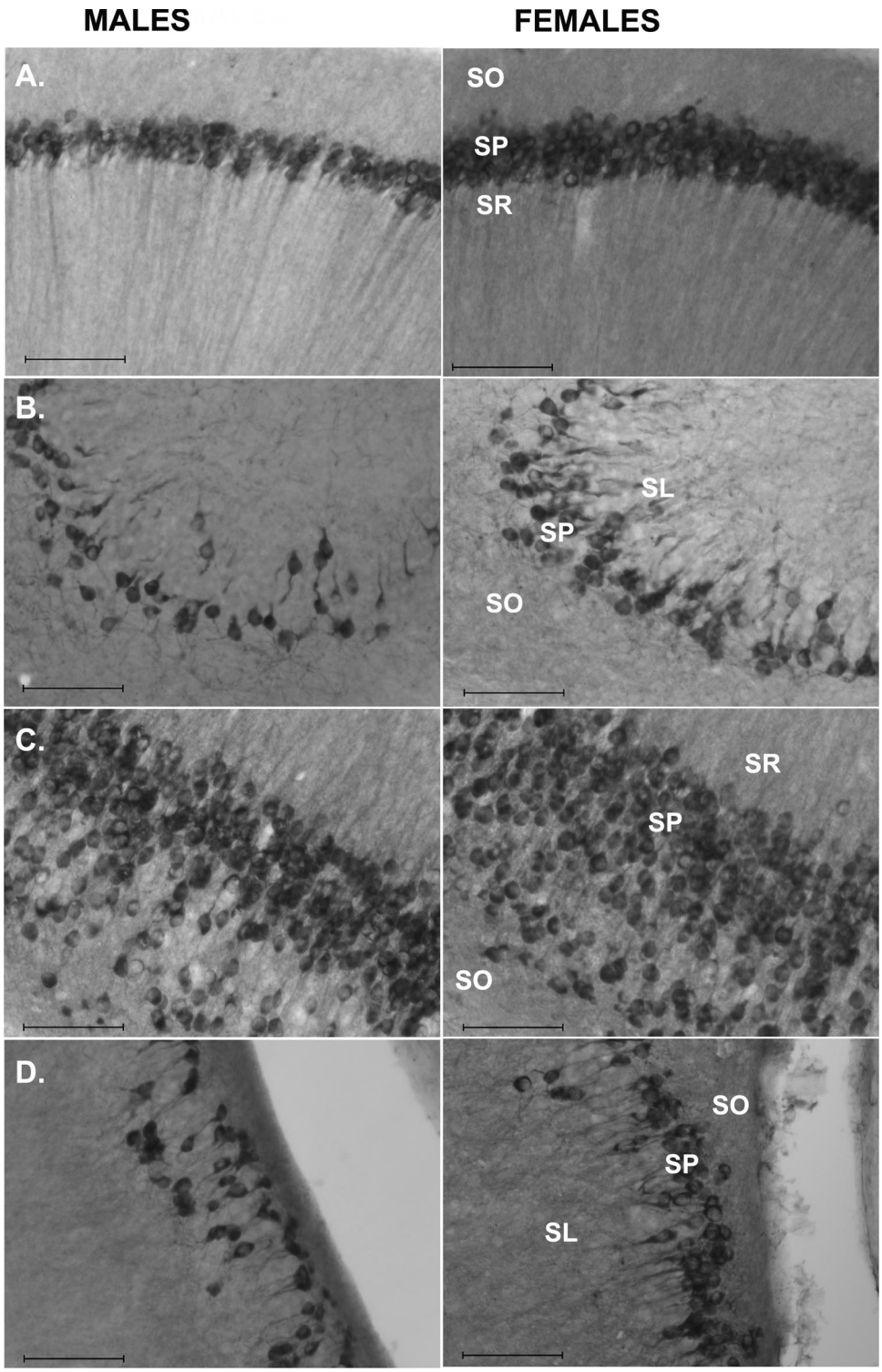

Figure 7. Hippocampal β-amlyloid pathology in 3 × Tg-AD mice. As suggested by the quantitative evaluation of β-amyloid immunoreactivity, hippocampal β-amyloid pathology appeared to be more pronounced in female compared to male mice. This phenomenon as observed in the dorsal CA1 (A) and CA3 (B) areas. A similar sex difference was also observed in the ventral hippocampus, although it was mainly detected in CA3 (D), whereas it was weak in CA1 (C). Microphotographs were obtained from one male and one female isolated mutants. Scale bar = 100 μm; SO = stratum oriens; SP = stratum pyramidale; SR = stratum radiatum; SL = stratum lucidum.

Table 1
Summary of The Main Behavioral Results

	Effect of mutant genotype				Effects of social isolation			
	Modulating factors				Modulating factors			
Behavioural test	Description	Direction	Housing	Sex	Description	Direction	Genotype	Sex
Elevated plus maze	Anxiogenic in grouped females	↑	Yes	Yes	Anxiogenic in wild type females	↑	Yes	Yes
Elevated plus maze	—	—	—	—	Hyperactivity	↑	No	No
Open field	Initial hypoactivity	↓	No	No	Hyperactivity	↑	No	No
Y maze	—	—	—	—	Hyperactivity (on both sample and test phase)	↑	No	No
Acoustic startle reflex	Marked enhancement	↑	No	No	Slight enhancement	↑	No	No
Water maze (cued task)	Improved performance; this effect is reduced in isolated conditions	↑	Yes	No	Overall impairment; it attenuates the genotype effect	↓	Yes	No
Y maze	Impaired performance	↓	No	No	Impaired performance	↓	No	No
Water maze (acquisition)	—	—	—	—	Impaired acquisition	↓	No	No
Water maze (probe tests)	—	—	—	—	Impaired performance on Probe 1	↓	No	No

Note. The present findings demonstrated that 3 × Tg-Ad genotype and social isolation both affected several behaviors and these effects often differed in direction and magnitude. In animals of both sexes the 3 × Tg-Ad mutation reduced locomotion in the open field, enhanced acoustic startle response, and facilitated performance in the cued version of the water maze. Mutant mice also displayed impaired Y maze learning, but normal acquisition and retention of a reference memory task in the water maze. These symptoms were accompanied by increased anxiety levels, but only in females. In male and female mice social isolation enhanced acoustic startle response, impaired performance in the cued version of the water maze, and induced hyperactivity. Furthermore, isolation impaired spatial memory in both water maze and Y maze. Social isolation did not exacerbate any of the noncognitive and cognitive AD-like symptoms in the 3 × Tg-AD mouse line, it actually eliminated the genotype differences on anxiety by inducing anxiogenesis in wild type animals. Isolated conditions also attenuated the impact of mutant genotype on the performance in the cued task, without altering any other effect of genotype.

not replicate previous findings demonstrating impairments in female 3 × Tg-AD mice only (Pietropaolo, Sun, et al., 2008). The presence of genotype effects on anxiety-like behavior also contradicted previous data (Pietropaolo, Sun, et al., 2008). Yet, the genotype difference on anxiety levels was observed here solely in grouped conditions and this may contribute to the discrepancy with our previous study, in which mice were housed singly from the age of 3 months in the presence of a locked wheel (Pietropaolo, Sun, et al., 2008). In conclusion, sex differences in the 3 × Tg-AD behavioral phenotype are not consistently detected across studies, and they appear to be critically affected by housing conditions. On the other hand, hippocampal β-amyloid pathology was more severe in mutant female mice. The latter finding was in agreement with previous results from the same (Nelson et al., 2007; Pietropaolo, Sun, et al., 2008) and other AD mouse models (Callahan et al., 2001; Savonenko et al., 2005; Wang, Tanila, Puolivali, Kadish, & van Groen, 2003), supporting the higher vulnerability of females to AD pathology (Fratiglioni et al., 1997; Gao, Hendrie, Hall, & Hui, 1998), perhaps related to the protective effects of testosterone against β-amyloid production (Gouras et al., 2000). Further studies are warranted to investigate possible compensatory mechanisms preventing females from displaying more marked AD-like symptoms despite the presence of more severe brain pathology.

The present findings confirmed the most robust effects of social isolation, that is, hyperactivity and enhanced acoustic startle reactivity. Both effects of social isolation have been well documented previously in mice (Abramov et al., 2004; Benton & Brain, 1981; Dai et al., 2005; Pietropaolo, Singer, et al., 2008; Sakaue et al., 2003), and were observed here in both sexes. Isolation also impaired the performance in the cued version of the water maze in mice of both sexes. To our knowledge, this is the first study to demonstrate this effect of social isolation because no difference between isolated and grouped animals was previously described in either mice (Ibi et al., 2008; Voikar et al., 2005) or rats (Schrijver et al., 2002). Differences in the genetic background may help to explain the uniqueness of our findings. Nonetheless, it should be noticed that in all previous studies investigating the isolation effects on water maze performance the visible test was administered at the end of the water maze experiment (Ibi et al., 2008; Schrijver et al., 2002; Voikar et al., 2005), with some exceptions in which the cued task was not performed at all (Hellemans et al., 2004; Lapiz et al., 2001; Moragrega et al., 2003). Here we adopted the more conventional procedure to perform the cued task before spatial training (Chen et al., 2000; Vorhees & Williams, 2006). This specific procedural difference may critically contribute to the discrepancy between our results and previous findings because differences in the motivation to escape may become undetectable after several days of training in the water maze. Beside the impact on the cued task, our water maze test also demonstrated an effect of isolation on the performance during acquisition and on the first probe test. This finding, in association with the impairment observed on Y maze performance, supports the view that isolation may induce hippocampal dysfunction (Ibi et al., 2008; Lu et al., 2003; Westenbroek, Den Boer, Veenhuis, & Ter Horst, 2004).

In addition to its extensive behavioral impact, social isolation affected only few AD-like symptoms. First, isolation rearing attenuated the improvement in the performance of mutant mice in the cued task of the water maze. One possible interpretation is that

social isolation selectively reduced the mutants' hyperresponsiveness to the adverse stimulation provided by the water maze. Alternatively, it is possible that the effect of isolation on the genotype difference in the cued task reflected a nonspatial cognitive impairment because the animals needed to learn to associate the visual stimulus with the escape platform. It is thus possible that social isolation affected the performance in the cued task through behavioral mechanisms that are independent of those underlying the effects of the triple mutations.

Second, isolation eliminated the difference observed between wild type and mutant female mice in the elevated plus maze. The latter effect was actually due to an anxiogenic effect of isolation in wild type females. This finding is in agreement with previous results showing no effect of isolation on anxiety in male mice (Moragrega et al., 2003; Pietropaolo, Singer, et al., 2008; Voikar et al., 2005) and may suggest that female mice are more sensitive to the effects of housing manipulations on emotionality (Pietropaolo, Mintz, Feldon, & Yee, 2007).

More interesting, the elevated plus maze was the only test revealing an impact of sex differences on isolation. The lack of sex differences on the isolation effects on locomotor activity and acoustic startle response was in contrast with previous studies in other mouse lines (Abramov et al., 2004; Guo et al., 2004; Pietropaolo, Singer, et al., 2008). Yet, it should be noticed that most of the previous studies have employed shorter exposure to postweaning isolation (4 to 7 weeks). It is therefore possible that the discrepancies between our findings and previous results may be due to the longer duration of isolation and/or the younger age at which animals were tested. Furthermore, certain effects of isolation observed here may be unique to the 3 × Tg-AD mouse line because the genetic background is known to critically influence the efficacy of social isolation in both rats (Weiss et al., 2000) and mice (Voikar et al., 2005).

Social isolation did not alter hippocampal β-amlyloid pathology. Hence, the effects of isolation on mutant phenotype did not correspond to changes in the severity of β-amlyloid pathology, as previously demonstrated by studies employing other environmental manipulations, for example, wheel running, and environmental enrichment in this (Pietropaolo, Sun, et al., 2008) and other mouse models of AD (Jankowsky et al., 2005; Jankowsky, Xu, Fromholt, Gonzales, & Borchelt, 2003; Nichol, Parachikova, & Cotman, 2007). Yet, further investigation with alternative measurements of β-amlyloid pathology, for example, levels of soluble β-amlyloid, are warranted to fully address this issue. Furthermore, it is possible that isolation may affect AD brain pathology without directly affecting β-amlyloid deposition, for example, by exacerbating inflammatory processes (Barrientos et al., 2003; Gibb, Hayley, Gandhi, Poulter, & Anisman, 2008) or altering the levels of neurotrophins (Bjornebekk, Mathe, Gruber, & Brene, 2007; Scaccianoce et al., 2006; Zhu et al., 2006). It is also possible that social isolation may reduce the protection of the brain against AD-related damage, for example, by reducing hippocampal neurogenesis (Ibi et al., 2008; Lu et al., 2003; Westenbroek et al., 2004), as already demonstrated in the Tg2576 AD-mouse model (Dong et al., 2004). This interpretation suggests that environmental deprivation may impair the reserve capacity of the brain to react to pathological insults, in line with the cognitive reserve hypothesis (Stern, 2002; Zhang et al., 1990).

In conclusion, the present study clearly demonstrated that 3 × Tg-AD genotype and social isolation exerted noncognitive and cognitive effects, and these effects were largely independent of sex. Our findings demonstrated the differential susceptibility of the 3 × Tg-AD mouse line to environmental manipulations, by highlighting the limited impact of social isolation on the genetically determined AD-like symptoms, in contrast to the marked effects reportedly induced by physical exercise. Nonetheless, further investigation is warranted to evaluate whether the impact of isolation on the behavioral phenotype of the 3 × Tg-AD mouse model may become more prominent with further aging, including the possibility of the emergence of more extensive and severe cognitive deficits.

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