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Age- and sex-dependent development of adrenocortical hyperactivity in a transgenic mouse model of Alzheimer's disease

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Abstract

In this study, we investigated mice of the TgCRND8 line, an APP transgenic mouse model of Alzheimer's disease (AD), with respect to behavioral, endocrinological, and neuropathological parameters. Our results show that transgenic and wild-type mice did not differ in their general health status, exploratory and anxiety related behavior as well as in the activity of their sympathetic-adrenomedullary system. Significant differences, however, were found regarding body weight, amyloid plaque formation, and the activity of the hypothalamic-pituitary-adrenocortical (HPA) axis. Continuous monitoring of glucocorticoid (GC) concentrations over a period of 120 days, utilizing a noninvasive technique to measure corticosterone metabolites in fecal samples, revealed that transgenic animals showed adrenocortical hyperactivity, starting very early in males (from day 30) and later in females (around day 90). It is hypothesized that these changes in the activity of the HPA axis are linked to amyloid-β associated pathological alterations in the hippocampus, causing degenerations in the negative feedback regulation of the HPA axis leading to hypersecretion of GC. Thus, the development of adrenocortical hyperactivity might be a key-element in the understanding of AD.

Keywords: Alzheimer's disease; Amyloid-β; Transgenic mouse model TgCRND8; Health check; Explorative and anxiety related behavior; HPA axis; SAM system; Adrenal tyrosine hydroxylase activity; Plasma corticosterone; Fecal corticosterone metabolites; Adrenocortical hyperactivity; Age- and sex-specific effects

1. Introduction

Alzheimer's disease (AD) is the most prevalent neurodegenerative disorder in humans worldwide and has become generally accepted as the most common basis for senile dementia [7,75,86]. It is characterized by progressive severe cognitive, language and behavioral impairment associated with a dramatic loss of most cortical and subcortical functions ultimately leading to death. Distinct neuropathological features are proposed to cause synaptic dysfunction, neuritic dystrophy and neuronal degeneration observed in certain brain regions including cortex, hippocampus, amygdala, anterior thalamus and basal forebrain of Alzheimer patients [2,35,58,75,86,87,104]. Two pathological hallmarks of AD are (i) the accumulation of insoluble extracellular protein aggregates (senile plaques), consisting mainly of amyloid-β (Aβ) peptide derived from amyloid-precursor protein (APP), and (ii) intracellular deposits of paired helical filaments composed of hyperphosphorylated tau protein (neurofibrillary tangles) [7,27,75,86,101]. Although two forms of AD can be distinguished ('sporadic' and 'familial' form, differing in age at onset of the disease), the underlying mechanisms appear in most regards to be identical [7,75,86]. Besides other factors like Down's syndrome (trisomy 21), age and missense mutations in specific genes encoding for APP, presenilin (PS) 1 and 2 or apolipoprotein E (ApoE) have been identified as major risk factors associated with AD [7,23,27,75,86]. Since the world population ages and the percentage of individuals affected by AD increases rapidly with age (from about 5–10% in persons older than 65 years to nearly 50% in individuals older than 85 years), there is an increasing demand for successful

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therapeutic treatment [7,27,86,101]. However, at present AD can only be treated symptomatically, but neither be cured nor prevented [7,27,86].

To investigate the pathogenesis and possible treatment of AD, transgenic mouse models expressing normal or mutated APP and/or PS are now increasingly used [34,38,75,84,106]. However, valid animal models should exhibit as many symptoms of AD as possible. Therefore, a 'good' mouse model of AD should develop neuropathological as well as behavioral AD-like alterations in an age-dependent manner and should encompass other features observed in Alzheimer patients like changes in the activity of the hypothalamic–pituitary–adrenocortical (HPA) axis [11,28,33,52,63,67,70,71,73,92]. The HPA axis is one of the organism's major stress axes controlling the production and secretion of glucocorticoids (mainly cortisol in humans and corticosterone in mice) from the adrenal gland [3,31,100]. Additionally, sex-specific effects with females being more prone to AD than males have been described in various studies [41,85,86,93,98] and should therefore be considered in animal models, too. In most studies investigating mouse models of AD, however, age and sex are not taken into account as variables, although recent studies strongly indicate distinct effects on behavioral tests (e.g. progressive impairments in various cognitive tests, disturbed activity patterns in the home cages, or increased locomotor activity in the open field test [42,45,99,103]), numbers of microglia and astrocytes in the brain [59], as well as effects on Aβ production and metabolism [6,98,109]. These effects are probably mediated by changes in concentrations of endogenous hormones like estrogens that have also been shown to be a crucial factor bringing about sex-specific differences in human Alzheimer patients leading to the higher prevalence of AD in women [14,20,29,37,55,65,83,85,93,95,98,107].

In this study, we used a double mutant (Swedish and Indiana) human APP_{695} model of AD (TgCRND8), generated by Westaway and colleagues [8]. Transgenic mice of this line show Aβ depositions (developing into dense core plaques) and neuritic pathology in several brain regions including cortex and hippocampus already at the age of three months. Additionally, plaque formation is accompanied by impaired acquisition and learning reversal in the Morris water-maze task proving AD-like cognitive deficits in these animals [8,39]. Notably, Aβ peptide immunization reduced plaque formation as well as cognitive deficits in this model [39]. Thus, this transgenic mouse line offers the potential to investigate aspects of AD pathogenesis, prophylaxis, and therapy within short time frames [8,39].

However, besides aspects of learning and memory, other behavioral alterations or endocrine changes like glucocorticoid hypersecretion, as observed in Alzheimer patients, have only been addressed in a few murine models of AD so far [68,77,78,79]. Therefore, the aim of our study was twofold: (i) to test mice of the TgCRND8 line with respect to exploratory and anxiety related behavior and (ii) to investigate if endocrine parameters like activity of the HPA axis and the sympathetic-adrenomedullary (SAM) system are altered in these mice.

In a first set of experiments, all transgenic mice as well as their wild-type littermates underwent a general health check to ensure that behavioral findings were not compromised by deteriorated physical conditions of the animals. Subsequently, they were tested for differences in spontaneous exploration, locomotor activity, and anxiety (barrier test, open-field test, and elevated plus-maze test) to reveal possible impacts of the transgene on the animals' behavior.

In a second approach, transgenic and wild-type mice were investigated regarding their body weight development, amyloid plaque formation and the activity of their stress axes (HPA and SAM). Additionally, possible sex-specific effects were considered and, for the first time, the activity of the HPA axis was monitored continuously in individual animals during the course of the developing disease. This was achieved by utilizing a recently established noninvasive technique to measure corticosterone metabolites in fecal samples of mice [96,97].

2. Methods

2.1. Animals and general housing conditions

To investigate behavioral, endocrine, and neuropathological aspects in a murine model of Alzheimer's disease (AD), we used transgenic mice of the TgCRND8 line generated by David Westaway and colleagues [8,39]. These mice express a double mutant form of the human amyloid precursor protein (APP) 695 transgene (K670N/M671L and V717F = 'Swedish' and 'Indiana' mutation) under regulation of the Syrian hamster prion promoter (PrP) on a hybrid C3H/HeJ-C57BL/6 strain background. The animals used derived from our local stock (founder animals were obtained from the 'Centre for Research in Neurodegenerative Diseases, University of Toronto') and were bred in pairs consisting of a wild-type female and a transgenic male. To distinguish between transgenic (hemizygotic, APP+/−) and wild-type animals (APP−/−), genomic DNA was extracted from tail tissue (sampling was performed on day 21 ± 1 of life) and a DNA fragment of 1kb within the PrP gene promoter controlling the βAPP_{695} cassette was identified after PCR amplification (this gene is absent in wild-type mice). Details about housing conditions and the number of mice used in each experiment are given in the respective section (see Sections 2.2–2.4). Generally, all animals were housed in transparent polycarbonate cages (standard Macrolon cages type III, 38 cm × 22 cm × 15 cm) with sawdust as bedding material (Allspan, Höveler GmbH & Co. KG, Langenfeld, Germany). The animal housing room was maintained under standard laboratory conditions (light–dark cycle: 12:12 h, lights on at 7 a.m.; temperature: $21 \pm 1\,^{\circ}C$; relative humidity: $50 \pm 10\%$). Commercial mouse

diet (Altromin no. 1324, Altromin GmbH, Lage, Germany) and bottled tap water were available ad libitum.

The presented work complies with current regulations covering animal experimentation in Germany and the EU (European Communities Council Directive 86/609/EEC). All experiments were announced to the competent local authority and were approved by the 'Animal Welfare Officer' of the University of Muenster.

2.2. Behavioral investigations

2.2.1. Housing conditions and general health check

In total, 19 mice were used for the behavioral investigations (11 APP +/− mice: four males and seven females; eight APP−/− mice: three males and five females). After weaning at 21 ± 1 days of age, the animals were housed in same sex groups of two to four littermates in standard cages as described above. At day 50 ± 1 a general health check was performed with all transgenic and wild-type mice to ensure that behavioral findings were not the result of deteriorated physical conditions of the animals. Health and neurological status were assessed using a 10-minute protocol including several tests as described in standard checklists [15,80]. The animals were inspected for physical appearance and underwent neurological testing including acoustic startle, visual placing, grip strength, and reflex functions.

2.2.2. Tests for exploration and anxiety

Since cognitive deficits of APP +/− mice in a spatial learning task (Morris water-maze) were already proven from eleven weeks of age on [8,39], we focused on three tests of exploratory and anxiety related behavior: the barrier test, the open-field test, and the elevated plus-maze test.

2.2.2.1. Barrier test. Spontaneous exploratory behavior was measured at 90 ± 1 days of age by means of the barrier test [76]. A standard cage (38 cm × 22 cm × 15 cm) was divided by a perspex barrier (3 cm high) into two equal compartments. At the beginning of a test, the mice were placed in one of the compartments (according to a pseudo-random schedule) and the latency was measured until either the mice climbed over the barrier (all four paws in the other compartment) or a maximum time of five minutes elapsed without the mice climbing over the barrier.

2.2.2.2. Open-field test. In the open-field test, mice have the opportunity to explore a square-shaped arena for a fixed amount of time [1,102]. The open-field we used was a moderately lit (75 lx) square arena of 80 cm × 80 cm with walls of 40 cm height. On day 91 ± 1 each mouse was given one test session of ten minutes during which the animals' locomotor behavior was measured using an automated tracking system (a video camera connected to a personal computer with a tracking software, see http://www.phenotyping.com/digital.html).

2.2.2.3. Elevated plus-maze test. Anxiety related behavior was measured at day 92 ± 1 by means of the elevated plus-maze when mice have the choice to move in opposing arms, which are either shielded or open [51,69]. Preference for open arms is thought to reflect exploration, and preference for shielded arms is thought to indicate anxiety. The maze was elevated 50 cm above the floor and had arms 30 cm long and 5 cm wide. The maze was lit (75 lx) by a bulb suspended above its center. At the beginning of a trial, the mice were placed into the center of the maze facing randomly one of the arms. Each entry into an open or closed arm was counted and the time animals spent in either type of arm was measured for ten minutes utilizing the automated tracking system mentioned above (due to technical problems, the test sessions of two transgenic females had to be excluded from the analysis).

2.3. Endocrinological investigations

2.3.1. Housing conditions and sample collection

To monitor endocrine patterns over the course of the developing disease, ten mice of each sex and each genotype were investigated for 120 days (i.e. in total, 40 mice were used for the endocrinological investigations). From weaning on day 21 ± 1 the animals were housed individually to avoid socially induced effects on their endocrine system (e.g. dominance relationships are known to affect stress and reproductive hormone concentrations [10,13,25,88]). In intervals of 15 ± 1 days (i.e. on days 30, 45, 60, 75, 90, 105, and 120) the animals were weighed and placed for 24 h in a cage identical to their home cage containing fresh bedding material (the males' behaviour was also videotaped during this period). All feces voided during these 24 h periods (starting at 9 a.m.) were collected and frozen at −30 °C until assayed for corticosterone metabolites (see Section 2.3.2) (corticosterone is the major glucocorticoid in mice [91]).

Three days after the last fecal sampling (i.e. day 123 ± 1 of life) the animals were decapitated at 9 a.m. and trunk blood (about 0.2 ml) was collected in heparinized capillaries to determine circulating corticosterone concentrations. After separation of cellular constituents by centrifugation (five minutes at 14,800 × *g*), plasma was frozen at −30 °C until analysis. To avoid stress effects of the handling procedure on the investigated endocrine parameters, blood sampling was performed in a separate room adjacent to the animal housing facility and within a maximum time of three minutes from disturbing the animal's cage [4,18,30,91].

Additionally to the blood sampling, both adrenals were dissected immediately after decapitation (the dissection was completed within five minutes), cleaned from fat and adherent tissue and snap-frozen on dry ice in 5 mM Tris–HCl buffer (pH 7.2). Until assayed for tyrosine hydroxylase activity (see Section 2.3.2) the adrenals were stored at −70 °C.

Since five mice (two transgenic males, two transgenic females, and one wild-type female) died before reaching the age of 120 days, the sample size was reduced to eight for

both sexes in the transgenic groups and nine in the wild-type female group.

2.3.2. Hormone analysis

To monitor the activity of the hypothalamic-pituitary-adrenocortical (HPA) axis, the collected fecal samples were analyzed for immunoreactive corticosterone metabolites (CM) using a recently established 5α-pregnane-3β,11β,21-triol-20-one enzyme-immunoassay (EIA). Details regarding development, biochemical characteristics, and biological validation of this assay are described by Touma et al. [96,97]. Before EIA analysis the fecal samples were homogenized and aliquots of 0.05 g were extracted with 1 ml of 80% methanol. For a detailed description of assay performance see [96]. Briefly, the EIA used a double-antibody technique and was performed on anti-rabbit-IgG-coated microtiter plates. After overnight incubation (at 4 °C) of standards (range: 0.8–200 pg/well) and samples (in duplicate) with steroid antibody and biotinylated label, the plates were emptied, washed and blotted dry, before a streptavidin horseradish peroxidase conjugate was added. After 45 min incubation, plates were emptied, washed, and blotted dry. The substrate (tetramethylbenzidine) was added and incubated for another 45 min at 4 °C before the enzymatic reaction was stopped with 2 mol/l sulphuric acid. Then, the optical density (at 450 nm) was recorded with an automatic plate reader and the hormone concentrations were calculated. The intra- and inter-assay coefficients of variation were 9.1 and 14.0%, respectively.

The blood samples were also analyzed by EIA to determine circulating corticosterone concentrations. Specifications of the corticosterone EIA used, are described by Palme and colleagues [66]. Assay performance was in principle similar to the EIA described above. However, the standard curve of the corticosterone EIA ranged from 2–500 pg per well and the plasma samples (0.02 ml) were extracted with diethyl ether (5 ml) before EIA analysis. The intra- and inter-assay coefficients of variation were 10.0 and 13.4%, respectively.

As an indicator of the activity of the sympathetic-adrenomedullary (SAM) system [13,81,100] the dissected adrenals were analysed for tyrosine hydroxylase (TH) activity using a radio-enzymatic technique as described by [60,105], with slight modifications. Briefly, adrenals were thawed on ice and homogenized in 0.15 ml 5 mM Tris–HCl buffer (pH 7.2). After centrifugation (30 min at 20,000 × *g* and 4 °C), TH activity was determined in triplicate from the supernatant. Aliquots were incubated (for 30 min at 37 °C) with ^{14}C-labelled tyrosine (^{14}C-Tyr) as substrate, before stopping the enzymatic reaction by adding 10% trichloroacetic acid and Dopa-carrier solution. Precipitated protein was eliminated by centrifugation and subsequently, ^{14}C-Tyr and ^{14}C-Dopa were separated by column-chromatography. Columns of equilibrated Al_2O_3 slurry (in Pasteur pipettes closed with glass wool) were used and washed successively with NH_4CH_3COOH buffer (pH 6.1), demineralized water, and acetic acid before eluting ^{14}C-Dopa with HCl–MeOH (1:1 mixture). The amount of recovered ^{14}C-Dopa was then quantified by liquid scintillation counting.

2.4. Neuropathological investigations

2.4.1. Brain dissection and processing

The morphological investigations were performed on the same individuals used to investigate the endocrinological parameters mentioned in the previous section (see Section 2.3). Immediately after decapitation, brains were removed and bisected in the mid-sagittal plane. One hemisphere was fixed in 4% buffered formaldehyde for two days followed by paraffin-embedding, whereas the other half was snap-frozen and stored at −80 °C.

2.4.2. Histology and immunohistochemistry

Sagittal brain sections of 2 μm were cut using a microtome. Two serial sections (immediately lateral from the mid-sagittal fissure) and two further ones at a distance of 140 μm from the first sections were affixed to Dako ChemMateTM Capillary Gap Microscope Slides. All samples were pretreated with formic acid and automatically stained in a TechMate Instrument (DAKO) with 6F/3D anti-Aβ monoclonal antibody to residues 8–17 (Dako, antibody dilution 1:100). Counterstaining was performed with hematoxylin. For further steps, the Dako StreptABC complex-horseradish peroxidase conjugated “Duet” anti mouse/rabbit antibody kit was used and development was done with 3,3′-diaminobenzidine (DAB) as chromogen. The whole staining procedure was performed simultaneously for all sections.

2.4.3. Determination of amyloid plaques

To quantify amyloid deposition, plaques were counted in four different brain regions (cortex, corpus callosum, hippocampus and basal ganglia) of all four sections. Total plaque burden was calculated by addition of the sixteen single values obtained for each animal.

Since Ingram and co-workers showed that glia inclusions, present in normal brain aging, might be mistaken for amyloid plaques [40], the brains of two APP −/− animals were also immunostained for Aβ in order to achieve a baseline of non-specific staining. As expected, no plaques or plaque-like structures were observed in the brains of all wild-type mice investigated.

2.5. Statistical analysis

Due to the relatively small sample size and since parts of the obtained data were not normally distributed, analyses were performed using non-parametric statistical tests [90]. All tests were calculated with the software package SPSS (version 11.0.1). ANOVA on ranks was used to evaluate differences between more than two related samples. Two independent samples were compared using the Mann–Whitney *U*-test (two-tailed), while the Spearman’s

rank-order correlation was calculated to elucidate the degree of association between two variables. In all tests, differences were considered significant if their probability of occurring by chance was less than 5%.

3. Results

3.1. Behavioral investigations

3.1.1. Health check and behavioral tests

The assessment of the general health state, gross sensory functions, reflexes, and motor abilities did not reveal any significant difference between APP +/− and APP −/− mice. All animals passed the health check, assuring that the behavioral tests were not compromised by non-behavioral parameters.

The performed tests for exploratory and anxiety related behavior revealed no significant differences between APP +/− and APP −/− mice (see Table 1). The latency to climb over the barrier in the barrier test, as a measure of spontaneous exploration, did not differ significantly between the two genotypes. Additionally, transgenic and wild-type mice showed a similar amount of locomotor activity measured as total ambulation in the open-field test. Furthermore, the proportion of time spent on the open arms in the elevated plus-maze test, serving as a measure of anxiety, revealed no difference between the two groups.

3.2. Endocrinological investigations

3.2.1. Fecal corticosterone metabolites (CM) and body weight (BW)

Significant differences between APP +/− and APP −/− mice were found regarding the time course of CM concentration measured in the feces (see Fig. 1). In the males, APP +/− mice had significantly higher CM concentrations than APP −/− mice from day 45 onwards (see Fig. 1a). Fecal CM levels in transgenic males were about four times higher than in wild-type males (overall medians, APP +/− males: about 125 ng/0.05 g; APP −/− males: about 32 ng/0.05 g). On average, this difference was also visible at day 30, although at this time the P-value did not reach statistical significance (see Fig. 1a). In the females, a different picture emerged (see Fig. 1b). Here, transgenic and wild-type animals did not differ in their concentrations of excreted CM at days 30, 45, 60, and 75. From day 90 on, however, increased CM concentrations were observed in APP +/− females compared to APP −/− females, reaching significance at day 120 (at days 90 and 105 P-values missed significance levels marginally, see Fig. 1b). Females of both genotypes also showed higher CM concentrations than the males (APP +/− males versus females: Mann–Whitney U-test, $n_{\text{males}} = 8$, $n_{\text{females}} = 8$, sample point at $T = 120$, $U = 9$, $P < 0.05$; APP −/− males versus females: Mann–Whitney U-test, $n_{\text{males}} = 10$, $n_{\text{females}} = 9$, sample points at $T = 45$, 60, 75, 90, 105, and 120, $U = 0$–11, $0.001 < P < 0.01$; see Fig. 1a and b).

Regarding the development of BW, significant variations were observed for mice of both genotypes and sexes (males: ANOVA on ranks, APP +/− $n = 8$, $\chi^2_r = 28.2$, df = 6, $P < 0.001$, APP −/− $n = 10$, $\chi^2_r = 56.5$, df = 6, $P < 0.001$, see Fig. 2a; females: ANOVA on ranks, APP +/− $n = 8$, $\chi^2_r = 33.7$, df = 6, $P < 0.001$, APP −/− $n = 9$, $\chi^2_r = 53.6$, df = 6, $P < 0.001$, see Fig. 2b). BW increased continuously in APP +/− and APP −/− mice. However, at all sample points wild-type mice were significantly heavier than transgenic animals (see Fig. 2a and b). At 120 days of age, wild-type males and females reached a BW of about 33 and 29 g, respectively, while males and females of the transgenic group weighed only about 19 g.

3.2.2. Plasma corticosterone and adrenal tyrosine hydroxylase (TH) activity

Increased glucocorticoid concentrations were also found in the blood samples of APP +/− compared to APP −/− animals (see Fig. 3). At day 123 transgenic males had significantly higher plasma corticosterone concentrations than wild-type males (medians: APP +/− = 55.4 nmol/l, APP −/− = 20.5 nmol/l, see Fig. 3). In the females, the same trend was observed (medians: *APP* +/− = 321.2 nmol/l, *APP* −/− = 120.9 nmol/l), but the P-value missed statistical significance marginally (see Fig. 3). Comparisons between the sexes also revealed significant differences (APP +/− males versus females: Mann–Whitney U-test, $n_{\text{males}} = 8$, $n_{\text{females}} = 8$, $U = 13$, $P < 0.05$; APP −/− males versus

Table 1
Data obtained in three behavioral tests for exploratory and anxiety related behavior

Behavioral test	Parameter measured	Medians and ranges		Statistics Mann–Whitney U-test
		APP +/−	APP −/−	
Barrier test	Latency (s)	300	124.5	$U = 27$, $P = 0.152$
		41–300	18–300	$n_{\text{APP}+/-} = 11$, $n_{\text{APP}-/-} = 8$
Open-field test	Path length (m)	52.8	45.3	$U = 36$, $P = 0.545$
		28.9–85.6	41.1–71.6	$n_{\text{APP}+/-} = 11$, $n_{\text{APP}-/-} = 8$
Elevated plus-maze test	Proportion of time spent on open arms (%)[a]	4.2	0.4	$U = 23$, $P = 0.229$
		0–12.7	0–29.8	$n_{\text{APP}+/-} = 9$, $n_{\text{APP}-/-} = 8$

[a] Time spent on open arms × 100/(time spent on open arms + time spent on closed arms).

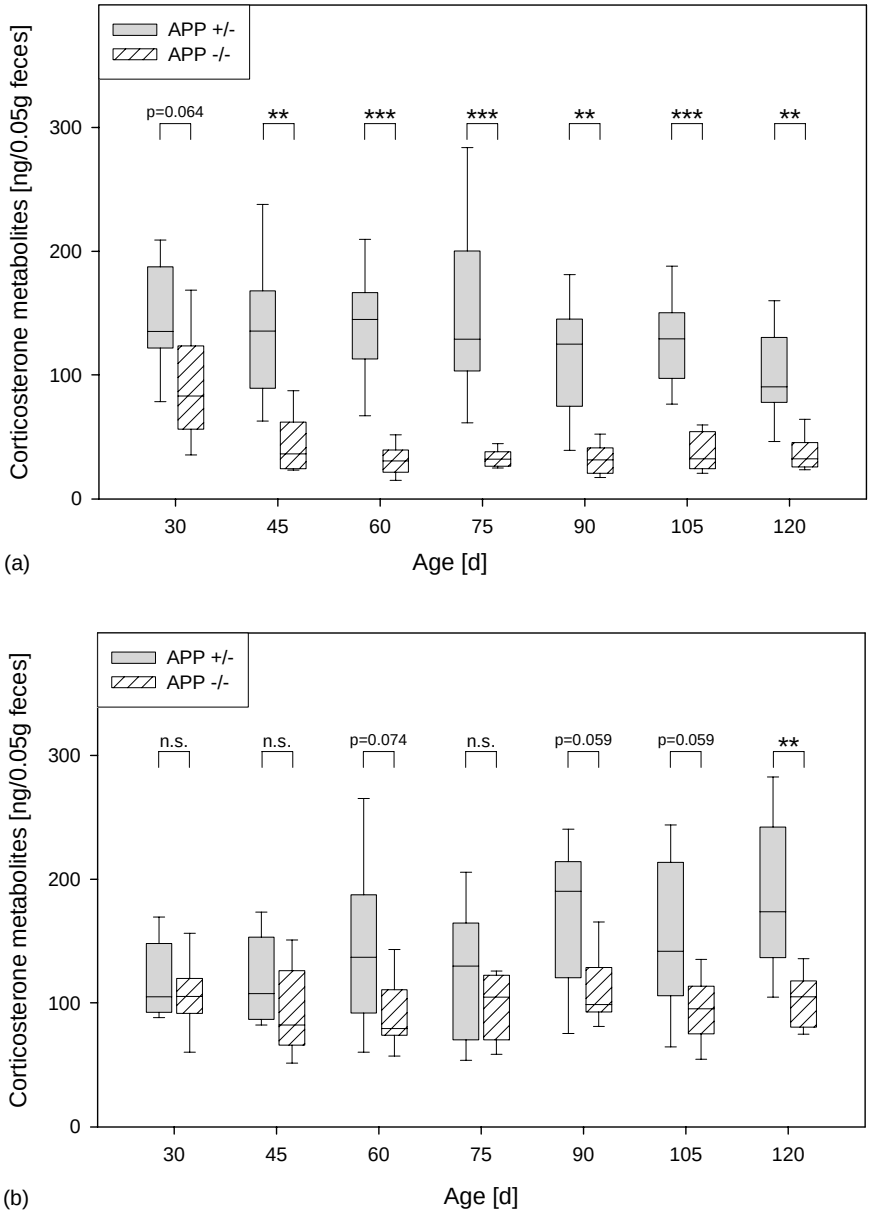

Fig. 1. Time course of corticosterone metabolite concentrations measured in the feces of male (a) and female (b) mice of the TgCRND8 line (transgenic animals: APP +/−, wild-type animals: APP −/−). Data are given as box-whisker plots showing medians (lines in the boxes), 25% and 75% percentiles (boxes) and 10 and 90% ranges (whiskers) (statistics: Mann–Whitney *U*-test (two-tailed), males: $n_{\mathrm{APP}+/-} = 8$, $n_{\mathrm{APP}-/-} = 10$, $U_{30} = 19$, $U_{45} = 5$, $U_{60} = 1$, $U_{75} = 2$, $U_{90} = 6$, $U_{105} = 0$, $U_{120} = 4$, $^{**}P < 0.01$, $^{***}P < 0.001$; females: $n_{\mathrm{APP}+/-} = 8$, $n_{\mathrm{APP}-/-} = 9$, $U_{30} = 32$, $U_{45} = 22$, $U_{60} = 17$, $U_{75} = 26$, $U_{90} = 16$, $U_{105} = 16$, $U_{120} = 7$, n.s. $p \gg 0.05$, $^{**}P < 0.01$).

females: Mann–Whitney *U*-test, $n_{\mathrm{males}} = 10$, $n_{\mathrm{females}} = 9$, $U = 16$, $P < 0.05$), with females showing clearly higher corticosterone levels than males (see Fig. 3).

In contrast to the differences found between transgenic and wild-type mice regarding the activity of the hypothalamus–pituitary–adrenocortical system, the activity of the sympathetic-adrenomedullary (SAM) system does not seem to differ between APP +/− and APP −/− animals. The measured adrenal TH activities, used as an indicator of the SAM activity, were similar for both groups in males and females (see Fig. 4).

3.3. Neuropathological investigations

3.3.1. Number and localization of amyloid plaques (AP)

At day 123 a large number of AP was detected in the brains of all APP +/− mice irrespective of sex. The majority of AP was localized in the cortex, but considerable amounts (range: 2–30 AP) were also observed in the corpus callosum, the hippocampus and the basal ganglia (see Fig. 5). Regarding the number of AP in the brains of male and female APP +/− mice, no significant differences were found (see Fig. 5). Total plaque burden was similar for both sexes (APP +/−

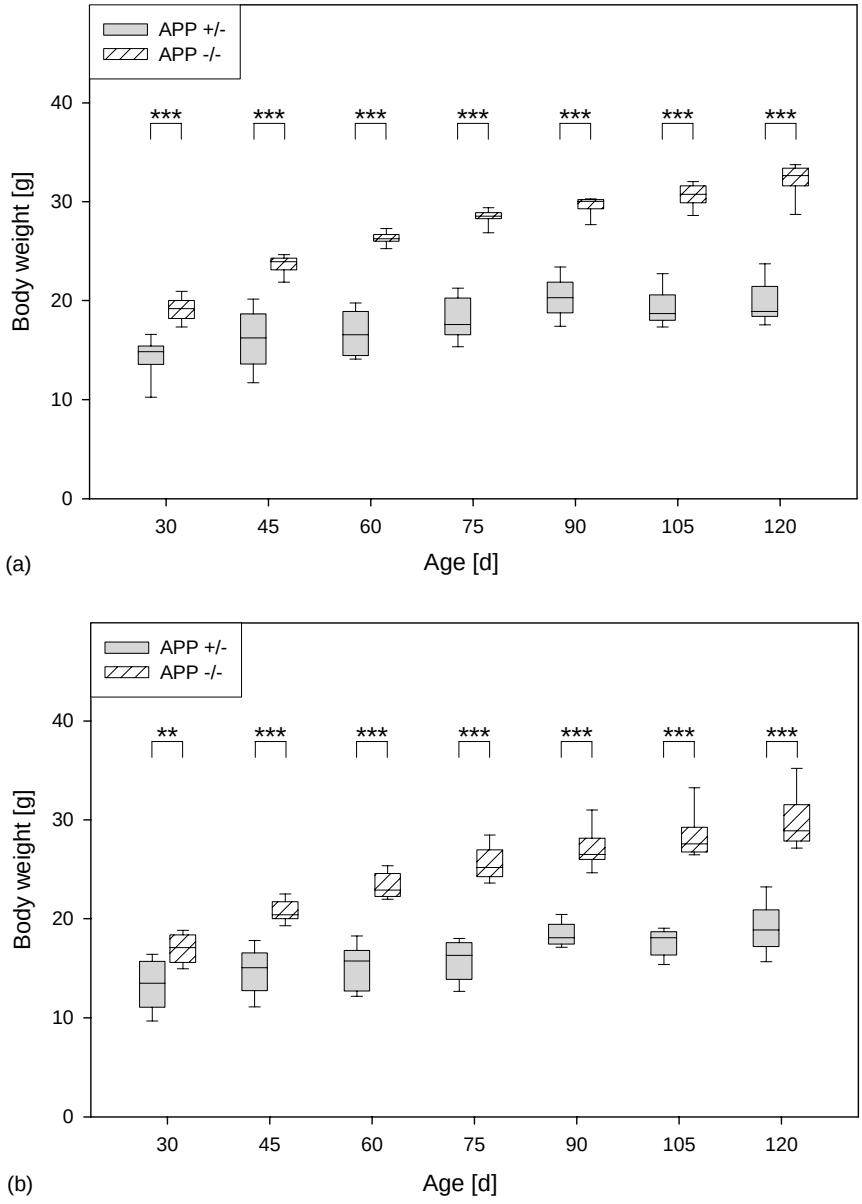

Fig. 2. Development of body weight in male (a) and female (b) mice of the TgCRND8 line (transgenic animals: APP +/−, wild-type animals: APP −/−). Data are given as box-whisker plots (for a description, see legend of Fig. 1) (statistics: Mann–Whitney *U*-test (two-tailed), males: $n_{APP+/-} = 8$, $n_{APP-/-} = 10$, all $U < 1$, $^{***}P < 0.001$; females: $n_{APP+/-} = 8$, $n_{APP-/-} = 9$, all $U < 1$, $^{**}P < 0.01$, $^{***}P < 0.001$).

males median = 129, APP +/− females median = 132.5, Mann–Whitney *U*-test, $n_{males} = 8$, $n_{females} = 8$, $U = 31$, $P \gg 0.05$) and also the distribution of AP did not differ in the various brain regions (see Fig. 5).

3.3.2. Correlation between number of AP and endocrine parameters

In males and females, the total number of AP detected at day 123, as well as the number of plaques observed in the four different brain regions, did not correlate significantly either with the matched plasma corticosterone concentrations, adrenal TH activity or with the CM concentrations measured in feces at all time points (Spearman's rank-order correlation, males: $r_s = -0.671$–0.595, all $P \gg 0.05$; females: $r_s = -0.667$–0.643, all $P \gg 0.05$).

4. Discussion

The results of the first part of our study revealed no significant differences between transgenic and wild-type mice regarding all behavioral parameters assessed. Around day 90, when amyloid plaques (AP) are reported to be well developed in transgenic animals [8], mice of both genotypes showed similar levels of spontaneous exploratory, locomotor and anxiety related behavior, as measured in the barrier test,

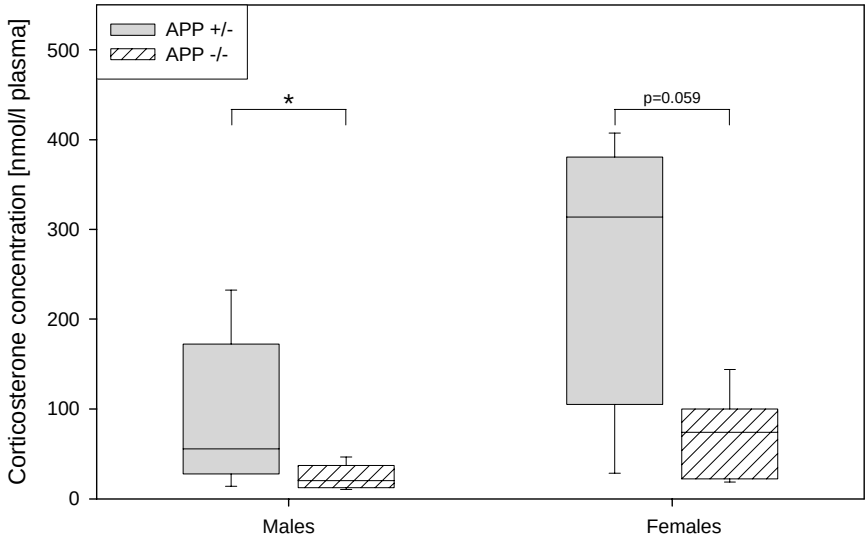

Fig. 3. Plasma corticosterone concentrations measured at day 123 of life in male and female mice of the TgCRND8 line (transgenic animals: APP +/−, wild-type animals: APP −/−). Data are given as box-whisker plots (for a description see legend of Fig. 1) (statistics: Mann–Whitney *U*-test (two-tailed), males: $n_{APP+/-} = 8$, $n_{APP-/-} = 10$, $U = 17$, $^*P < 0.05$; females: $n_{APP+/-} = 8$, $n_{APP-/-} = 9$, $U = 16$, $P = 0.059$).

open-field test, and elevated plus-maze test (see Table 1). Furthermore, the animals' health state, gross sensory function, reflexes, and motor abilities were normal and did not differ between APP +/− and APP −/− mice. Therefore, it can be assumed that the reported deficiencies in learning and memory of APP transgenic animals at eleven weeks of age [8,39] are not due to either differences in exploratory drive, emotional state, physical condition or to the observed significant differences in body weight between APP +/− and APP −/− animals (see Fig. 2a and b). Thus, a causal relationship between the described neuropathological alterations and the occurrence of cognitive deficits is likely. Indications for sex-specific effects, as reported in other studies investigating exploration, anxiety and learning in different strains of laboratory mice [12,46,76] as well as in APP transgenic mice [45], were not found in any of our behavioral tests or in the general health check. However, since in our study the sample sizes were too small to test groups separately, sex-specific effects cannot be ruled out completely.

In the second part of our investigation, we showed for the first time that transgenic male and female mice of the TgCRND8 line exhibit adrenocortical hyperactivity, an endocrine hallmark of AD [11,28,33,52,63,64,67,71,73,92], in a sex-specific manner. At 123 days of age, plasma

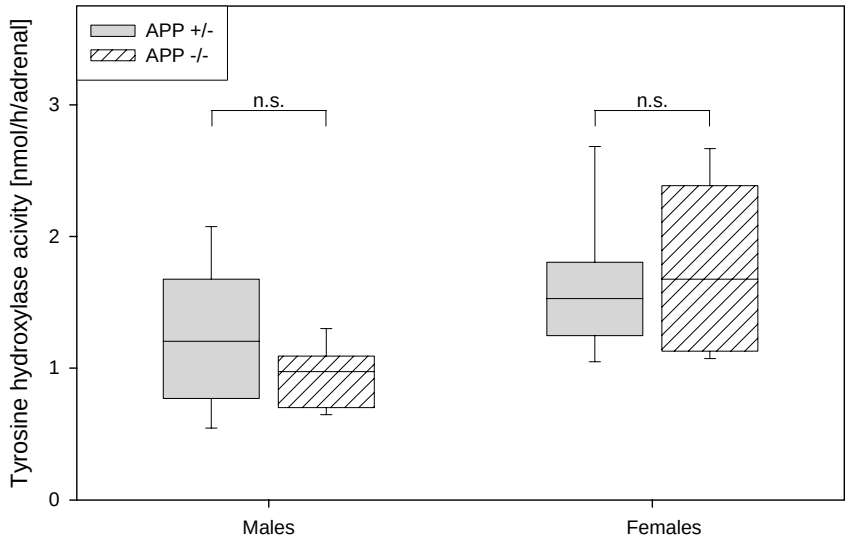

Fig. 4. Adrenal tyrosine hydroxylase activity measured at day 123 of life in male and female mice of the TgCRND8 line (transgenic animals: APP +/−, wild-type animals: APP −/−). Data are given as box-whisker plots (for a description see legend of Fig. 1) (statistics: Mann–Whitney *U*-test (two-tailed), males: $n_{APP+/-} = 8$, $n_{APP-/-} = 10$, $U = 32$, n.s. $P \gg 0.05$; females: $n_{APP+/-} = 8$, $n_{APP-/-} = 9$, $U = 30$, n.s. $P \gg 0.05$).

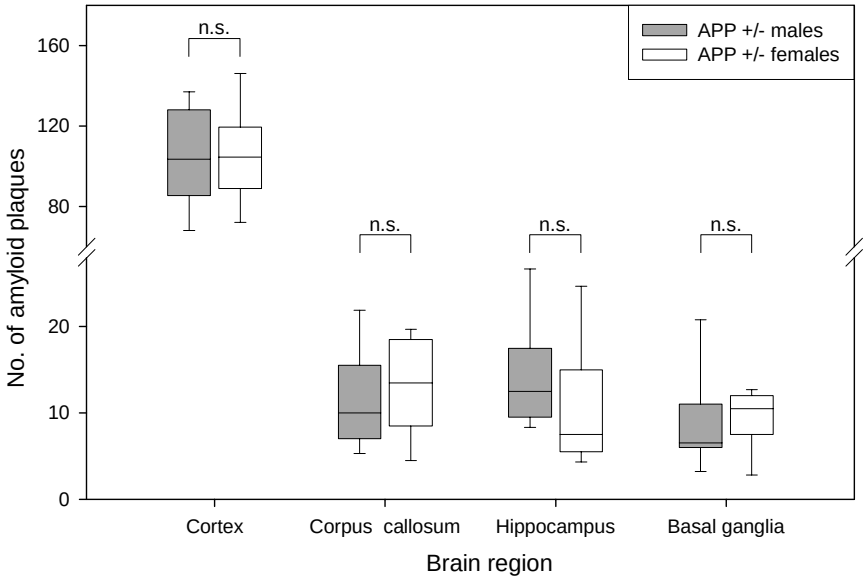

Fig. 5. Number of amyloid plaques observed at day 123 of life in different brain regions of transgenic (APP +/−) male and female mice of the TgCRND8 line. Data are given as box-whisker plots (for a description, see legend of Fig. 1) (Statistics: Mann–Whitney *U*-test (two-tailed), $n_{\text{males}} = 8$, $n_{\text{females}} = 8$, all $U > 18$, n.s. $P \gg 0.05$).

corticosterone concentrations of both sexes were elevated in APP +/− mice compared to APP −/− animals (see Fig. 3). However, the continuous monitoring of fecal corticosterone metabolites (CM) over the course of 120 days, revealed significant differences regarding the time this endocrine feature occurred in males and females (see Fig. 1a and b). In males, significantly increased CM concentrations were already observed very early in life (from day 45 onwards, see Fig. 1a), while in females, clear differences between APP +/− and APP −/− animals firstly appeared at day 90 (see Fig. 1b). This phenomenon of glucocorticoid (GC) level elevation with age has been shown in a variety of species including humans and is widely discussed in the context of neuroendocrinology of stress and aging as well as in dementias like AD [32,33,36,44,47,54,62,68,74,77–79,82,108]. For example, Landfield and co-workers [43,47,48,74] showed that GC or chronic stress can accelerate markers of brain aging (but see also Finch and associates who have shown that caloric restriction, which increases GC, reduces some neuronal markers of aging and AD [14,57]).

Associated changes of elevated GC levels in the brain have been found in certain regions, namely the hippocampus, having the highest concentration of GC receptors (type I and II) and playing a central role in the feedback regulation of the HPA axis [17,33,36,44,49,54,62,82]. Already in the late 1970s, Landfield and colleagues started working in this field and formulated the 'glucocorticoid hypothesis of brain aging', which was later modified by others [43,47,48,74,82]. Sapolsky and McEwen for example proposed a mechanism named the 'glucocorticoid cascade hypothesis' [82] that causally relates hippocampal degeneration with cognitive deficits and impairments in the feedback regulation of the HPA axis leading to elevated concentrations of circulating GC (see also [33,36,44,47,49,53,54,62,74]). Briefly, periods of elevated GC secretion, e.g. during chronic stress, down-regulate the number of GC receptors in hippocampal neurons. At some point, the down-regulation of receptors is irreversible and sufficient to dampen hippocampal feedback inhibition of the adrenocortical axis resulting in increased production of hypothalamic-corticotrophin-releasing factor and therefore GC hypersecretion emerges. This precipitates further down-regulation of receptors and further hypersecretion until permanent loss of hippocampal neurons themselves occurs, and irreversible commitment to the cascade begins. Transferring this to our findings, it seems likely that the observed occurrence of adrenocortical hyperactivity in the APP transgenic mice is associated with progressive pathological changes in the hippocampus probably caused by neurotoxic effects of Aβ and/or the process of plaque formation [7,86,101,106]. However, to clarify this, more sophisticated investigations of neurological damages in the hippocampus are needed detecting for example progressive loss of GC receptors, synapses or neurons. Furthermore, the adrenocortical response of APP +/− and APP −/− mice should be investigated in e.g. ACTH challenge and Dexamethasone suppression tests to evaluate if the observed elevation of corticosterone levels indeed is mediated by an impaired negative feedback inhibition of the HPA axis at the hippocampal level, as known to develop in Alzheimer patients [11,28,61,64,67,70,73].

Regarding the observed alteration in HPA axis activity, distinct sex-differences existed, as adrenocortical hyperactivity occurred much earlier in males than in females (see Fig. 1a and b). This might be explained by the well-known neuroprotective effects of estrogens probably bringing about sex-specific effects in animal

models for AD as well as in human Alzheimer patients [19,20,21,22,29,37,55,56,65,72,83,85,89,95,98,107]. Furthermore, transgenic as well as wild-type females showed clearly higher corticosterone concentrations in plasma and fecal samples compared to the respective males (see Figs. 1 and 3). This is in accordance with findings of other studies reporting females to have higher levels of circulating GC [26,32,50], which might be due to higher levels of gonadal steroid binding proteins also having affinities to corticosterone [5].

In contrast to the differences found between APP transgenic and wild-type mice concerning the activity of the HPA axis, no significant differences were observed regarding their adrenal tyrosine hydroxylase (TH) activity at day 123 in males and females of both genotypes (see Fig. 4). Adrenal TH activity was used as a marker for the activity of the SAM system [13,24,81,100], which was obviously not influenced by the expression of the transgene, indicating a normal activity of this second stress axis. However, possible differences may be found when other parameters of the SAM system are addressed, as Pascualy et al. [67] reported increased basal norepinephrine concentrations and increased cardiovascular responsiveness to sympathoneural stimulation in AD patients (see also [16]).

The neuropathological features investigated, i.e. total plaque burden and number of plaques found in the different brain regions, did not differ between the sexes at day 123 (see Fig. 5), although females showed adrenocortical hyperactivity later in life than males. This might be due to the fact that at this time corticosterone levels have been already clearly increased for some time in both sexes (see Fig. 1), indicating progressive brain damages mediated by excessive GC secretion and/or the expression and metabolism of the transgene, i.e. females already caught up to the levels observed in males. According to this, we would expect differences in neuropathological features between the sexes at an earlier stage, when adrenocortical hyperactivity has not yet developed in the females, but is already established in the males (e.g. around day 70). However, this hypothesis remains to be tested. Another point might be that plaque load was shown not always to be predictive of functional impairments in humans (cf. [9,87,94]). Therefore, other neuropathological parameters like synapse loss in specific brain areas could be better predictors of disturbances in HPA axis activity and correlate with cognitive deficits (cf. [86,87]).

In conclusion, our study underlines that the APP transgenic mouse line TgCRND8 is a very promising animal model to study AD. We could show that the reported cognitive deficits in spatial learning and memory are not brought about by differences in general health status nor by different levels of exploration or anxiety in transgenic and wild-type animals. Furthermore, we demonstrated adrenocortical hyperactivity, an endocrine hallmark of AD, in APP transgenic animals of both sexes. However, the continuous monitoring of GC revealed for the first time that these changes in HPA axis activity occurred much earlier in males than in females, that is, in an age- and sex-dependent manner. Thus, our data strongly suggest that the development of adrenocortical hyperactivity might be a key-element in the understanding of AD.

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