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Research report

Sex-dependent worsening of NMDA-induced responses, anxiety, hypercortisolemia, and organometry of early peripheral immunoendocrine impairment in adult 3xTg-AD mice and their long-lasting ontogenic modulation by neonatal handling

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ABSTRACT

The neuroimmunomodulation hypothesis for Alzheimer's disease (AD) postulates that alterations in the innate immune system triggered by damage signals result in adverse effects on neuronal functions. The peripheral immune system and neuroimmunoendocrine communication are also impaired. Here we provide further evidence using a longitudinal design that also studied the long-lasting effects of an early life sensorial intervention (neonatal handling, from postnatal day 1–21) in 6-month-old (early stages of the disease) male and female 3xTg-AD mice compared to age- and sex-matched non-transgenic (NTg) mice with normal aging. The behavioral patterns elicited by the direct exposure to an open field, and the motor depression response evoked by NMDA (25 mg/kg, i.p) were found correlated to the organometry of peripheral immune-endocrine organs (thymus involution, splenomegaly, and adrenal glands' hypertrophy) and increased corticosterone levels, suggesting their potential value for diagnostic and biomonitoring.The NMDA-induced immediate and depressant motor activity and endocrine (corticosterone) responses were sensitive to sex and AD-genotype, suggesting worse endogenous susceptibility/neuroprotective response to glutamatergic excitotoxicity in males and in the AD-genotype. 3xTg-AD females showed a reduced immediate response, whereas the NTg showed higher responsiveness to subsequent NMDA-induced depressant effect than their male counterparts. The long-lasting ontogenic modulation by handling was shown as a potentiation of NMDA-depressant effect in NTg males and females, while sex × treatment effects were found in 3xTg-AD mice. Finally, NMDA-induced corticosterone showed sex, genotype and interaction effects with sexual dimorphism enhanced in the AD-genotype, suggesting different endogenous vulnerability/neuroprotective capacities and modulation of the neuroimmunoendocrine system.

1. Introduction

The functional interplay between the neuro system and immune system is essential to ensure homeostasis preservation and health [1–3]. Their crosstalk significantly contributes to the progress of aging and associated neurodegenerative disorders and *vice versa* [4,5]. Age-related peripheral chronic inflammation modulates the central nervous system and results in neuroinflammation, neuro-oxidation, behavioral alterations, and cognitive deficits [3,6–8]. In this context, Alzheimer's disease (AD) can be understood in the context of aging of this neuro-immune-endocrine communication [9,10]. We have proposed that peripheral immune cells' function and redox state can help measure AD progression [11]. Thus, alterations in the innate immune system triggered by several damage signals result in neuroinflammation and adverse effects on neuronal functions at peripheral and central levels *i.e.*, [9,11,12]. The crosstalk between behavior and the immune system is an early phenomenon. It appears since the premorbid and prodromal stages of AD [10,11,13] when "Behavioral and psychological symptoms of dementia" (BPSD) have already started, but the cognitive function still seems normal [14]. The behavioral clusters distinguished by behavioral

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assessment scales at the clinical level include anxieties and phobias, activity and affective disturbances, aggressiveness, alterations of diurnal rhythms, paranoia and delusion, and hallucinations [15]. These, also called neuropsychiatric symptoms (NPS) of dementia, are quite prevalent among AD patients [16]. They are regarded as the most important source of burden for family and professional caregivers [17], both dramatically affecting their quality of life [18] and usually leading to patient institutionalization [19].

The 3xTg-AD mouse model mimics many critical hallmarks of AD neuropathology [20]. Thus it presents beta-amyloid plaques and neurofibrillary tangles with a temporal- and regional-specific profile closely mimicking their development in the human AD brain; synaptic dysfunction, including LTP deficits; and the characteristic behavioral deficits in learning and memory in an age-dependent manner [20–26]. This animal model has provided evidence for the pathogenic role of intraneuronal Aβ in AD [20,22]. Also, this model has shown how this early event relates to other neuropathological aspects, such as the affectation of the basolateral amygdala [27] and the early appearance of BPSD-like symptoms [11,28]. These early BPSD-like symptoms are mostly related to emotionality, stress coping strategies, and exploratory behaviors, similar to that observed in patients at the pre-clinical stages of the disease [29]. At all pre-pathological stages, disinhibitory behavior and lack of ability to cope with mild stressors such as novelty in most behavioral tests are common features of the 3xTg-AD behavioral phenotype [14,30,32] and worse with the progress of disease [9,14,33,34].

Regarding the neuro-immune-endocrine network, the impairment in the nervous and the peripheral immune system communication that occurs with aging is accelerated and more pronounced in the 15–18 months old 3 ×Tg-AD mice [9–11]. A disabled choroid plexus-CSF system at these ages facilitates the derangements [35]. 3xTg-AD mice exhibit premature immunosenescence and confirm the involvement of central but also systemic immunity and inflammation in behavioral and cognitive deficits [9–11,13,36]. In addition to the thymus weight, established as an indirect indicator of the functional immunological state [37], we propose that the organometry of peripheral immune (thymus and spleen) and endocrine (adrenal glands) systems can be relevant to the understanding and monitoring of the disease [10,11,38]. Thus, we reported that the total weight of these peripheral immunoendocrine organs and their index (relative weight *vs.* the body weight) correlated with the sex-dependent impairment of the neuro-immunoendocrine network. These organometric alterations were described in premorbid, prodromal, and early stages of the disease [10,11,13,39] and were found to worsen at advanced neuropathological stages [9,13] and in correlation with worse NPS-like behavioral profiles [10].

Impaired neuro-immune communication benefits from lifestyle strategies such as physical exercise or social and cognitively enriched environments [40,41]. On the other hand, ontogenic development of the nervous system and the neurobehavioral output are known to be highly dependent on the interactions between the organism and its environment, whose relevance may even last till old age [40]. Neonatal handling (H) is a tactile and proprioceptive sensorial stimulation administered after birth (from neonatal day 1, PND1) until weaning [40,41]. This early intervention was one of the first rodent models in developmental psychobiology to investigate the positive and negative effects of early-life experiences on behavior, physiology, and neural function across the lifespan [41]. Neonatal handling is known to induce long-lasting positive effects on anxiety- and stress-related profiles in adulthood [40,42–45] and to show positive effects on the hypothalamic-pituitary-adrenal (HPA) axis functioning [43]. In a precedent work, we studied the long-lasting beneficial effects of neonatal handling on the behavioral phenotype of 6-month-old male and female 3xTg-AD as compared to age- and sex-matched non-transgenic mice [31]. Animals confronted three environments differing on the anxiogenic levels, with the open field being the best to trigger bizarre and other anxiety-like behaviors compared to the corner test or the dark-light box. Whether the behavioral benefits could also translate to their neuroimmunoendocrine network remained to be elucidated. Therefore, the first aim of the present work was to study further whether the disruption of the neuroimmunoendocrine network could benefit from such ontogenic intervention.

Moreover, neonatal H in rodents exerts some protection against early hypoxic-ischemic conditions [46], age-related cognitive deficits and also excitotoxic insults [44]. Excitotoxicity is a major degenerative process in which glutamate is the main neurotransmitter [47]. The dysfunction of the glutamatergic *N*-methyl-D-aspartate receptor (NMDAR) related neurotransmission is known to lead to cognitive impairment and behavioral changes in AD [48]. Therefore, our second aim was to evaluate the long-lasting effects of neonatal handling on NMDA-induced locomotor response assay, a behavioral paradigm for phenotype-based excitotoxicity screening in rats and mice [49]. In this model, low doses of NMDA (25 mg/kg, *i.p.*) induce an activity response useful for investigating functional interactions between regulatory systems [49,50]. Our precedent literature has extensively described that the immediate NMDA-induced motor depression effect is due to the release of adenosine [51,52], the endogenous protective mechanism to avoid NMDA-induced excitotoxic effects [53]. Therefore, in the present work, we analyzed the status of the neuroimmunoendocrine network of those 6-month-old males and females 3 ×Tg-AD mice and age- and sex-matched non-transgenic counterparts using the organometry of the immune and endocrine system, the corticosterone levels and a new detailed analysis that defined their behavioral response patterns under the anxiogenic conditions of the open-field and the NMDA-evoked locomotor response.

2. Materials and methods

2.1. Animals

Triple-transgenic (3xTg-AD) mice harboring PS1M146V, APPSwe, and tauP301L transgenes were genetically engineered at the University of California Irvine, as previously described [20]. Briefly, two independent transgenes (encoding human APPSwe and human tauP301L, both under control of the mouse Thy1.2 regulatory element) were co-injected into single-cell embryos harvested from homozygous mutant PS1M146V knock-in (PS1KI) mice. The PS1 knock-in mice were originally generated as a hybrid C57BL/6 × 129.

A total of 77 experimental subjects, 20 male and 21 female homozygous 3xTg-AD mice and 18 male and 18 female non-transgenic (NTg, C57BL/6 J × 129) mice from a breeding program of these two homozygous colonies established in our laboratory at the Medical Psychology Unit, Universitat Autònoma de Barcelona, were used in this study. Animals were kept in groups of 3–4 mice per cage (Macrolon, 35 × 35 × 25 cm) filled with 5 cm of clean wood cuttings and nesting materials, under standard laboratory conditions (12 h light:dark, cycle starting at 8:00 h, food and water available *ad libitum*, 22 ± 2∘C, 50–60% humidity). The experiments were performed from 9 a.m. to 6 p.m. under ilumination of 20 lux in accordance with the Spanish legislation on Protection of Animals Used for Experimental and Other Scientific Purposes 2010/63/EU Council on this subject. The study complies with the ARRIVE guidelines developed by the NC3Rs and aims to reduce the number of animals used [54].

2.2. Early neonatal handling (H) and experimental design

A longitudinal study with a factorial design was carried out to evaluate the functional impact of genotype (G), sex (S) and neonatal handling treatment (T) factors on behavior and neuro-immunoendocrine system in adulthood (6 months of age) that in the case of the 3xTg-AD mice corresponds to early stages of the disease.

The subjects came from 30 litters (6–8 pups) randomly distributed

into handled (H) and non-handled (nH) treatment groups. For each genotype, the final experimental design consisted of the following groups: non-handled males, non-handled females, handled males, and handled females (n = 9–11, in each experimental group).

Neonatal handling was administered 4 times, every 8 min, twice a day (mornings, 10–12 a.m., and afternoons, 4–6 p.m.), from the postnatal day (PND) 1 to PND21 before weaning at PND23. In each session, the home cages were transferred to a neighbor test room maintained at 25ºC and allowed to habituate for 30 min before the start of the protocol. Then, the mother was gently removed from their pups before gently handling them (one by one) to receive a first tactile stimulation on their back done with the thumb. Immediately after, each pup was placed (individually) in plastic compartments (35 cm × 15 cm × 25 cm) lined with paper towels [31]. Then, the pups were softly handled and received 2 more tactile stimulations on their back before a fourth tactile stimulation immediately before their return (one by one) to the nest. Once all the pups were back in the nest, the mother was returned to it. In the control groups, the pups were left undisturbed except for weekly cage cleaning.

Six months later, the long-lasting effects of the treatment on the behavioral response in adulthood were evaluated. Since early-neonatal handling has been reported to exert anxiolytic effects and neuroprotection [44], the animals were assessed under anxiogenic conditions [31] and using the NMDA-evoked locomotor response as a behavioral paradigm to screen excitatory aminoacids excitotoxicity (25 mg/kg, i.p) [49].

2.3. Behavioral assessment

2.3.1. Motor activity under anxiogenic conditions

Mice were placed in the center of an open field (OF) (homemade woodwork, white box, 55 × 55 × 25 cm). Neophobia was measured during the first minute of the test. The time course of the locomotor horizontal (crossings) and vertical (rearings) exploratory activity in confronting and habituating to the fearful context was recorded for 5 min.

2.3.2. NMDA-induced motor activity paradigm

The long-term neuroprotective effects of neonatal H on the NMDA-induced excitotoxicity were assessed at the behavioral level through the NMDA-induced motor response paradigm when animals reached 6 months of age [49]. N-methyl-D-aspartic acid (Sigma) was dissolved in 5.5% glucose and adjusted to pH 7.4 with NaOH [49]. Animals were weighed, and immediately after the i.p. administration of 25 mg/kg of NMDA, animals were individually placed in infrared photocells activity meter cages (40 × 40 × 40 cm), and the horizontal motor activity was recorded for 45 min under light conditions. The motor activity pattern was recorded and analyzed by a computerized system (SMART, Panlab, S.L., Barcelona, Spain) and analyzed at different time points and intervals.

2.4. Glucocorticoid analysis and organometry of the immunoendocrine system

Immediately after the end of the behavioral assessments, mice were euthanized and samples of about 1 mL of whole trunk blood were collected into heparinized tubes and centrifuged immediately at 10,000g for 2 min. The plasma obtained was stored at –20∘C. Determination of corticosterone content (ng/mL) was analyzed using a commercial kit (Corticosterone EIA Immunodiagnostic Systems Ltd, Boldon, UK) and ELISA EMS Reader MF V.2.9–0.

The total weights of the peripheral immunoendocrine organs (spleen, thymus, and adrenal glands) and their index (RW, relative weight *vs.* body weight) were measured as an indirect indicator of their functional state [10,11].

2.5. Statistics

Statistical analyses were performed using SPSS 17.0 software. All data are presented as mean ± SEM or percentage. To evaluate the effects of genotype (G), sex (S), and neonatal handling treatment (T), a 2 × 2 × 2 factorial analysis design was applied, as detailed in the text and figures. Differences were studied through Multivariate General Linear model analysis, followed by post-hoc Duncan's test comparisons, as depicted in the figures (g, genotype; s, sex, and *, neonatal handling treatment). Differences between two independent samples were depicted with the Student t-test. The relationship between two different variables was analyzed with Pearson's correlations. In all cases, $p < 0.05$ was taken as statistically significant.

3. Results

3.1. Motor activity under anxiogenic conditions and its modulation by neonatal handling

The time course of the horizontal and vertical activities in the OF is illustrated in Fig. 1. Horizontal activity patterns exhibited sex [S, $F(1,69) = 10.276$; $p < 0.01$], genotype [G, $F(1,69) = 12.484$; $p < 0.001$], and a sex × genotype interaction [S×G, $F(1,69) = 4.647$; $p < 0.05$] effects. Female sex and 3xTg-AD genotype showed a reduced horizontal activity as compared to their respective counterparts, already from the first minute of the test, when the fearful response is elicited. In contrast, all the groups of animals treated with H increased the horizontal component [T, $F(1,69) = 12.464$; $p < 0.001$]. Sex per genotype interaction effects pointed to the similarity of horizontal patterns between male and female 3xTg-AD mice, except for the first minutes of the test [s, $p < 0.05$], in contrast to more clear sex differences in the NTg genotype. In the vertical activity, sex × genotype interaction effects were found [S×G, $F(1,69) = 10.544$ $p < 0.01$], since male and female 3xTg-AD exhibited identical responses, whereas in the NTg genotype, males developed more rearings during the test than NTg females. Sex [S, $F(1,69) = 16.638$; $p < 0.001$] and genotype [G, $F(1,69) = 62.334$; $p < 0.001$] effects were also found. Both sexes of 3xTg-AD mice showed a reduced number of rearings compared to the NTg mice. Neonatal handling did not exert any effect in this vertical component.

3.2. NMDA-induced motor response and its modulation by neonatal handling

Fig. 2 shows the NMDA-induced motor activity response analyzed minute by minute, in the interval of time that refers to the phase of motor depression (minutes 1–7). This detailed analysis enabled the distinction of an initial spontaneous activity (min 1 of the test) and the following motor activity depression (min 2–7).

During the depression of motor activity (minutes 2–7) sex, and handling effects were observed. Thus, a higher depressant effect was shown by NTg females compared to NTg males (minute 4, S, [$F(1,35) = 4.232$; $p < 0.05$]) and was potentiated by handling (minute 6, T, [$F(1,35) = 5.760$; $p < 0.05$]) (Fig. 2A-B).

The initial spontaneous activity was equal in all the NTg animals, independently of their sex and handling condition. In contrast, a treatment x sex interaction effect [T × S, $F(1,37) = 9.402$; $p < 0.01$] was found in 3xTg-AD animals, with females showing a 2-fold reduction of spontaneous activity during the first minute (g, $p < 0.05$), that was reverted by neonatal H (see Fig. 2 C). Treatment effect [T, $p < 0.05$] was also found at several other minutes of the test, with genotype effects (^{ggg}, $p < 0.001$; g, $p < 0.05$) and treatment effects (**, $p < 0.01$) pointing at handled 3xTg-AD females as those with the lowest decrease of activity, while the other three groups exhibited a faster NMDA-induced motor depressant response. No differences were found in the subsequent intervals or total activity, as shown in Fig. 2D.

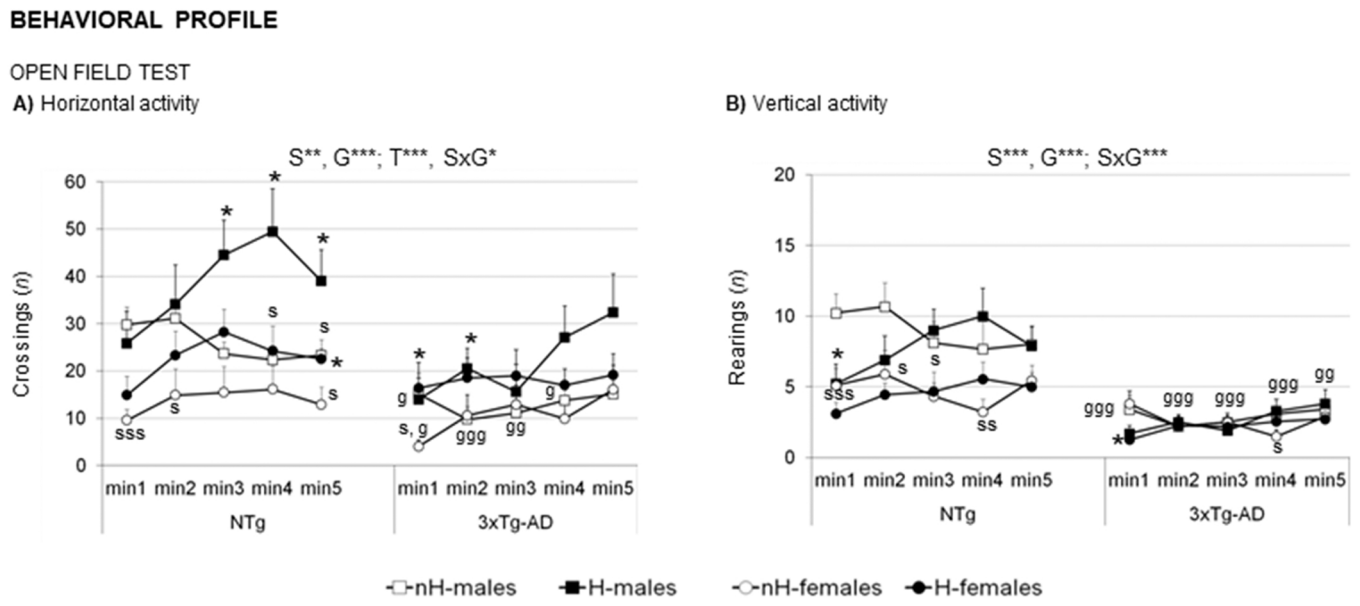

Fig. 1. Behavioral response under anxiogenic conditions and effects of neonatal handling. Data are expressed by mean ± SEM. (A) Horizontal activity, as measured by the number of crossings. (B) Vertical activity, as measured by the number of rearings. ANOVA 2 × 2 × 2: S, sex effect; G, genotype effect; T, handling treatment effect; S×G, sex × genotype interaction; * $p < 0.05$, * * $p < 0.01$, * ** $p < 0.001$ Post-hoc Duncan's test: ^{s} $p < 0.05$, ^{ss} $p < 0.01$, ^{sss} 611 $p < 0.001$ *vs.* the corresponding male group; ^{g} $p < 0.05$, ^{gg} $p < 0.01$, ^{ggg} $p < 0.001$ *vs.* the corresponding NTg group; * $p < 0.05$ *vs.* the corresponding non-handled (nH) group.

3.3. Peripheral immuno-endocrine system and its modulation by neonatal handling

The corticosterone levels (ng/mL) (Fig. 2E) showed sex [S, F(1,69)= 31.255; $p < 0.001$], genotype [G, F(1,69)= 8.363; $p < 0.01$] and sex × genotype interaction [SxG, F(1,69)= 4.584; $p < 0.05$] effects. Higher levels of corticosterone were found in females and 3xTg-AD mice.

The organometry of peripheral immunoendocrine organs of both sexes of 6-month-old NTg and 3xTg-AD mice is illustrated in Fig. 3. Overweight, with a sexual dimorphism reported for 3xTg-AD mice, was also observed in the present work. Sex [S, F(1,69)= 55.661; $p < 0.001$], genotype [G, F(1,69)= 17.008; $p < 0.001$] and sex × genotype interaction [S×G, F(1,69)= 11.771; $p < 0.001$] effects were found. Also, sex-dependent differences were shown in all the relative weight of the peripheral organs [S, see statistics for each organ in the figure; $p < 0.05$]. Splenomegaly was clearly observed in male and female 3xTg-AD mice, both in the absolute [G, F(1,69)= 29.158; $p < 0.001$] and the relative [G, F(1,69)= 14.232; $p < 0.001$] weights of the spleen.

The long-lasting effects of neonatal H were selectively shown in 3xTg-AD at the endocrine level showing a Treatment x Genotype interaction effect [TxG, F(1,69)= 5.013; $p < 0.05$] with the normalization/restoration of the size of the adrenal glands in the 3xTg-AD animals.

3.4. Behavioral correlations of organometry of the peripheral immunoendocrine system and the anxious-like profile

Further analysis searched for behavioral correlations of the organometry with behavioral response to anxiogenic open field test, NMDA-induced response, and corticosterone levels, as indicated in Table 1. The results evidenced that "number of rearings in the OF" was correlated with "absolute and relative weights of the spleen" [$r < -0.315$, $p < 0.01$ and $r < -0.296$, $p < 0.01$, respectively], "absolute weight of the thymus" [$r < -0.381$, $p < 0.05$] and "absolute and relative weights of the adrenal glands" [$r < -0.504$, $p < 0.001$, and $r < -0.408$, $p < 0.01$, respectively]. The immediate (Counts Minute 1) and the total NMDA-induced response correlated with the absolute weight of the spleen [$r < 0.242$ and $r < 0.237$, $p < 0.05$, respectively) and total counts also with body weight ($r < 0.272$, $p < 0.05$). Corticosterone levels correlated with total NMDA-induced response ($r < -0.292$, $p < 0.01$). When only non-handled animals are considered, corticosterone levels correlated with the immediate NMDA-induced response ($r < -0.344$, $p < 0.05$) and the absolute and relative weight of adrenal glands ($r < 0.477$, $p < 0.01$, and $r < 0.543$, $p < 0.001$, respectively).

4. Discussion

The impairment of the neuroimmunoendocrine system and its long-lasting modulation by neonatal handling in male and female 3xTg-AD mice at 6 months of age (mimicking the onset of AD) were studied as compared to age- and sex-matched control mice. Behavioral responses were assessed under two experimental conditions: i) the direct exposure to an anxiogenic open field, and ii) the excitatory stimulation by a low NMDA dose that results in a motor activity depression effect, a behavioral paradigm for screening the functional interplay of NMDA-adenosine-dopamine systems [49] related to excitotoxicity. After that, corticosterone levels were measured, and organometry of the peripheral immunoendocrine system was analyzed as absolute and relative weights, taking into account the sexual dimorphism in weight and overweight of male 3xTg-AD mice [28,38]. Furthermore, the long-lasting modulation by neonatal handling on these neuro-immuno-endocrine measures and NMDA-evoked response was also studied.

The anxious-like behavioral response elicited by the open field was increased in the 3xTg-AD mice compared to the NTg counterparts, as shown by the reduced locomotor activity of these animals. This was in agreement with previous reports from our and other laboratories [14,28, 31–34,38]. The time-course of locomotor activity under anxiogenic conditions showed sex and genotype differences. The female sex is more prone to exhibit anxiety-like behaviors, exacerbated in 3xTgAD mice at 6 months of age, corresponding to the onset of disease with NPS-like symptoms [25,38]. Thus, an increased response in the open field was found in 3xTg-AD females compared to their sex and genotype counterparts. Neonatal handling was able to reverse this behavior, restoring activity to normal levels. It is known that stress-coping strategies can be benefited from several kinds of environmental interventions such as exercise, enrichment, and handling [44]. The exacerbation of the anxious response as part of the expression of NPS symptoms of AD has

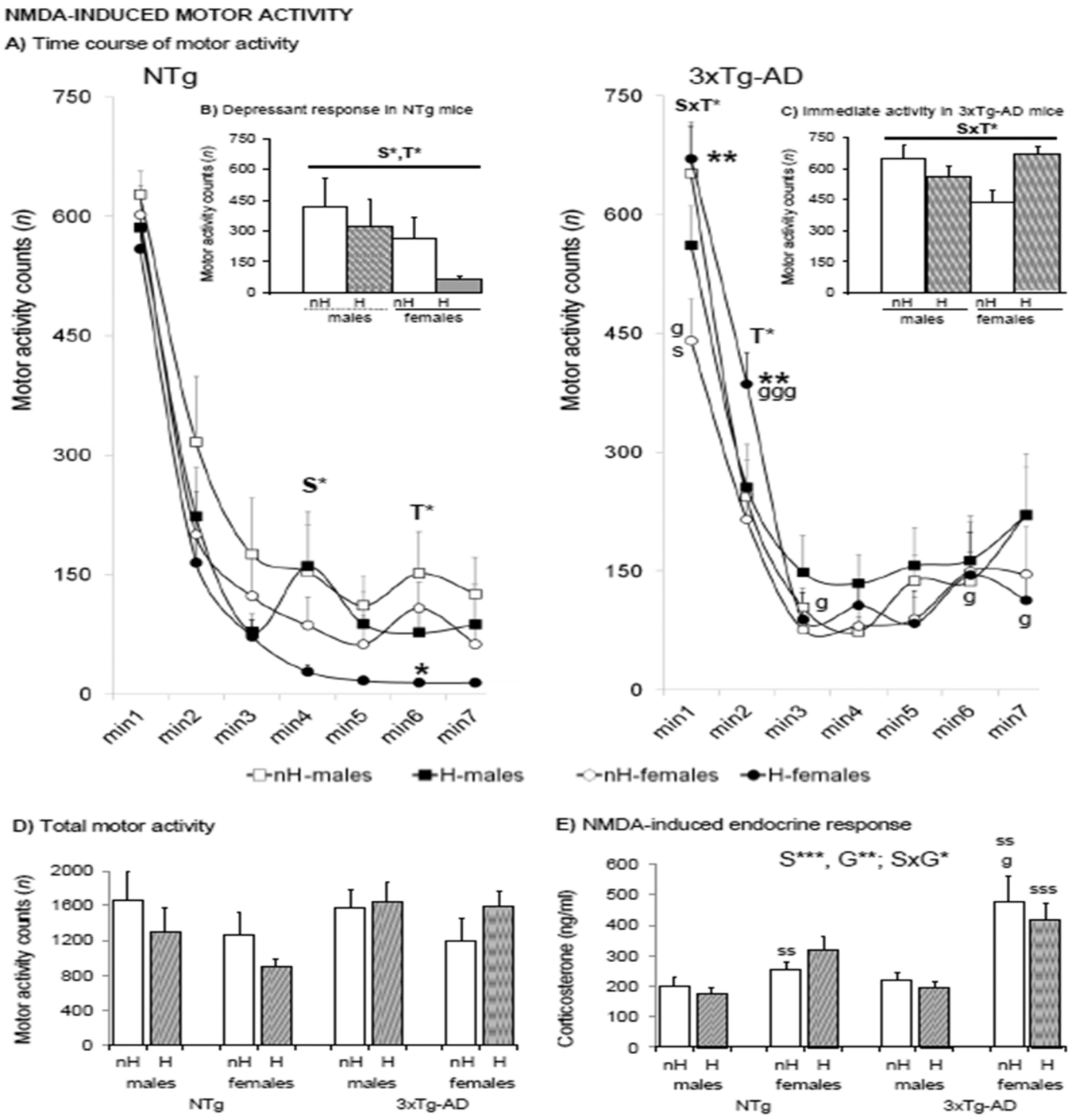

Fig. 2. NMDA (25 mg/kg i.p.)-induced motor activity, corticosterone levels, and effects of neonatal handling. Data are expressed by mean ± SEM. (A) Time course of motor activity. Left, NTg mice. Right, 3xTg-AD mice. (Inset B) The long-lasting effects of neonatal handling in NTg mice induced a depressant motor response, mostly in females. (Inset C) The long-lasting effects of neonatal handling in 3xTg-AD were shown as a reversal of the reduction of the depressant motor response, mostly shown in the immediate activity of female 3xTg-AD mice during the first minute of the test. (D)Total motor activity and (E) corticosterone levels (ng/mL). Statistics; ANOVA 2 × 2 × 2: S, Sex effect; G, genotype effect; T, Treatment effect, S×G, Sex × Genotype interaction; S×T, Gender × Treatment interaction $^{*}p < 0.05$, $^{**}p < 0.01$. *Post hoc*: $^{s}p < 0.05$, $^{ss}p < 0.01$, $^{sss}p < 0.001$ *vs.* the corresponding male group; $^{g}p < 0.05$, $^{ggg}p < 0.001$ *vs.* the corresponding NTg group; $^{*}p < 0.05$, $^{**}p < 0.01$ *vs.* the corresponding non-handled group.

also been shown to be improved by voluntary [55] but not forced [38] exercise, handling [30,31], and environmental enrichment [56].

The anxious performance was observed during the first minute of the test, where the initial freezing response is elicited, and during the subsequent intervals of exploratory behavior. In our precedent works, we demonstrated that freezing and bizarre behaviors in the open-field test are conspicuous at this adult age in both genotypes, but they result in a substantial reduction of horizontal and vertical locomotor activity in the 3xTg-AD mice [10,31,32]. In one of these studies using 6 months-old male mice, this reduced vertical activity in the OF correlated with the animal's thymus index (its relative weight *vs.* body weight) [10]. Also, among new environments with different anxiogenic conditions, the open field was the most sensitive test to show genotype and sex differences and to demonstrate, for the first time, the benefits of neonatal handling

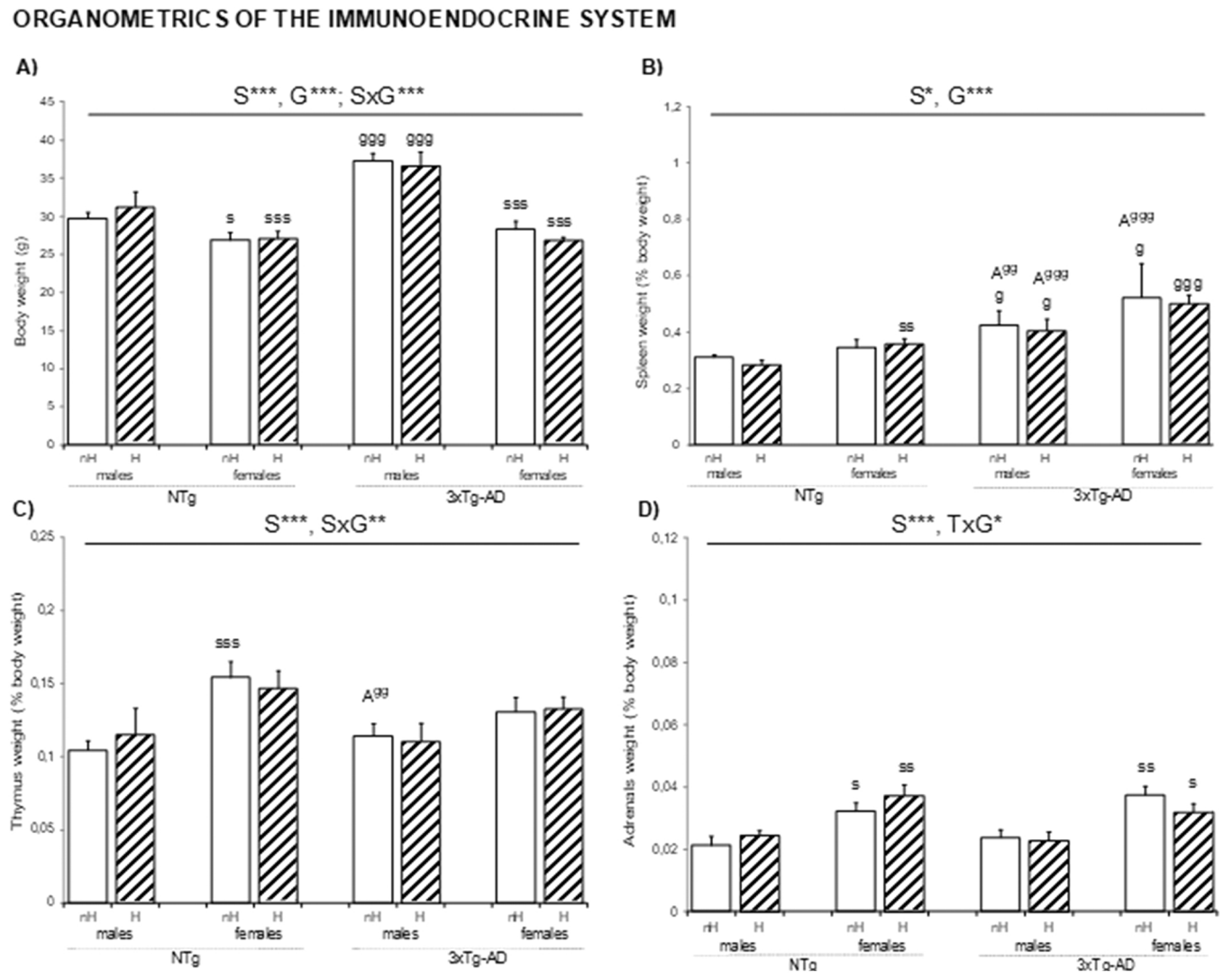

Fig. 3. Body weight, peripheral immunoendocrine system, and effects of neonatal handling. Data are expressed by mean ± SEM of each 6-months-old group ($n = 9$–11). (A) body weight, (B) spleen, (C) thymus and (D) adrenal glands relative weights. Open bars: non-handled animals, closed bars: handled animals. A: absolute weight. Statistics: ANOVA 2 × 2 × 2: S, Sex effect; G, Genotype effect; S×G, Sex × Genotype interaction; T × G, Treatment × Genotype interaction; * $p < 0.05$, * * $p < 0.01$, * ** $p < 0.001$. *Post-hoc*: ^{s} $p < 0.05$, ^{ss} $p < 0.01$, ^{sss} $p < 0.001$ *vs.* the corresponding male group; ^{g} $p < 0.05$, ^{gg} $p < 0.01$, ^{ggg} $p < 0.001$ *vs.* the corresponding NTg group.

in this animal model of AD [31]. In this regard, here we conducted a detailed analysis of the time-course of the locomotor activity in the open field and its correlates with the immunoendocrine system. In the present work using males and females, we demonstrate that both reduced horizontal and vertical activities negatively correlated with the spleen, thymus, and adrenal glands indexes, indicating a close relationship between them. In this respect, other animal models, such as PAM for premature aging, show decreased locomotor activity, increased emotionality/anxiety, immunosenescence, and decreased life span compared to their control counterparts [8]. The results also support the role of the innate immune system in the neuropsychiatric symptoms associated with AD in parallel to what is postulated for psychiatric disorders [5]. At this early stage, both ongoing central and peripheral inflammatory processes have been shown to precede the onset of clinical AD-like disease [57] and correlate with cognitive decline [36]. Other research groups have also confirmed in the 3xTg-AD mice the functional relevance of the peripheral immune system and impaired neuroimmune communication as part of the neuroinflammation process shown in AD patients, providing further evidence of sex-dependent impaired autoimmunity [58,59]. Abnormalities of plasma cytokines and spleen in old 3xTg-AD mice [60], enhanced susceptibility to acute infection with more severe comorbidity [61], and worse autoimmune adaptive response [62] have also been demonstrated. Beneficial effects of curtailing immune susceptibility in an AD model have also been proposed [63].

At low doses, the systemic administration of NMDA induces a biphasic effect on motor activity [49]. A first episode of motor depression is followed by a period of normal or stimulated motor activity. While NMDA and other excitatory amino acids are known to release endogenous adenosine from rat cortical slices [51], our microdialysis studies demonstrated that the motor depression induced by NMDA was due to its ability to release adenosine at the striatal level [52]. This increased release of adenosine-induced by NMDA is an endogenous protective mechanism to avoid NMDA-induced excitotoxic effects [53]. Similar to the nicotine-evoked locomotor response used as a behavioral paradigm for toxicity screening [64], NMDA-induced motor response is used as a behavioral paradigm to study excitotoxicity [49,50]. The lack of a control group for injection can be considered a major limitation in both paradigms. However, the effect of injection results in a motor stimulation acute effect, the opposite of the motor depression effect

Table 1
Correlations between neuro-immuno-endocrine systems. Pearson's correlations (in bold) are statistically significant: * $p < 0.05$, * * $p < 0.01$, * ** $p < 0.001$. Pearson's correlations (in brackets) represent those statistically significant values when only non-handled animals are considered. AW, absolute weight (g), RW, relative weight (% *vs.* body weight).

	Body weight (g)	Spleen (AW,g)	Spleen (RW,%)	Thymus (AW,g)	Thymus (RW,%)	Adrenal glands (AW,g)	Adrenal glands (RW,%)	Corticosterone (ng/mL)
Organometry								
Spleen (AW, g)	0.220*							
Thymus (AW, g)	(0.365*)							
Thymus (RW, %)	-0.372***							
Adrenal glands (AW, g)								(0.477**)
Adrenal glands (RW, %)	-0.421***				0.307**			(0.543***)
Open field test								
Neophobia - Crossings						-0.294**	-0.320**	
Crossings Minutes 2 to 5			-0.253*			-0.272*	-0.275*	
Crossings Minutes 3 to 5			-0.242*			-0.257*	-0.267*	
Crossings Minutes 4 to 5						-0.287**	-0.302**	
Crossings Minute 5					-0.258*	-0.299*	-0.334**	
Rearings Minutes 1 to 5		-0.315**	-0.296**	(-0.381*)		(-0.504***)	(-0.408**)	
Rearings Minutes 2 to 5		-0.314**	-0.300**			-0.307**	-0.272*	
Rearings Minutes 3 to 5		-0.292**	-0.289**			-0.292**	-0.269*	
Rearings Minutes 4 to 5		-0.236*	-0.236*	-0.233*		-0.288**	-0.255*	
Rearings Minute 5				-0.278*	-0.278*	-0.259*	-0.230*	
NMDA-induced response								
Counts Minute 1		0.242*						(-0.344*)
Total activity counts (*n*)	0.272*	0.237*						-0.292**
HPA axis								
Corticosterone (ng/ml)	-0.275*					(0.477**)	(0.543***)	

induced by NMDA [49,50,52]. In the present work, we analyzed the time-course of NMDA-induced motor activity response, and the results indicated differences in NMDA-adenosine interplay depending on sex. Thus, females showed a protective response more effective than males (higher motor depression), potentiated by handling.

Regarding the AD-transgenic phenotype, the motor depression effect was not as strong as in the NTg animals, suggesting that NMDA-adenosine-dopamine interplay in 3xTg-AD mice is not as well regulated as in their wild-type strain. Therefore, the behavioral response pattern induced by a low dose of NMDA in NTg mice depends on the sex, with females exhibiting half of the total horizontal motor activity compared to males. Moreover, handling emphasized these effects potentiating the initial motor depression in both sexes and increasing the motor activity of males during the normal activity period. On the contrary, in 3xTg-AD mice, sex- and handling-dependent differences were mostly observed in the spontaneous motor activity, whereas in the NMDA-induced response, the main factor effects pointed at an S×T interaction. Therefore, the present results suggest worse protective actions of adenosine induced by the administration of NMDA in 3xTgAD mice and that the female sex is prone to show the neuroprotective benefits of handling.

These behavioral results on the long-term effects of early-neonatal handling in male and female 3xTg-AD mice and their wild-type C57BL/6 J × 129 counterparts provide further evidence of the benefits of this sensorial (tactile and proprioceptive) stimulation [30,31,44,65,66]. These were despite the action of handling pups just for 1 min per day during PND1 to PND10 has been reported to change the maternal odor preference in rat pups [67] and also mother-pups bonding by decreasing mother-pups contact and changing maternal behavior, especially daily licking and nest-building [67]. Interestingly, the olfactory bulb, a cholinergic structure affected in AD, plays a fundamental role in the olfactory learning mechanism of the nest odor that also involves maternal behavior, becoming very important for mother-pups bonding formation [67,68]. Therefore, in the present experimental design would have been interesting to dissect the contribution of maternal separation *per se*, that is, to have a third experimental group of pups separated from their mother but not receiving sensorial stimulation. Also, maternal behavior in the handling and non-handling groups could have been interesting to record. Although they were not monitored, we have previously shown that male and female 3xTg-AD mice exhibit worse nesting behavior than their sex- and age-matched counterparts in individual, parental (a couple) [69], and also social structures [66]. Most importantly, handled animals of both sexes and genotypes are better in social nest building than non-handled mice [66]. Finally, one should also refer to 'environmental programming' in terms of the epigenetic modification of gene expressions during the first week of postnatal life [70]. Thus, early environmental regulation of forebrain and hippocampal glucocorticoid receptor gene expression has been

described, as well as its downstream effects on the offspring HPA axis, neuroendocrine and behavioral stress responsivity throughout life [70–72]. However, it can not be discarded that neonatal handling may exert a potential modification of Thy1.2 promoter activity, which would confound its effects on this 3xTg-AD model beta-amyloid and tau genetic targets.

The increased size of adrenal glands, one of the three hallmarks of stress, with females usually prone to it, was also confirmed in female NTg mice. Interestingly, here we report for the first time this sex dimorphism in the 3xTg-AD mice. Moreover, the correlation analysis indicated a strong relationship between the weight of adrenal glands and the behavioral patterns exhibited in the open-field test and the corticosterone levels measured in plasma. In this regard, dissection and weight of the adrenal cortex and medulla would provide more precise data related to their glucocorticoid and adrenaline release functions, respectively, than the total weight of the adrenal glands. Similarly, as discussed later, the levels of glucocorticoids correlated with the motor-depressant patterns exhibited in the behavioral paradigm of excitatory stimulation with NMDA. Dysregulation of the stress-responsive HPA axis is another feature of AD at the neuroendocrine level [73]. Adrenal responsivity and hypercortisolemia have been reported in moderate to severe AD and major depression [74–76]. Thus, these changes had been explained by increased cortisol levels secondary to increased adrenal sensitivity to adrenocorticotropic hormone (ACTH) due to the abnormal negative feedback function [77]. Recently, a meta-analysis has also shown that basal hypersecretion of cortisol, but not circadian dysrhythmia, is characteristic of individuals with AD [78]. The authors argue that mechanistically, the hypersecretion would agree with the theorized AD-driven deterioration of the hippocampus and subsequent reduction in HPA-axis inhibition. Interestingly, glucocorticoids increased both amyloid and tau pathology in 3xTg-AD mice [79]. However, normal serum glucocorticoid levels are found in early AD function [80], as also reproduced in young 3–4-month-old 3xTg-AD mice mimicking prodromal stages of the disease [39]. Despite these normal levels, an activated central HPA axis with altered mRNA levels of the mineralocorticoid receptor, the glucocorticoid receptor, and ACTH in several stress- and emotionality-related brain regions suggests ongoing neuroendocrine regulation precedes the onset of severe AD-like pathology and behavioral deficits [39].

In the present work in mice, the correlations found between organometry, corticosterone levels, and behavioral parameters related to anxiety-like behaviors confirm the close relationship between the neuro and the endocrine systems. As compared to previous reports from our laboratory using 6-month-old mice of these colonies of NTg and 3xTg-AD mice [38] (range: 25–50 ng/mL in males, 75–100 ng/mL in females), the plasmatic levels of corticosterone in the NMDA-induced paradigm were increased and showed sex, genotype, and interaction effects. Besides, the female sex also showed higher glucocorticoid levels than males, in agreement with a previous report [11], indicating a sexual dimorphism exacerbated by the excitotoxic challenge in the 3xTg-AD genotype. Similarly, handling potentiated these sex differences in NTg animals. The size of adrenal glands showed sex- and genotype patterns that, in the case of non-handled animals, mirrored those of corticosterone, as shown by the strong correlations with absolute and relative adrenal glands weight, not found in handled animals. The enhancement of plasmatic glucocorticoid levels in female 3xTg-AD mice suggests that the HPA axis activity is enhanced in female 3xTgAD mice under the same NMDA excitotoxic stimuli. This sex-dependent hyperactivity of the HPA axis agrees with previous reports in old 3xTg-AD [9] and TgCRN8mice [81], where elevated corticosterone metabolite levels correlated with a worse ability to cope with stressors [82]. Interestingly, at 6 months of age, we described that intra-neuronal ß-amyloid accumulation in the glutamatergic pyramidal neurons of the 3xTg-AD mice basolateral amygdala is involved in the amygdala-dependent emotional responses [27]. Previous reports on handled animals have shown they are characterized by lower stress-induced anxiety; however, handled and control animals did not differ in their general way of coping with stressors [83]. Sex differences in the effects of neonatal handling on the animal's response to stress and the vulnerability to depressive behaviors were also reported [84], as well as the modulatory effect of neonatal handling and sex in brain monoamines and plasma corticosterone levels following repeated stressors in adulthood [85].

However, a part of the impact of the HPA axis hyperactivity/glucocorticoid cascade on the hippocampus and amygdala [86], the changes in NMDA-induced motor depression are a direct behavioral output of striatal release of adenosine in front of NMDA-induced excitotoxic insult, as shown with microdialysis [52]. Besides the increases in the hypothalamic-pituitary-adrenal (HPA) axis activity [87], the role of stressful stimuli in the pathophysiology of the dopaminergic system [88] increasing extracellular DA concentrations in mesolimbic, mesostriatal, and mesocortical dopaminergic pathways in psychogenetically selected animals that differ in coping strategies to aversive conditions has been demonstrated [89]. On the other hand, increases in dopamine (DA) content and reduced striatal D2 receptors in the striatum of AD patients have been related to disruptive behaviors [90].

In the present work, despite the benefits at the behavioral level, neonatal handling could not modulate the weight of organs but could even exert opposite effects depending on the genotype. These interaction effects of early life events found in the weight of adrenal glands agree with its known effects on the HPA axis and are similar to those we have reported in this animal model with forced exercise also at 6 months of age [38]. Similarly, other authors and we have shown that other environmental strategies, such as environmental enrichment, where improvement of the functional activity of the peripheral immune system has been demonstrated, were not able to modify the biometric parameter [91,92].

In conclusion, the present results confirm the co-existence of a sex-dependent behavioral-immuno-endocrine impairment in 3xTg-AD mice. The anxious behavior in the open field exhibited by mice with normal aging and its increased manifestation as early NPS-like symptoms exhibited by 6-month-old male and female 3xTg-AD correlated with changes in the organometry of peripheral immunoendocrine organs. The involution of thymus, splenomegaly, and hypertrophy of adrenal glands were recorded as indirect indicators of the immunological and endocrine status of the 3xTg-AD mice. The results in male and female 3xTg-AD mice suggest that at the onset of the disease, the weight of adrenal glands was the most sensitive to exhibit sex- and genotype-dependent neuroimmunoendocrine impairment and the long-lasting modulatory effects of early-life stimulation. The present behavioral results suggest not only an impaired neuroimmunoendocrine network, with worse coping with stress strategies, HPA axis glucocorticoid response but also sex- and genotype-dependent NMDA excitatory-evoked vulnerability/neuroprotection responses mediated by adenosine. Finally, similarly to the methods of visualization in anthropometry and computer organometry useful in the diagnostics of metabolic syndrome [93] and assessing liver and spleen parameters among subjects with Schistosoma infection [94,95], the present results in male and female 3xTg-AD mice at early stages of AD suggest that changes in the organometry of the peripheral immunoendocrine system (involution of the thymus, splenomegaly, and hypertrophy of adrenal glands) could be putative early biomarkers of its impairment easily to be monitored by the implementation of ultrasonographic examinations in the population at risk.

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Institutional review board statement

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee of Department de Medi Ambient i Habitatge, Generalitat de Catalunya.

CRediT authorship contribution statement

Raquel Baeta-Corral: Methodology, Data curation, Software, Writing – original draft, Analysis and statistics, Scientific discussion. **Lydia Giménez-Llort:** Conceptualization, Methodology, Software, Supervision, Funding acquisition, Writing – review & editing, Behavioral performance, Analysis and statistics. **Monica de la Fuente:** Writing – review & editing. . All authors have read and agreed to the published version of the manuscript.

Data Availability

Data will be made available on request.

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Conflicts of interest

The authors declare no conflict of interest. The funders had no role in the study's design, in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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