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Environmental enrichment eliminates the anxiety phenotypes in a triple transgenic mouse model of Alzheimer's disease

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Abstract Although the impacts of environmental enrichment (EE) in several genetic models of Alzheimer's disease (AD) have been documented, the focus has remained predominantly on cognition. Few have investigated the expression of emotional phenotypes that mimic the notable affective features in AD. Here, we studied the interaction between EE and the coexpression of three genetic risk factors (mutations) for AD. In a longitudinal design, 3×Tg-AD mutants and wild type controls were compared at 6–7 months and subsequently at 12–13 months of age. Under standard housing, phenotypes of heightened anxiety levels were identified in the 3×Tg-AD mice in the elevated plus maze and open-field tests. Such trait differences between genotypes were substantially diminished under EE housing, which was attributable to the anxiolytic impact of EE on the mutant mice as much as the anxiogenic impact of EE on the wild type mice. In contrast, the phenotypes in learned fear were not significantly modified by EE in the tests of Pavlovian freezing and conditioned active avoidance conducted at either age. Rearing under EE thus has uncovered a novel distinction between innate and acquired expressions of fear response in the 3×Tg-AD mouse model that might be relevant to the mental health management of AD.

Keywords Animal models · Anxiety · Emotion

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Recent years have seen a number of studies investigating the impact of environmental/housing manipulations in genetic mouse models of Alzheimer's disease (AD; for reviews, see Nithianantharajah & Hannan, 2009, 2011). This interest is due to the relevance of gene–environment interactions in the etiopathology of AD, and the possible therapeutic value of environmental stimulation (Andel et al., 2005; Crowe, Andel, Pedersen, Johansson, & Gatz, 2003; Vespa, Gori, & Spazzafumo, 2002). Indeed, converging lines of evidence suggest that a stimulating lifestyle that engages physical exercise and intellectual and social activities can slow down the progress of the disease and attenuate the severity of the symptoms in AD patients (Butler, Ashford, & Snowdon, 1996; Katzman, 1993; Mortimer, Borenstein, Gosche, & Snowdon, 2005; Palleschi et al., 1996; Rovio et al., 2005; Vespa et al., 2002; Wilson et al., 2002). One common tool to translate and explore these findings in preclinical animal models is environmental enrichment (EE).

Under EE, mice or rats are exposed to multiple forms of enhanced stimulation, including motor, sensorial, cognitive, and social stimulation in their daily living (Hebb, 1947; Renner & Rosenzweig, 1987). EE is typically implemented by group-housing animals in large cages containing varying sets of toys, tunnels, and running wheels (Renner & Rosenzweig, 1987). Rearing under EE is known to induce marked neuroprotective effects and improve emotional and cognitive function in wild type animals (Diamond, 2001; Rosenzweig & Bennett, 1996; van Praag, Kempermann, & Gage, 2000; Will, Galani, Kelche, & Rosenzweig, 2004), especially when initiated early in life—typically from

weaning (Kohl, 2002; Renner & Rosenzweig, 1987). These observations support the idea that environmental stimuli can critically modify the phenotypic expression of even highly inheritable traits (Pigliucci, 2001; Waddington, 1957). Such "phenotypic plasticity" would be particularly relevant to familial AD with identifiable genetic risk factors, and it can be studied in genetically modified mouse models bearing one or more of the relevant susceptibility genes or mutations.

Indeed, beneficial effects of EE on brain and behavior have been demonstrated in several genetic mouse models of AD (for reviews, see Nithianantharajah & Hannan, 2009, 2011). AD models are mainly based on single or combined mutations in genes encoding the amyloid precursor protein (APP) and the presenilins (PS1, PS2) (Gotz et al., 2004; Janus & Westaway, 2001)—enzymatic subunits involved in the amyloidogenic cleavage of APP (Rosenberg, 2000). These investigations have mostly focused on cognitive phenotypes, often with a special emphasis on spatial memory and related hippocampus-dependent AD-like pathology. Although this bias is perfectly understandable, it has led to a relative neglect over emotional and affective symptoms in AD (Spalletta et al., 2004). Anxiety, enhanced fear, and irritability are notable behavioral and psychological concerns that accompany the disease, which severely erode the patients' and their carers' quality of life (Aalten et al., 2003; Frisoni et al., 1999; Hope, Keene, Fairburn, McShane, & Jacoby, 1997; Lawlor & Bhriain, 2001).

Some of these psychological disturbances have been successfully modeled using the triple-transgenic mouse line (3×Tg-AD; Espana et al., 2010; Garcia-Mesa et al., 2012; Garcia-Mesa et al., 2011; Gimenez-Llort et al., 2007; Marchese et al., 2014; Pietropaolo, Feldon & Yee 2008a; Pietropaolo, Sun, et al., Pietropaolo, Sun, Li, Brana, Feldon and Yee 2008b; Pietropaolo et al., 2009; Sterniczuk, Antle, LaFerla, & Dyck, 2010), harboring APP (APPSwe), PS1 (PS1M146V), and tau (tauP301L) transgenes, supporting the genetic link to their aetiology. The 3×Tg-AD model is the only genetic mouse model to date that recapitulates the two defining histopathological hallmarks of AD: β-amyloid (βA) deposits and tau–neurofibrillary tangles (Oddo et al., 2003). Furthermore, the timing of appearance of these two pathological hallmarks in the 3×Tg-AD model closely resembles what observed in AD patients. βA is first detected in the hippocampus and amygdala of 3×Tg-AD mice (at 6 months of age), with tau pathology emerging later in life in these limbic areas, typically near the age of 12 months (Billings, Oddo, Green, McGaugh, & LaFerla, 2005; Oddo et al., 2003). A comparison between these two specific ages in this mutant line is therefore relevant to the relative contribution of the (βA) deposits and tau–neurofibrillary tangles to the progression of AD (Billings, Green, McGaugh, & LaFerla, 2007; Billings et al., 2005; Gimenez-Llort et al., 2007; LaFerla, Green, & Oddo, 2007; Oddo, Billings, Kesslak, Cribbs, & LaFerla, 2004; Oddo et al., 2007).

Here, we investigated the behavioral effects of early EE in the 3×Tg-AD mouse model, with a particular focus on the expression of anxiety and fear. The same cohort of subjects underwent behavioral evaluations at 6 and then 12 months of age—in the absence and presence of tau pathology, respectively, whereas prominent βA deposits were expected at both ages. The expression of anxiety induced by multiple environmental cues in a novel situation was assessed using the elevated plus maze and open-field tests. Stronger memory of negative experiences has been described in AD patients (Fleming, Kim, Doo, Maguire, & Potkin, 2003), and it has been shown that anxiety may interfere with the acquisition of novel conditioned fear associations in humans (Bishop, 2007). We therefore included here two standard tests of learned fear—Pavlovian conditioned freezing and two-way signaled active avoidance—because they are known to be robustly sensitive to the effects of the 3×Tg-AD mutation from our previous studies (Pietropaolo, Feldon, & Yee, 2008; Pietropaolo, Sun, et al., 2008; Pietropaolo et al., 2009).

Method

Subjects

Mutant 3×Tg-AD and wild type female mice of matched genetic backgrounds were used. Female mice were chosen here since 3×Tg-AD mutants of this sex are known to show the most prominent emotional AD-like symptoms, at least in at the early phases of the pathology (Gimenez-Llort et al., 2007; Pietropaolo, Sun, et al., 2008; Pietropaolo et al., 2009). Furthermore, female triple mutants are often preferred for behavioral longitudinal studies (Davis, Easton, Eacott, & Gigg, 2013), since they show a more rapid and aggressive progression of the disease (Hirata-Fukae et al., 2008; Pietropaolo, Sun, et al., 2008; Pietropaolo et al., 2009). Finally, female animals are used in most enrichment mouse studies (Brown et al., 2003; Rhodes et al., 2003) so as to avoid excessive intermale aggressive behavior, which is known to be exacerbated by enriched rearing among male mice (Haemisch & Gartner, 1994; Haemisch, Voss, & Gartner, 1994).

Both the mutant and control wild type mouse lines were originally generated and maintained by LaFerla and colleagues, and the full descriptions of their generation has been reported previously (Oddo et al., 2003). Briefly, founder mutant 3×Tg-AD mice were generated by microinjection of human APPSwe and tauP301L transgenes in single-cell embryos obtained from PS1M146V homozygous knock-in mice (PS1–KI). These founder mice were backcrossed to PS1–KI mice. Because the APP and tau transgenes cointegrate at the same site, the 3×Tg-AD mice all have the same genetic background. Mice with the identical genetic background of 129SvJ/C57BL6 hybrids (based on the initial PS1–KI mouse

line) were used as controls. Separate breeding pairs of homozygous 3×Tg-AD mice and the corresponding wild type controls were obtained directly from Dr. Frank LaFerla (University of California, Irvine). They were maintained and bred in the SPF (specific-pathogen-free) facility in the Laboratory of Behavioural Neurobiology (Swiss Federal Institute of Technology, Zurich) following the same strategies originally employed; the offspring were used as experimental subjects employed in the present report. Genotype status was reconfirmed by standard PCR analysis of DNA isolated from tail tips collected after the animals' sacrifice (see Billings et al., 2005, and Oddo et al., 2003, for PCR details).

3×Tg-AD mutant mice and wild type mice were obtained from eight independent litters (four for each genotype) born within days of each other. The animals were weaned on postnatal day (PND) 21 and transferred to a separate temperature (22º ± 1ºC) and humidity (55% ± 5%) controlled room for group housing in either standard or enriched conditions. Mutant and wild type mice were equally assigned to one of the two housing conditions (n= 9), each comprising two cages of four or five mice of the same genotype. Members from a given litter were randomly allocated to one of the two possible housing conditions, in order to minimize possible confounding due to litter effects (Zorrilla, 1997). We further ensured that the animals in a given cage originated from multiple litters. The initial sample sizes were nine mice per group. For tests that were repeated between the two time points (6 vs. 12 months of age), the relevant statistical analyses only included subjects that had successfully completed both tests. Some animals were dropped either because of ill health or unexpected death. Thus, the final data set in all analyses comprised 15 (standard = 8, enriched = 7) wild type and 13 (standard = 5, enriched = 8) mutant mice, except that 17 (standard = 8, enriched = 9) mutant mice were included in the conditioned freezing experiment performed only at the age of 6 months.

The animals were kept in the corresponding housing conditions throughout the entire experimental period and were maintained under ad lib food and water and a 12:12 h reversed light:dark cycle (lights off: 0700–1900 h). The ambient light intensity in the animal vivarium during the light phase was 250 lux.

Housing

Environmental enrichment The animals were caged in groups of ~4–5 in transparent Makrolon cages (Tecniplast, Milan, Italy), each measuring 54 × 31 × 18 cm, equipped with a stainless steel wired lid and sawdust bedding (Schill AG, Muttenz, Switzerland). Food chow (Provimi Kliba SA, Kaiseraugst, Switzerland) and two water bottles were provided on the top of the cage. The type of environmental enrichment employed here was previously described in detail (Pietropaolo, Feldon, Alleva, Cirulli, & Yee, 2006). Briefly, each cage was enriched with plastic and wooden tunnels, mouse houses or igloos, and small plastic toys that could be easily manipulated by the animals. A plastic running wheel with a solid back and a solid running surface, measuring 10 cm in diameter and 6 cm deep (Rolf C. Hagen Corp., Mansfield, MA), was mounted under the metal lid of each cage. The cages were renewed weekly, when bedding was refreshed and randomly selected enrichment items were changed or relocated between different positions locations within the cage. This enrichment procedure represents the most widely used across a variety of studies showing marked effects on both brain and behavior (for a review, see Renner & Rosenzweig, 1987).

Standard caging The animals were also kept in groups of ~4–5 in cages measuring 41 × 20 × 19 cm (Tecniplast, Milan, Italy), each equipped with a stainless steel wired lid and sawdust bedding. Food chow and water were provided as described above. Sawdust bedding was renewed weekly.

Behavioral procedures

All animals were submitted to testing on multiple tasks, which are described below in chronological order. Four tests were included in this study: (1)elevated plus maze, (2)open-field test, (3)Pavlovian conditioning, and (4)two-way active avoidance. Tests 1 and 2 were first performed when the animals were ~180–185 days old, and then repeated when they were 360–365 days old. Conditioned fear was evaluated at ~190 days old by Pavlovian conditioned freezing, and then at ~370 days old by two-way signaled active avoidance. To minimize possible undesirable transfer effects between tests, tests that relied mainly on observations of spontaneous behavior, namely the elevated plus maze and open-field tests, were conducted first. A similar testing sequence has been previously adopted for the behavioral phenotyping of the 3×Tg-AD mouse line (Pietropaolo, Feldon, & Yee, 2008; Pietropaolo, Sun, et al., 2008; Pietropaolo et al., 2009).

Behavioral testing was always carried out during the dark phase of the cycle—the animals' active phase; and the experimenter was blind to the animals' genotype, but not to the housing condition of individual subjects. All manipulations described here had been approved by the Cantonal Veterinary Authority of Zurich, and were in accordance to the directives of the European Union (Directive 86/609/EEC).

Elevated plus maze

The construction and dimensions of the elevated plus maze have been fully described elsewhere (Pietropaolo, Feldon, & Yee, 2008; Pietropaolo et al., 2009). 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.

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. The following anxiety-related measures were calculated: percentages of entries into open arms = [number of entries into open arms / number of entries into open and enclosed arms] × 100%, and percentage of time spent in open arms = [time spent in open arms / time spent in open and enclosed arms] × 100%.

Open-field test

Details of the four identical open fields used here have been fully described before (Pietropaolo, Feldon, & Yee, 2008; Pietropaolo et al., 2009). A digital camera was mounted directly above the 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.

The mice were tested in squads of four. They were gently placed in the center of the appropriate arena and allowed to explore undisturbed for 1 h. Afterward, they were returned to the home cage and the arenas cleansed with water and dried prior to the next squad. To evaluate anxiety-like behavior, the time spent within as well as the number of entries into the central region (13 × 13 cm) of the open-field arena was recorded. These measures are considered useful in indexing phobia toward exposed and unfamiliar open space (Prut & Belzung, 2003). In addition, locomotor activity was evaluated by distance travelled recorded in the entire open field across consecutive 5-min bins.

Pavlovian conditioned freezing (at 6 months of age)

Two sets of chambers were used to provide two distinct contexts; their detailed description has been provided elsewhere (Pietropaolo, Feldon, & Yee, 2008). Each chamber contained a miniature digital camera mounted 30 cm directly above the center of the area of interest. The output of the camera was fed to a multiplexer (YSQ-430, Sony, Japan) before being transmitted to a computer (Power Mackintosh 7600/120) running the NIH Image software (version 1.61) for real-time analysis. The algorithm of the freezing response detection procedure has been validated and fully described before (Richmond et al., 1998). Briefly, successive digitized images (192 × 144 = 27,648 pixels, at 8-bit grayscale) obtained at a rate of 1 Hz were compared. The number of pixels differed between adjacent frames was then computed. If this was less than 0.05% of the total number of pixels in a frame, the animal was considered to be freezing in that 1-s interval.

The procedure consisted of three distinct phases: (1)conditioning, (2)test of conditioned context freezing, and (3)test of conditioned tone freezing across days.

On Day 1, all animals were given three conditioning trials in context A. Each trial consisted of a 30-s tone stimulus (conditioned stimulus, CS) followed immediately by a 1-s 0.3-mA scrambled foot-shock (unconditioned stimulus, US). The first trial was administered 3 min after the animals were placed into the chambers. Successive trials were administered every 3 min. The conditioning session was concluded with a final 3-min interval.

On Day 2, the animals were returned to context A. They were placed in the test chamber for a period of 8 min.

On Days 3–7, CS-freezing to the tone stimulus was assessed in context B. The tone stimulus was administered 2 min after the animals were placed into the test chamber. The tone remained on for a period of 8 min.

The expression of freezing or immobility was expressed as percentages of time freezing. The three phases were separately analyzed.

Two-way active signaled (conditioned) avoidance test (at 12 months of age)

The apparatus consisted of four identical two-way shuttle boxes that have been described in details before (Pietropaolo, Feldon, & Yee, 2008). The conditioning phase lasted for two consecutive daily sessions. Each animal was placed in the shuttle box and underwent 50 avoidance trials administered at variable intertrial intervals (ITIs; mean of 40 s, ranging from 25 to 55 s). A trial began with the onset of the CS—that is, a 85-dB white noise. If the animal shuttled within 5 s of CS onset, the CS was terminated and the animal avoided the electric shock on that trial. Avoidance failure led immediately to a 0.3-mA electric foot shock at presented in coincidence to the CS. This might last for a maximum of 2 s but could be terminated by a shuttle response during this period (i.e., an escape response).

The number of avoidance and escape responses as well as the number of the failures to escape the shock was analyzed in successive blocks of ten trials to assess avoidance learning. In addition, the number of shuttles performed during the ITIs was also computed and evaluated as an index of general locomotor activity.

Statistical analysis

All data were analyzed by parametric analysis of variance (ANOVA) with genotype and housing as the between-subjects factors; behavioral data included also age as the within-subjects factor. Additional within-subjects factors (e.g., trials, days) were also included whenever appropriate. Post-hoc comparisons were performed using Fisher's LSD test based on the overall error variance associated with the relevant factor. All statistical analyses were carried out using SPSS for Windows (Release 13.0; SPSS Inc., Chicago IL,

USA) implemented on a PC running the Microsoft Windows 7 operating system.

Results

Elevated plus maze

Anxiety generally increased with age, and the impacts of genotype and housing strongly interacted in manners that were similar at both ages (Fig. 1a). Mutant mice appeared much more anxious than wild type mice only under standard housing conditions. For animals maintained under enriched housing, the genotypic difference in anxiety was weak, and if anything, reversed. Thus, enriched housing seemed anxiogenic in wild type, but anxiolytic in mutant mice, yielding a clear cross interaction (Fig. 1b). These interpretations were confirmed by the 2 × 2 × 2 (Genotype × Housing × Ages) ANOVA of the percentages of entries into the open arms of the plus maze, which yielded significant effects of age [$F(1, 24) = 5.38$, $p < .05$] and genotype [$F(1, 24) = 7.49$, $p = .01$], and a Genotype × Housing interaction [$F(1, 24) = 23.21$, $p < .001$; post-hoc: $p < .05$]; the three-way interaction was far from statistical significance. These effects did not appear to be confounded by parallel differences in overall activity levels in the elevated plus maze. Analysis of the total number of arm entries—a common index of locomotor activity in this test—did not reveal any significant effects of genotype, enrichment, or their interaction [all n.s.; Fig. 1c], except for the significant age effect [$F(1, 24) = 22.05$, $p < .05$], which indicated a reduction of locomotor activity from 6 to 12 months of age.

Open-field test

Anxiety The open-field experiment yielded a pattern of results highly comparable to the outcomes of the elevated plus maze test. A general anxiogenic effect of age was demonstrated by the reduced exploration of the central zone of the open field from 6 to 12 months of age (Figs. 2a and c). Against this background effect of age—which was evident in both mutant and WT controls—the strong anxiogenic phenotype of the 3×Tg-AD mutants seen under standard housing condition was almost absent in animals kept in enriched housing. This is most readily discerned when the data are collapsed across ages (Figs. 2b and d): We once again observed an anxiogenic effect of enriched housing in the wild type mice, but an anxiolytic effect in the mutants. These impressions were supported by the 2 × 2 × 2 (Genotype × Housing × Ages) ANOVA of the number of entries into the central area, which yielded significant effects of age [$F(1, 24) = 4.13$, $p = .05$] and genotype [$F(1, 24) = 12.61$, $p < .01$], and the Genotype × Housing interaction [$F(1, 24) = 21.57$, $p < .001$]. The opposing effects of enrichment between genotypes were further confirmed by post-hoc comparisons, as is illustrated in Fig. 2b (all ps < .05). A highly comparable pattern of results was obtained in the analysis of the time spent in the central area (see Figs. 2c and d), which yielded significant effects of age [$F(1, 24) = 5.67$, $p < .05$] and genotype [$F(1, 24) = 17.22$, $p < .001$]. Although the Genotype × Housing interaction only assumed a trend, without reaching statistical significance [$F(1, 24) = 3.45$, $p = .08$], further restricted analyses revealed a highly significant genotype effect in the standard [$F(1, 11) = 25.47$, $p < .001$], but not in the enriched [$F(1, 13) = 2.24$, n.s.] condition (Fig. 2d). This is consistent with the conclusion above (based on entries into the central area) that EE had effectively attenuated the genotype difference in anxiety expression observed under standard housing (Fig. 2b).

Locomotor activity Activity was initially high as the animals were introduced to the novel open-field environment, but it gradually declined and stabilized at lower levels, suggesting locomotor habituation (Figs. 3a and b). This effect was evident in all experimental conditions. Differences due to the age of testing or genotypes were notable, especially at the onset of

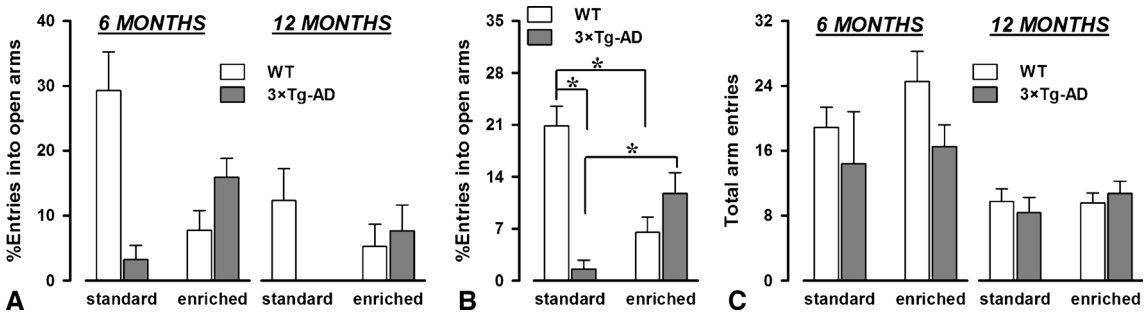

Fig. 1 Anxiety in the elevated plus maze. Anxiety tended to increase with age, regardless of experimental grouping(A). Independent of age, the genotypic difference in anxiety was reversed between the two housing conditions, because enriched housing appeared anxiogenic in wild type but anxiolytic in 3×Tg-AD mice(B). Panel B illustrates specifically the significant Genotype × Housing interaction and the outcomes of the post-hoc comparisons on the nature of this interaction. No enrichment or genotype effects emerged from the analysis of locomotion—that is, the number of total arm entries—leading only to an effect of age(C). The wild type mice included eight standard and seven enriched, whereas the 3×Tg-AD animals included five standard and eight enriched. The data depicted as means+ *SEM*s. *Pair-wise comparison significant at $p < .05$

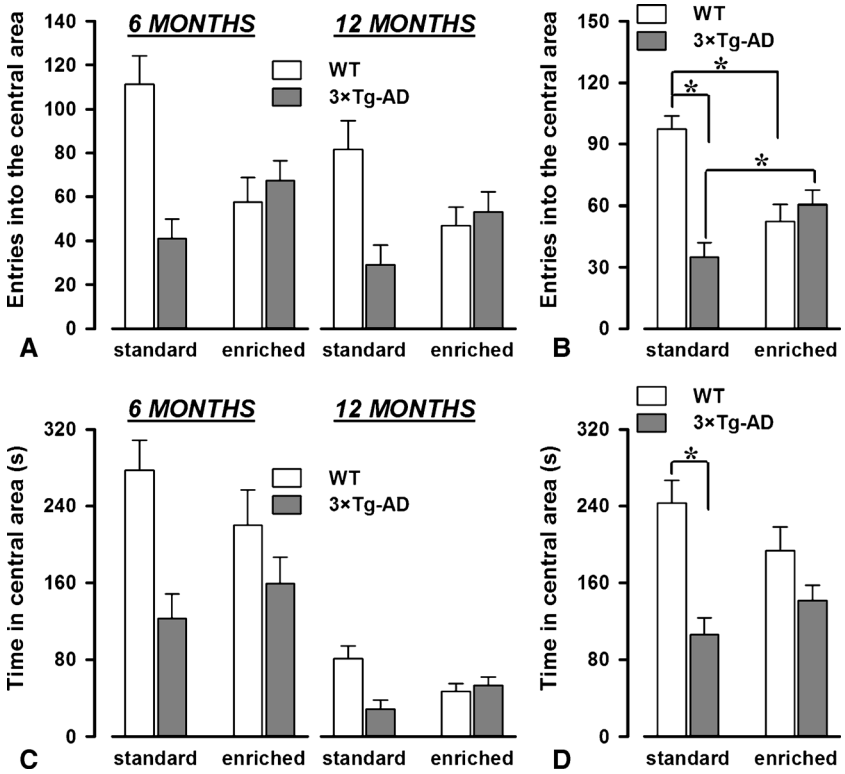

Fig. 2 Anxiety in the open-field test. Anxiety levels in the open field were higher at 12 than at 6 months of age in all mice (A, C). Furthermore, independently of age, 3×Tg-AD mice showed an anxiogenic phenotype in the open field that was mostly eliminated by environmental enrichment (B, D). The wild type mice included eight standard and seven enriched, whereas the 3×Tg-AD animals included five standard and eight enriched. Error bars refer to *SEM*s. $^{*}p < .05$

the test. In the first 5-min bin of the test, activity was substantially lower when at 12 months relative to 6 months of age (Fig. 3a) and in the mutant relative to the wild type mice, regardless of the age at testing (Fig. 3b). These independent effects appeared transient, since activity levels became more comparable subsequently. Indeed, no statistically significant Genotype × Housing interaction was detected on locomotor activity (Fig. 3c), suggesting that the effects observed on anxiety in the open field could not be solely attributed to activity changes.

A 2 × 2 × 2 × 12 (Genotype × Housing × Ages × Bins) ANOVA of the distance moved yielded evidence for habituation, with a highly significant effect of bins [$F(11, 264) = 40.38$, $p < .0001$], as well as its interactions with age [$F(11, 264) = 3.67$, $p < .0001$] and with genotype [$F(1, 24) = 4.38$, $p < .0001$] (see Figs. 3a and b). The latter interaction was

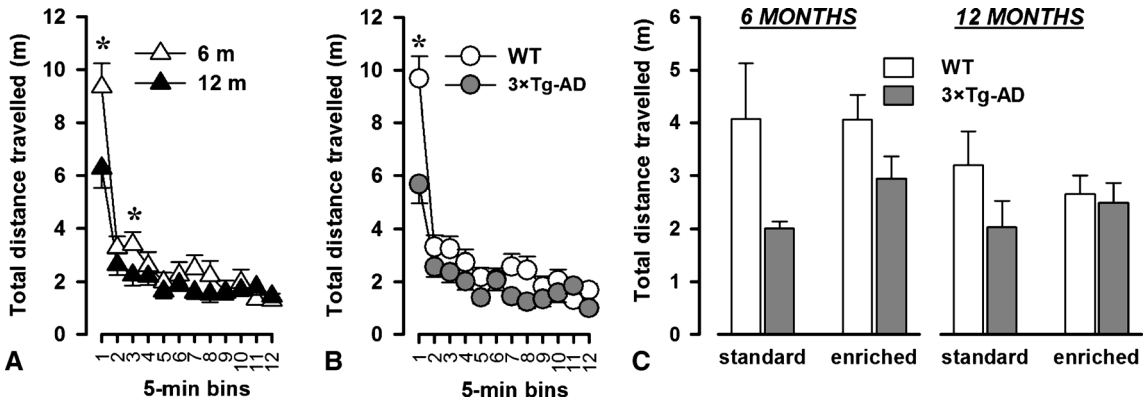

Fig. 3 Locomotion in the open-field test. All mice displayed habituation to the open-field arena, but this phenomenon was less pronounced at the older age, due to the lower levels of initial locomotor activity(A). Regardless of housing conditions and age, 3×Tg-AD mice displayed lower levels of locomotor activity, but this was observed mostly during the first 5 min of the test(B). No overall difference in locomotion was observed between the housing conditions (C). The wild type mice included eight standard and seven enriched, whereas the 3×Tg-AD animals included five standard and eight enriched. Error bars refer to *SEM*s. $^{*}p < .05$

also associated with a significant genotype effect [$F(1, 24) = 5.51$, $p < .05$; Fig. 3c]. The outcomes of post-hoc comparisons at successive bins are illustrated in Figs. 3a and b ($p < .05$).

In view of the presence of the independent effects of age and genotype, we further examined the dependency of our critical finding regarding the anxiety measure—entries into the center—on overall locomotor activity, performing an analysis of covariance (ANCOVA) on this critical measure (obtained at each age) with total distance travelled (at the respective age) as covariate. The separate ANCOVA confirmed that the presence of the critical Genotype × Housing interaction remained highly significant, supporting the interpretation that the dependency of the anxiety phenotype on housing environment cannot be solely explained by the concomitant differences in general activity levels.

Pavlovian conditioned freezing (at 6 months of age)

Although the triple mutation and differential housing independently exerted significant modifications of Pavlovian conditioned freezing at various stages of the experiment, we observed no evidence for any gene–environment interaction.

Day 1: CS freezing Freezing observed during the CS tone presentation predictably increased across the three trials of tone–shock pairing, but 3×Tg-AD mice generally exhibited stronger freezing than did wild type mice (Fig. 4a). In support of these impressions, a 2 × 2 × 3 (Genotype × Housing × Trials) ANOVA of the percentages of time freezing yielded significant effects of trials [$F(2, 56) = 95.86$, $p < .0001$] and genotype [$F(1, 28) = 7.55$, $p < .05$].

Day 1: ITI freezing As expected, freezing in-between conditioning trials showed a parallel increase across successive ITIs (Fig. 4b). The mutant and wild type mice remained highly comparable until the last ITI period, when the wild type exhibited an unexpected drop of freezing level—as opposed to the mutants, who exhibited a consistent monotonic increase. This led to the emergence of a Genotype × ITI interaction [$F(3, 84) = 8.14$, $p < .0001$], in addition to the main effect of ITI [$F(3, 84) = 139.23$, $p < .0001$] in the 2 × 2 × 4 (Genotype × Housing × ITI) ANOVA of percentages of freezing time.

Day 2: Context freezing Conditioned fear to the training context was measured when the animals were returned to the test chamber 24 h after conditioning. Although the mutant and wild type mice were comparable in their overall levels of freezing across the 8-min test session, their temporal profiles differed somewhat: Mutant mice showed a weak decrease, whereas wild type mice exhibited an initial rise after the first 2 min, which then remained stabilized over the last 6 min (Fig. 4c). The 2 × 2 × 4 (Genotype × Housing × 2-Min Bins) ANOVA of the percentages of time freezing yielded only a significant Genotype × Bins interaction [$F(3, 84) = 6.71$, $p < .001$].

Days 3–7: Tone freezing Freezing to the tone CS was assessed across five days of extinction test, and within each day across four 2-min bins. The 3×Tg-AD mice, irrespective of housing conditions, showed more marked extinction of the conditioned freezing response, both between and within days (Figs. 4d and e). By comparison, the wild type animals exhibited very weak extinction. The 2 × 2 × 5 × 4 (Genotype ×

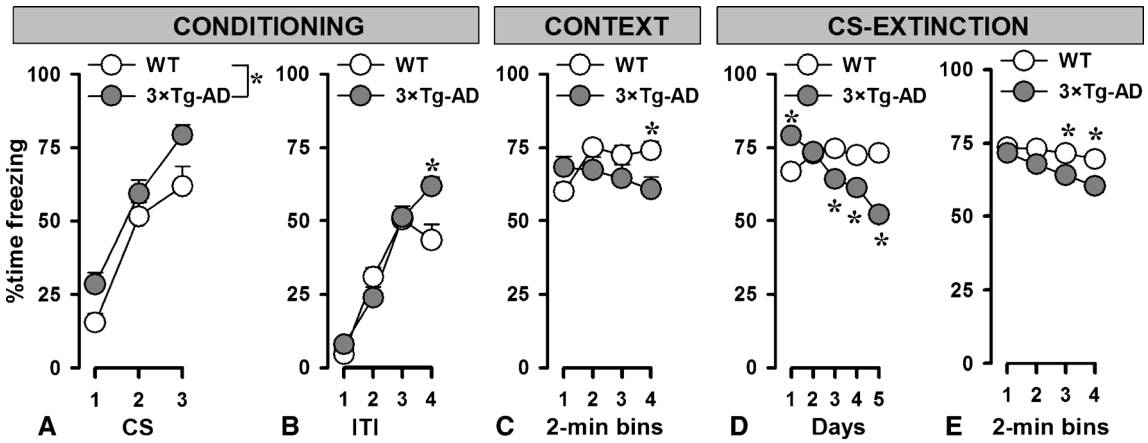

Fig. 4 Pavlovian conditioned freezing. The 3×Tg-AD mutation affected the acquisition and expression of the freezing response throughout all of the testing phases. In contrast, enriched housing did not exert any effect. Day 1: During the acquisition phase, 3×Tg-AD mice displayed higher levels of freezing response to the conditioned stimulus, CS(A), and they also showed higher levels of freezing during the last intertrial interval (ITI)(B). Day 2: The freezing response to the context of mutant mice tended to decrease with time, in contrast to the increasing trend observed in wild type animals (C). Days 3–7: The extinction of the conditioned freezing response to the CS was evaluated across 4 days and four 2-min bins within each day. The 3×Tg-AD mice showed more rapid and marked extinction both between(D) and within(E) days. The wild type mice included eight standard and seven enriched, whereas the 3×Tg-AD animals included eight standard and nine enriched. Error bars refer to *SEM*s. $^{*}p < .05$, from post-hoc comparisons based on the overall error variance associated with the relevant factor

Housing × Days × 2-Min Bins) ANOVA revealed significant effects of days [$F(4, 112) = 8.975$, $p < .0001$] and 2-min bins [$F(3, 84) = 15.18$, $p < .0001$], as well as significant Genotype × Days [$F(4, 112) = 18.61$, $p < .0001$] and Genotype × Bins [$F(3, 84) = 3.56$, $p < .05$] interactions. Interestingly, a similar pattern of results was observed during the pre-CS 2-min initial interval (data not shown): 3×Tg-AD mice displaying lower levels of freezing on the last 3 days of the extinction test than did wild type animals [Genotype × Days: $F(4, 112) = 4.81$, $p < .01$].

Two-way active avoidance (at 12 months of age)

Although the triple mutation and differential housing exerted significant modifications of the acquisition of signaled active avoidance at various stages of the experiment, we found no evidence for any gene–environment interaction in all performance measures.

Avoidance Avoidance learning, indexed by the number of successful avoidance responses per ten-trial block across the two days of acquisition training, showed a general increase across blocks that was most pronounced on Day 2. A 2 × 2 × 2 × 5 (Genotype × Housing × Days × Ten-Trial Blocks) ANOVA yielded significant effects of days [$F(1, 24) = 59.95$, $p < .0001$] and blocks [$F(4, 96) = 37.40$, $p < .0001$], as well as their interaction [$F(4, 96) = 35.49$, $p < .0001$]. By the end of the second day, clear effects of 3×Tg-AD mutation and enriched housing became apparent (Figs. 5a and b). The facilitating effect of the mutation was supported by the presence of the significant Genotype × Days [$F(1, 24) = 5.11$, $p < .05$] and Genotype × Blocks [$F(4, 96) = 10.06$, $p < .0001$] interactions. The similar effect of enriched housing was confirmed by the significant Housing × Days [$F(1, 24) = 7.58$, $p < .05$], Housing × Blocks [$F(4, 96) = 5.52$, $p < .001$], and Housing × Days × Blocks [$F(4, 96) = 3.16$, $p < .05$] interactions.

Escape failures An analysis of the number of escape failures—that is, trials in which the animals failed to avoid or escape from the shock—yielded a picture complementary to that obtained in the avoidance analysis above. The numbers of failures decreased across days [$F(1, 24) = 37.24$, $p < .0001$] and blocks [$F(4, 96) = 28.28$, $p < .0001$]. This was expected, given that avoidance performance improved over training. Importantly, the 3×Tg-AD mutation and enriched housing

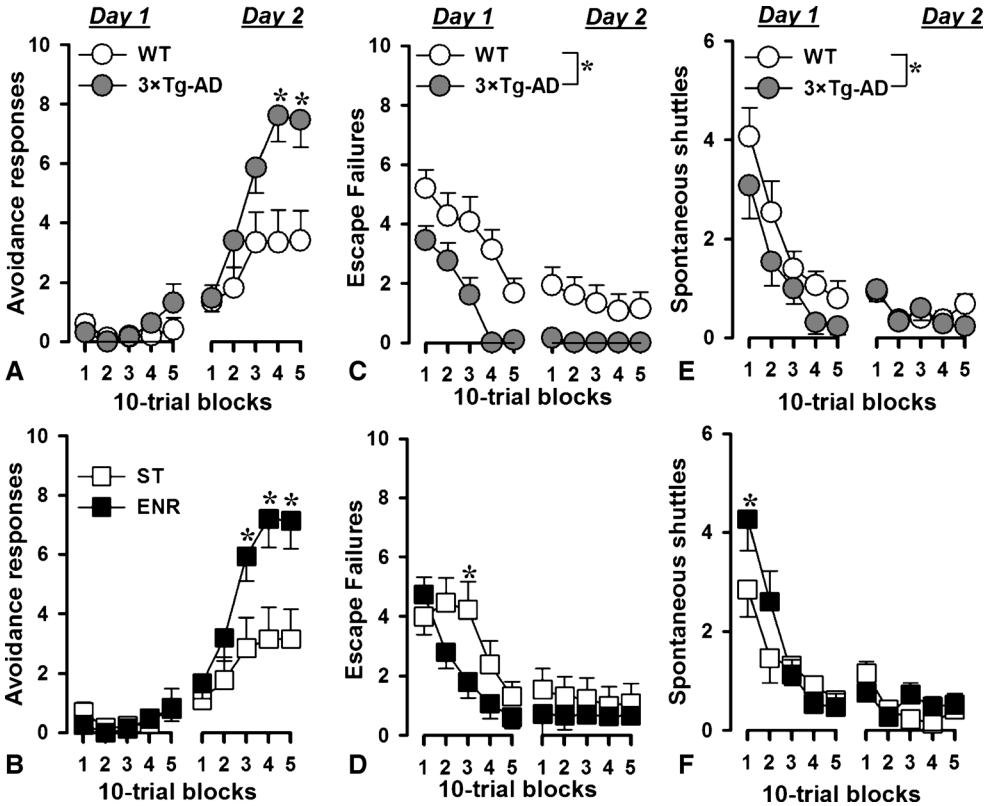

Fig. 5 Two-way avoidance learning. Both environmental enrichment and the 3×Tg-AD mutation improved the performance in a two-way active avoidance test. These effects were reflected by an increase in avoidance responses, mainly observed at the end of Day 2 (A–B). The analysis of failures provided a pattern of results complementary to that observed for avoidance responses (C–D). Finally, the effects of enrichment and genotype on avoidance performance were not paralleled by an increase in general locomotor activity (E–F). The wild type mice included eight standard and seven enriched, whereas the 3×Tg-AD animals included five standard and eight enriched. Error bars refer to *SEM*s. *$p < .05$, from post-hoc comparisons based on the overall error variance associated with the relevant factor

independently reduced the numbers of escape failures (Figs. 5c and d). This effect of the triple mutation was clearly observed on both days, whereas the effect of enriched housing was more prominent on Day 1 (possibly a floor effect). Hence, this measure unmasked an effect on active avoidance learning that was not apparent when only the avoidance response was considered (see Figs. 5a and b). The impact of genotype was confirmed by the presence of a significant genotype effect [$F(1, 24) = 8.63$, $p < .01$], which was associated with a trend toward a Genotype × Days × Blocks interaction [$F(4, 96) = 2.10$, $p = .09$]. The behavioral impact of housing, on the other hand, was confirmed by the presence of a significant Housing × Blocks interaction [$F(4, 96) = 3.96$, $p < .01$] and the Housing × Days × Blocks interaction [$F(4, 96) = 2.96$, $p < .05$].

Spontaneous shuttles during ITIs The number of shuttles made during the ITIs between successive avoidance trials provided a measure of spontaneous locomotor activity. The number of spontaneous shuttles tended to decrease across days [$F(1, 24) = 9.99$, $p < .01$] and blocks [$F(4, 96) = 20.35$, $p < .0001$] in all animals. Locomotor activity was reduced in mutant mice (Fig. 5e), and it was enhanced in enriched animals, although the latter effect was mostly observed on the first block of trials on the first day (Fig. 5f). These impressions were confirmed by an ANOVA of spontaneous shuttles, which yielded a main effect of genotype [$F(1, 24) = 4.30$, $p < .05$] and a Housing × Days × Blocks interaction [$F(4, 96) = 4.64$, $p < .01$].

Discussion

Here, we demonstrated that the postweaning exposure to an enriched environment (EE) could attenuate the behavioral abnormalities present in our 3×Tg-AD mutant mice. The modification of 3×Tg-AD phenotypes by EE was specific to anxiety-related phenotypes measured by the elevated plus maze and the open-field tests, and this pattern of gene–environment interactions was already evident when the mice were 6–7 months old, and persisted up to the age of 12–13 months. Comparable interaction, however, was not observed when conditioned fear was examined at either age, which suggest that not all expressions of disturbed fear emotional processing are similarly sensitive to the differential housing conditions compared here.

The present study confirmed and extended the previous reports of anxiety and conditioned fear phenotypes in the 3×Tg-AD mouse line (Espana et al., 2010; Garcia-Mesa et al., 2012; Garcia-Mesa et al., 2011; Gimenez-Llort et al., 2007; Marchese et al., 2014; Pietropaolo, Feldon, & Yee, 2008; Pietropaolo, Sun, et al., 2008; Pietropaolo et al., 2009; Sterniczuk et al., 2010). Increased anxiety has been described in triple mutant mice as early as at 6 months of age (Espana et al., 2010; Garcia-Mesa et al., 2011; Gimenez-Llort et al., 2007; Pietropaolo, Sun, et al., 2008; Pietropaolo et al., 2009) and appears as a stable phenotype extending into more advanced age (Garcia-Mesa et al., 2012; Gimenez-Llort et al., 2007; Pietropaolo, Feldon, & Yee, 2008; Sterniczuk et al., 2010). Enhanced conditioned fear has also been previously described in 3×Tg-AD mice at both early (Espana et al., 2010) and late (Pietropaolo, Feldon, & Yee, 2008) phases of the pathology. These are in board agreement with the present data in Pavlovian conditioned freezing (see Figs. 4a and b, and Day 1 of Fig. 4d) and active avoidance (see Fig. 5a). Although the conditioned fear phenotypes may seem surprising within the context of AD because they might be considered as enhanced learning, comparable observations have been reported in patients. Memory enhancement associated with a negative stimuli has been described in AD patients (Fleming et al., 2003), and clinical studies have shown that anxiety enhances the acquisition of novel conditioned fear associations (Bishop, 2007). The phenotype of fasting long- and short-term extinction of fear response observed at 6 months (see Figs. 4d and e) may appear subtle, but it has been demonstrated before (Pietropaolo, Feldon, & Yee, 2008), and its expression may be interpreted as a cognitive as much as an affective phenotype. As has been concluded by others (e.g., Espana et al., 2010), both explanations must be considered as possible accounts of these findings in patients.

Our data also extended the existing literature on the behavioral effects of postweaning EE documented in WT mice. The anxiogenic effects of EE in WT mice here are consistent with the previous findings in our (Pietropaolo et al., 2006; Zhu et al., 2006) and other laboratories (Abramov, Puussaar, Raud, Kurrikoff, & Vasar, 2008; Pietropaolo et al., 2004; van de Weerd, Baumans, Koolhaas, & van Zutphen, 1994), although some reports have demonstrated an opposite effect, or no effect, of EE in mice (Abramov et al., 2008; Akillioglu, Babar Melik, Melik, & Kocahan, 2012; Chapillon, Manneche, Belzung, & Caston, 1999; Chapillon, Patin, Roy, Vincent, & Caston, 2002; Roy, Belzung, Delarue, & Chapillon, 2001). This apparent inconsistency might in parts be attributed to the type of anxiety tests and the mouse strain employed. Indeed, our finding that EE modify anxiety in opposite directions between WT and the 3×Tg-AD mice (in identical test conditions within a single laboratory) indicates that genomic difference can be critical. The neurobiological mechanisms underlying this complex gene–environment interaction however is not known, and one may only speculate. Previously, we have hypothesized that increased hippocampal BDNF levels may be linked to the anxiogenic effect of EE we observed in wild type B6 mice (Yee, Zhu, Mohammed, & Feldon, 2007; Zhu et al., 2006), which might potentiate the functionality of the hippocampus as a key structure in the processing of anxiety-provoking stimuli (Gray & McNaughton, 1983; McNaughton & Gray, 2000). This is

consistent with the positive correlation between anxiety response in the elevated plus maze and baseline hippocampal BDNF levels in the same mouse strain (Yee et al., 2007). However, this relationship clearly does not apply to 3×Tg-AD mice. First, EE tended to reduce rather than increase anxiety behavior in the 3×Tg-AD mice. Second, similar to AD patients (Connor et al., 1997; Peng, Wuu, Mufson, & Fahnestock, 2005; Phillips et al., 1991), baseline hippocampal BDNF expression is expected to be reduced in 3×Tg-AD mice (Corona et al., 2010). Hence, the anxiogenic phenotype of the 3×Tg-AD mice as well as its unique response to EE is unlikely attributable solely to the expected changes in hippocampal BDNF levels. However, direct measures of BDNF levels would be necessary to support this interpretation.

Regarding the effects of enrichment on anxiety, it is also important to notice that the elevated plus maze and open-field tests also seem to be particularly sensitive to this form of gene–environment. One possibility is that these tests involve the expression of an active choice to take risk in exploring open spaces. Thus, anxiety is demonstrated within an approach-avoidance conflict, whereby anxiogenesis should promote avoidance. Such an approach–avoidance conflict is clearly lacking in the Pavlovian fear conditioning test and the expression of conditioned fear in this test is relatively insensitive to the effects of EE (see also Pietropaolo et al., 2006). On the other hand, EE clearly facilitated the acquisition of an avoidance response as previously reported in WT mice reared under an EE regime similar to ours (Donovick, Burright, Fuller, & Branson, 1975), although EE did not lead to an opposite effect in the 3×Tg-AD mice. Therefore, one must not overlook the test-specificity of the gene–environment interaction revealed in the present longitudinal study, which stood in clear contrast to its stable expression over time (6 vs. 12 months of age).

Gene × Environment interaction

Here, our search for possible gene–environment interactions was met with the clearest outcome in the elevated plus maze and open-field tests. The elevated plus maze results, in particular, showed that the effect of one manipulation was being reversed by the other. Thus, one may conclude that either (1) the 3×Tg-AD switched effects of EE from anxiogenesis (in WT mice) into anxiolysis (in mutants), or (2)EE switched the phenotypic expression of the 3×Tg-AD mutation from anxiogenesis (under standard housing) to anxiolysis (under enriched housing; see Fig. 1b). With the clear presence of a cross-interaction, as illustrated in Fig. 1b, one may readily conclude that the specific genetic and environmental manipulations were modifying each other's impact on behavior in a nonadditive manner. This interpretation is appropriate even though the gene–environment interaction observed in the open-field measure of anxiety did not reverse the difference in anxiety expression between the 3×Tg-AD and wild type mice reared under standard housing (see Fig. 2b). It is therefore worth noting that the effect of EE was not sufficient to "normalize" mutant behavior to the levels observed in wild type mice reared under standard housing conditions. Hence, any attempt to translate the present finding to human must first addresses whether standard housing of wild type mice is the appropriate approximation of the non-AD human population. Indeed, we may even consider the phenotypes revealed in the comparison of 3×Tg-AD mutant and wild type mice under standard housing as the product of interaction between the three AD-related mutations and an impoverished environment.

Our results also demonstrated that the effects of environmental enrichment on anxiety in AD mice were largely independent of the animals' age: enrichment was able to rescue the anxiety phenotype of mutant mice at both early and advanced stages of AD pathology. This suggests that environmental stimulation, at least when started at prepathological stages, may induce marked and long-lasting effects independent of the progression of AD pathology. Furthermore, the emergence of tau pathology expected by 12 months of age did not modify the interaction between enrichment and the 3×Tg-AD mutations. Hence, parsimony may lead us to suggest that the interaction was more closely linked to the stable expression of β-amyloid plaques from 6 to 12 months of age, even though we cannot completely dismiss the possibility that tau pathology might further contribute to the observed gene–environment interaction obtained at 12 months of age. Future studies are needed to disentangle the functional contributions of these two histopathological hallmarks of AD in the domain of emotionality and their sensitivity to environmental manipulations.

The present study has identified that the anxiety, but not the conditioned fear, phenotypes of the 3×Tg-AD mutation as being modifiable by enriched housing. Is this contrast between the two forms of fear-related behavior generalizable to other common forms of environmental manipulations? We have previously reported that the conditioned fear phenotypes of the 3×Tg-AD mice were resistant to modification by another environmental intervention—namely, social isolation (Pietropaolo, Sun, et al., 2008; Pietropaolo et al., 2009)—which was sufficient to alter the expression of the anxiety phenotypes. Although one should be cautioned against generalization to *all* forms of environmental impact, these findings strongly suggest that the mechanisms underlying the two categories of phenotypes (generalized anxiety to external threats versus learned fear to specific stimuli) are likely dissociable. This is consistent with the impression that individual differences in these two behavioral traits do not correlate with each other in wild type B6 mice (Dubroqua & Yee, unpublished data) despite overlaps of the brain circuits involved. Furthermore, the present results confirms that the anxiogenic

profile of triple AD mice is highly sensitive to environmental stimulation, in agreement with previous data showing beneficial behavioral effects of wheel running exercise at both early (Garcia-Mesa et al., 2011; Pietropaolo, Sun, et al., 2008) and advanced (Garcia-Mesa et al., 2012) pathological stages.

Future studies

To consolidate the key findings emerged in the present study, it would be necessary to address some of the limitations inherent to our present experimental design.

First, although the longitudinal design adopted here may allow a within-subjects comparison across age and facilitate comparison with evaluations in the clinic, it also introduces confounds in terms of age, re-testing and age-dependent progression of neuropathology. For instance, retesting in the elevated plus maze test of anxiety may increase the observed levels of anxiety (Almeida, Garcia, & de Oliveira, 1993), although this did not prevent us from detecting the anxiogenic phenotype of the triple mutant (see Sterniczuk et al., 2010). To address these confounds, studies with a between-subjects design (tested once per selected age) would be necessary.

Second, we included only female mice here to avoid persistent aggressive behavior often encountered with male mice under enriched housing conditions (Haemisch & Gartner, 1994; Haemisch et al., 1994). We are aware that this approach does not allow us to detect potential sex differences in the behavioral response to enriched rearing. However, one may suspect that our observations made in female mice here may be generalizable to the male sex because home cage voluntary wheel-running exercise (which is an integral component of the enriched rearing) has been shown to attenuate the anxiety phenotype of male 3×Tg-AD mutants (Garcia-Mesa et al., 2012; Garcia-Mesa et al., 2011).

Third, EE was introduced immediately at the time of weaning here, which maximizes its long-term impact (Kohl et al., 2002; Renner & Rosenzweig, 1987). Hence, our data may be more readily interpreted from the perspective of prevention rather than that of treatment of AD-related affective symptoms. For the latter perspective, comparisons between longitudinal EE regimes with different age of initiation are necessary.

Fourth, EE is a complex procedure involving the presence of multiple social, intellectual, and physical elements. Their individual contribution as well as interaction with each other to the overall impact of EE to modify genetic disposition can only be revealed if each element is separately manipulated one at a time. Recent data have shown that wheel running exercise by itself can be effective in modifying the anxiogenic phenotypes of the 3×Tg-AD mutants (Garcia-Mesa et al., 2012; Garcia-Mesa et al., Garcia-Mesa, Lopez-Ramos, Gimenez-Llort, Revilla, Guerra, Gruart and Sanfeliu 2011).

Conclusions

The present longitudinal study provides empirical evidence for an interaction between genetic risks for AD and environmental factors. Our results support the view that the exposure to stimulating environments can attenuate the severity of AD symptoms, at least partially. Further investigation with a lifelong developmental perspective is necessary to fully delineate how the complex interaction between genes and environment shapes the pattern and severity of expression of behavioral phenotypes determined by AD-related mutations.

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