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Restraint stress increases neuroinflammation independently of amyloid β levels in amyloid precursor protein/PS1 transgenic mice

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Abstract

Both hypercortisolemia and hippocampal damage are features found in patients diagnosed of Alzheimer's disease (AD) and epidemiological evidence supports a role for stress as a risk factor for AD. It is known that immobilization stress is followed by accumulation of oxidative/nitrosative mediators in brain after the release of proinflammatory cytokines, nuclear factor kappa B activation, nitric oxide synthase-2 and cyclooxygenase-2 expression. Long-term exposure to elevated corticosteroid levels is known to affect the hippocampus which plays a central role in the regulation of the hypothalamic–pituitary–adrenal axis. We therefore studied the effect of chronic immobilization stress on amyloid precursor protein/PS1 mice. Stress exposure increased AD-induced neuroinflammation characterized by astrogliosis, increased inflammatory gene transcription and lipid peroxidation. Importantly, immobilization stress did not increase the soluble or insoluble amyloid β levels suggesting that increased cortisol levels lower the threshold for a neuroinflammatory response, independently from amyloid β. Since inflammation may act as a factor that contributes disease progression, the stress-inflammation relation described here may be relevant to understand the initial mechanisms in underlying the risk enhancing action of stress on AD.

Keywords: Alzheimer's disease, astrocytes, behavioral stress, microglia, protein nitration.

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Stress exposure modifies the onset and progression of several neuropsychiatric diseases (post-traumatic stress disorder, major depressive disorder, anxiety disorders, schizophrenia) as well as of neurological diseases, like Alzheimer's disease (AD) (Lucas *et al.* 2006). It is accepted that brain inflammatory responses contribute to cell damage during these disorders. Studies carried out with several stress protocols (physical, psychological or mixed) show also a proinflammatory response in the brain and other organ systems mainly characterized by the release of cytokines, prostanoids, and free radicals.

Alzheimer's disease is characterized by the presence of two histopathological hallmarks, extracellular amyloid β (Aβ) deposition and neurofibrillary tangles of tau protein (Querfurth and LaFerla 2010). In addition, a severe neuroinflammation is also observed starting at early disease stages, resulting in a widespread micro- and astrogliosis localized to senile plaques, and the release of cytokines, chemokines and nitric oxide which is thought to contribute to disease progression (Glass *et al.* 2010). There are indications that neuroinflammation even drives Aβ deposition by increasing the production of Aβ, thereby pointing towards an even disease causing scenario (Sastre *et al.* 2006).

Stress mediators (cortisol in humans and corticosterone in rodents) provoke the release of excitatory amino acids (glutamate and aspartate) in certain brain areas (Moghaddam 1993), resulting in an continuous excitation of ionotropic NMDA receptors on neurons, eventually leading to neuronal death by excitotoxicity.

Further, free radicals generated during this process may damage cellular membranes (Coyle and Puttfarcken 1993), resulting in the damage of neurons and glial cells which

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Abbreviations used: AD, Alzheimer's disease; APP, amyloid precursor protein; Aβ, amyloid β; GFAP, glial fibrillar acidic protein; HPA, hypothalamic–pituitary–adrenal; IDE, insulin-degrading enzyme; MDA, malonyldialdehyde; NeuN, neuronal nuclei; NOS-2, nitric oxide synthase; TNF-α, tumor necrosis factor α.

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respond with the secretion of certain pro-inflammatory cytokines such as IL-1β or tumor necrosis factor α (TNF-α, resulting in the activation of microglia and leads to the infiltration of blood-borne inflammatory cells into the brain parenchyma (Dirnagl *et al.* 1999). Stress-released glutamate can activate transcription factors such as nuclear factor kappa B thereby causing the expression of the inducible isoform of nitric oxide synthase (NOS-2) and cyclooxygenase-2 (COX-2) (Madrigal *et al.* 2001b). In turn, expression of NOS-2 and COX-2 results in the generation and release of nitric oxide, which then may modify membrane phospholipids (Madrigal *et al.* 2001a).

Long-term exposure to elevated corticosteroid levels is deleterious to central nervous system structures, and in particular affects the hippocampus (Sapolsky 1990, 1996, 2000) which plays a central role in the regulation of hypothalamic–pituitary–adrenal (HPA) axis activity, thereby contributing to the formation of memory and learning. Both hypercortisolemia and hippocampal damage are features that can be observed in patients suffering from AD (Swaab *et al.* 1994; Weiner *et al.* 1997; Peskind *et al.* 2001; Pomara *et al.* 2003) and epidemiological evidence further points to a role for stress as a risk factor for AD (Wilson *et al.* 2005).

Based on these epidemiological data and the neuroinflammation that occurs in both, chronic stress and AD, we hypothesized, that chronic stress aggravates the course of AD and therefore investigated the effect of chronic immobilization stress on Aβ processing and deposition along with neuroinflammatory characteristics in a murine model of AD.

Material and methods

Animals

Heterozygous double transgenic male mice expressing a chimeric mouse/human amyloid precursor protein (APP) with the Swedish mutation (K595N/M596L) and a mutant human presenilin 1 Δexon 9 under the control of prion promoter were used (n = 10). The animals were on a C57BL/6J background. In addition, male C57BL/6J mice were used for the wild-type groups (n = 10). Mice were housed in groups under standard conditions at a temperature of 22°C (±1°C) and a 12-h light/dark cycle with free access to food and water. Transgenic and wild-type mice were 5 months at the beginning of the experiment (Fig. 1a). Animal care and handling were performed according to the declaration of Helsinki and approved by local ethical committees (approval ref. SAF07-63138). At the end of the study,

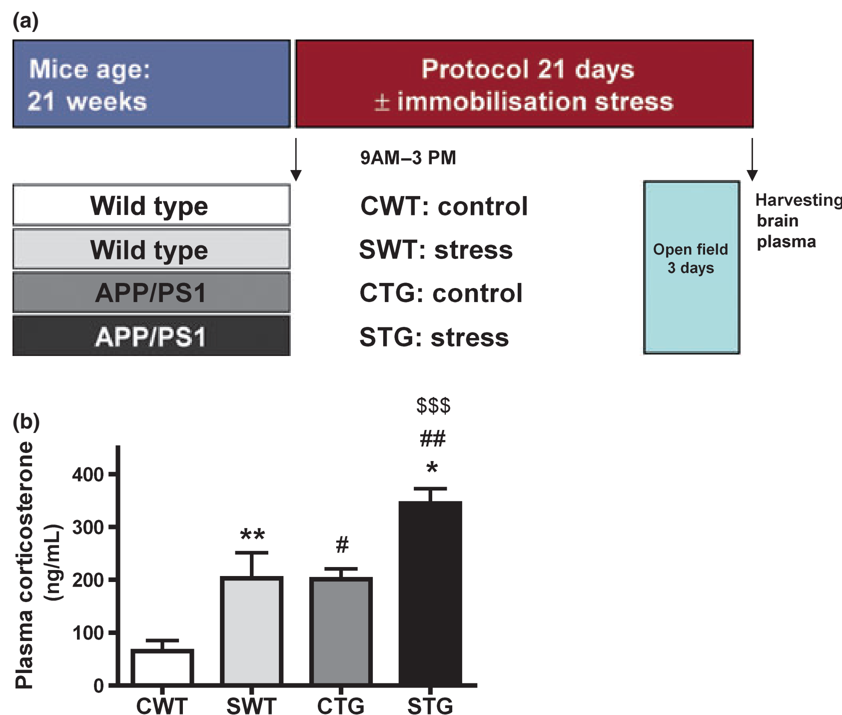

Fig. 1 Stress increase in plasma corticosterone levels. (a) Scheme depicting the experimental design. Five-month-old animals were subjected to the stress protocol for 21 days or left untreated. Tissue was extracted immediately after the last stress treatment. (b) Determination of plasma corticosterone levels in (wild-type) WT, APP/PS1 mice (CTG), WT mice submitted to chronic stress (SWT), and APP/PS1 mice submitted to chronic stress (STG) (n = 10 ± SEM; one-way ANOVA, followed by Newman-Keuls *post hoc* test: factor APP/PS1: CWT vs. CTG $^{\#}$ < 0.05, SWT vs. STG $^{\#\#}p < 0.01$; factor stress: CWT vs. SWT $^{**}p < 0.01$, CTG vs. STG $^{*}p < 0.05$; combination: CWT vs. STG $^{\$\$\$}p < 0.001$).

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mice were anaesthetized by isoflurane, killed and brains were removed and processed for further analysis. All experimental protocols followed the guidelines of the Animal Welfare Committee of the Universidad Complutense according to European legislation (DCUE86/609/CEE, RD 223/1988 and OEM 13/X/1989).

Restraining protocol

Mice were restrained, as previously described (Flint *et al.* 2003; Lauterborn 2004) in plastic tubes with modified caps, each with a hole to accommodate the tail of the mouse. Adequate ventilation was provided by holes drilled into the conical end of the tube and at the sides of the tubes. The tubes did not allow for forward, backward, or rotational movement. Because of the circadian rhythm inherent in corticosterone production, restrain stress was applied at 9:00 AM for all experiments. The protocol of stress used was a chronic model consisting of 6 h of immobilization for 21 consecutive days. Animals were killed right after the final stress protocol at day 21, and blood for plasma determinations was collected by cardiac puncture and anticoagulated by sodium citrate (3.15% w : v, 1 volume citrate per 9 volume blood).

Behavioral studies

Mice were singly housed for in a reversed light/dark cycle (lights off at 8:00 AM, lights on at 8:00 PM) in the testing room at least two weeks prior to the start of behavioral experiments. The tests were performed during the active phase of the animals. The experimenter was blind to the genotype and stress protocol.

Open-field exploration

Mice were placed in the center of the dimly lit (20–30 lux) chamber of the open field apparatus (61 × 61 × 61 cm). Movements of the animals were tracked by the automatic monitoring system Ethovision (Noldus, the Netherlands) for 5 min. The area was virtually divided into a center (a quadrates in the middle of the field with 40 cm edge lengths), a corridor (7.5 cm along the walls) and edges (four quadrates with 10 cm edge lengths), which partly overlapped with the corridor domain. The time spend in each domain, horizontal (distance travelled) and vertical activity (number of rearing), number of urination and defecation were evaluated. The experiment was repeated on three consecutive days.

Extraction of brain lysates

Snap-frozen forebrains (one hemisphere after removal of the cerebellum) were extracted as previously described (Jardanhazi-Kurutz *et al.* 2010). In brief, brains were homogenized in phosphate-buffered saline with protease inhibitor mixture (Sigma, Munich, Germany). The homogenate was extracted in 25 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.5% sodium dexoxycholate, 1% NP-40 for 30 min on ice, resulting in a fraction containing soluble proteins. After centrifugation at 100 000 *g* for 30 min at 4 °C the resulting supernatant was saved and the pellet was sonicated in 25 mM Tris–HCl pH 7.5, 2 % sodium dodecyl sulfate.

Western blot analysis of brain extracts and analysis of APP processing

Protein concentrations were determined using the BCA protein assay kit (Pierce, Bonn, Germany). Protein samples (10 – 50 μg) were separated by 4–12% NuPAGE (Invitrogen, Darmstadt, Germany) using 2-(N-morpholino)ethanesulfonic acid or 3-(N-morpholino)propanesulfonic acid buffer and transferred to nitrocellulose membranes and probed for insulin-degrading enzyme (IDE) using antibody PC730 (1 : 5000; Calbiochem, San Diego, CA, USA), and tubulin using antibody E7 (1 : 5000; Developmental Studies Hybridoma Bank, Iowa City, IA, USA). Primary antibody binding was followed by incubation with appropriate horseradish peroxidase-conjugated secondary antibodies. Immunoreactivity was detected by enhanced chemiluminescence reaction (Millipore, Schwalbach, Germany) and luminescence intensities were analyzed using Chemidoc XRS documentation system (Bio-Rad, Munich, Germany).

Immunohistochemistry

Serial sagittal sections were cut (10 μm) from cryo-conserved preserved hemispheres (Leica Cryostat CM 3050S), embedded in tissue freezing medium (Jung/Leica Microsystems #0201-08926, Solms, Germany) and mounted (Microscope Slides #K0123b, Engelbrecht, Germany). After drying for 30 min at 20°C for fixation, slides were incubated in 4% paraformaldehyde (Roti Histofix 4% #P087.4, Roth, Germany) for 20 min. Blocking was achieved by incubation in 5% normal goat serum for 1 h (Linaris #S-1000, Wertheim – Bettingen, Germany). Slides were rinsed three times for five minutes in PBS, 0.1% Tx-100. Immunostaining was performed overnight by incubation at 4°C with the following primary antibodies: 1) polyclonal antibody rabbit anti glial fibrillary acidic protein (1 : 900 in 2% normal goat serum in PBS, 0.1% Tx-100; DAKO Z0334, Denmark) or 2) anti-neuronal nuclei (NeuN) antibody (1 : 1000, Chemicon, Temecula, CA, USA, MAB377). Afterwards slides were incubated with Alexa Fluor 594-labeled secondary antibodies hosted in goat for 1 h (1 : 400 in PBS, 0.1% Tx-100; Invitrogen and #A11020 Germany). Slides were additionally stained with the nuclear marker 4′,6-diamidino-2-phenylindole stain (Roche Molecular Biochemicals, Indianapolis, IN, USA, 236276) to delineate the hippocampal and cortical regions. Slides were mounted in Mowiol 4-88 (Calbiochem/VWR #475904).

Sandwich ELISA for $A\beta_{1-40}$ and $A\beta_{1-42}$

Quantitative determination of $A\beta_{1-40}$ and $A\beta_{1-42}$ from brain extracts was performed using the human Amyloid $A\beta_{1-40}$ and $A\beta_{1-42}$ ELISA kit (The Genetics Company, Schlieren, Switzerland) according to the manufactures procedure. Samples were cleared by centrifugation at 100 000 *g* for 20 min and diluted to meet the concentration range of the standard curve.

Determination of corticosteroids

Corticosterone was measured in plasma obtained by cardiac puncture immediately after stress at 3:00 PM. After blood centrifugation at 1000 *g* for 15 min, all plasma samples were stored at −80°C before assay by using a commercially available kit by RIA of ^{125}I-labeled rat corticosterone (Coat-a-count rat corticosterone kit, Siemens Medical Solutions Diagnostics, Eschborn, Germany). A gamma counter (Perkin Elmer Wallac Wizard 1470, Pfaffenhofen, Germany) was used to measure radioactivity of the samples following the instructions of the manufacturer.

Lipid peroxidation

Lipid peroxidation was measured by the thiobarbituric acid test for malonyldialdehyde (MDA) as previously described (Das and Ratty

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1987). The cortex was homogenized in 10 volumes of 50 mM phosphate buffer and deproteinized with 40% trichloroacetic acid and 5 M HCl, followed by addition of 2% (wt/vol) thiobarbituric acid in 0.5 M NaOH. The reaction mixture was heated in a water bath at 90°C for 15 min and centrifuged at 12 000 *g* for 10 min. Absorption was measured at 532 nm.

Real-time quantitative polymerase chain reaction

RNA samples from tissues were extracted by disruption of one mouse forebrain in Trizol (Invitrogen, Germany) by using a sterile Ultra Turrax (IKA Labortechnik, Staufen, Germany). Total RNA was quantified spectrophotometrically and reverse transcribed into complementary DNA using the RevertAid First Strand cDNA Synthesis kit (Fermentas, St. Leon-Rot, Germany) according to the manufacturer's instructions. Real time qPCR was performed using the StepOnePlus™ Real-Time PCR System (Applied Biosystems, Darmstadt, Germany). The TaqMan gene expression assay and TaqMan universal PCR master mix (Applied Biosystems) was used for PCR amplification and real-time detection of PCR products. PCRs were carried out in 20 µL with 1 µL of the reverse transcribed product corresponding to 40 ng of total RNA, 1 µl of the gene expression assay mix and 10 µL of the master mix with the following temperature profile: 95°C for 10 min and 45 cycles of 95°C for 15 s and 60°C for 1 min. mRNA expression values were normalized to the level of GAPDH expression. Analysis of the expression of the genes was performed using StepOne software provided by Applied Biosystems.

Statistical analysis

All experimental data were analyzed by two-way ANOVA. If one of the independent variable was changed, it was followed by one-way ANONA followed by Newman-Keuls multiple comparison test. Statistical evaluation was performed with SPSS 17 and Graphpad Prism 5 (San Diego, CA, USA).

Results

Chronic stress increases plasma corticosterone levels

Chronic immobilization stress of mice increased plasma corticosterone levels in wild type (202.9 ± 48.45 in stress group, SWT vs. 65.12 ± 19.92 ng/mL in control group, CWT) and transgenic mice (see Fig. 1b). Interestingly, control transgenic mice showed significantly higher plasma corticosterone levels (201.4 ± 19.58 ng/mL) compared with age-matched wild-type controls. However, transgenic mice submitted to stress (STG) revealed even higher levels (335.4 ± 79.58 ng /mL plasma) compared with SWT, together suggesting a higher HPA activity in transgenic mice.

Aβ levels were not modified by stress, although IDE expression was decreased in transgenic mice submitted to stress

To study the effect of stress on APP and Aβ at an early time point, Aβ measured by ELISA in APP/PS1 mice. We were not able to detect differences in Aβ40 or Aβ42 either in

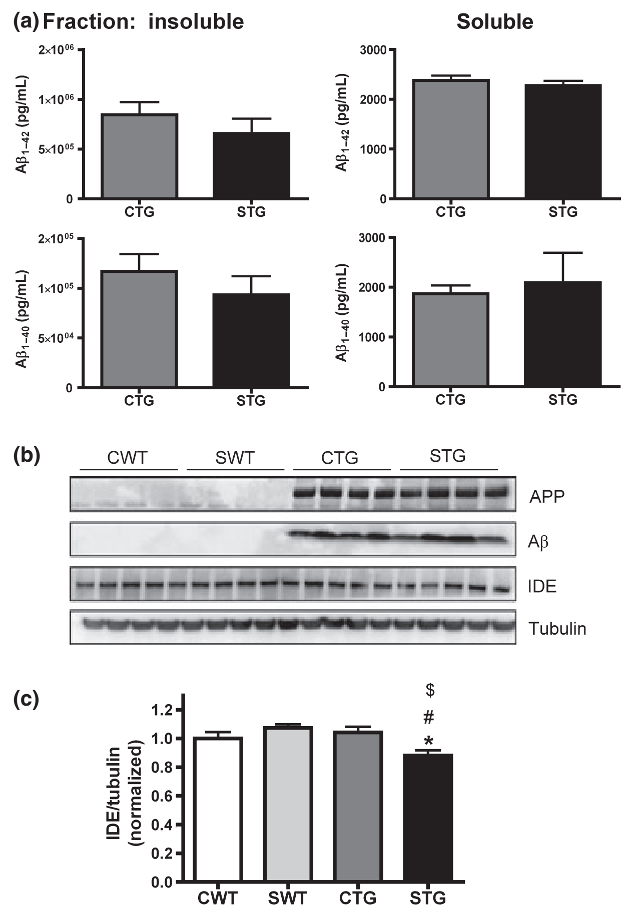

Fig. 2 Aβ levels are not modified by stress exposure but IDE is down-regulated in APP/PS1 mice subjected to stress. (a) Aβ 42 and Aβ 40 levels in soluble and insoluble fraction of whole brain homogenates from APP/PS1 mice subjected or not to stress $n = 5$. (b) Western blot determinations of Aβ processing show no differences in Aβ levels, APP or PS1, but IDE expression is slightly reduced in STG mice. (c) Densitometry of IDE expression ($n = 4 \pm$ SEM; intergroup comparison one-way ANOVA, followed by Newman-Keuls *post hoc* test: factor APP/PS1: SWT vs. STG $^{\#}p < 0.05$; factor stress: CTG vs. STG $^{*}p < 0.05$; combination: CWT vs. STG $^{\$}p < 0.05$).

soluble or insoluble fraction between stressed or non-stressed APP/PS1 mice (Fig. 2a). This was confirmed by western blot. In addition, there were no changes in the expression of APP (Fig. 2b). However, the expression of IDE, a zink-dependent metalloprotease involved in Aβ degradation, was decreased in transgenic mice in response to stress (Fig. 2b and densitometry of the band of interest in 2C). These results indicate that Aβ processing, at this age, is not affected by this stress protocol. Changes in IDE levels, however, may still be compensated for by other mechanisms.

Stress increased astrogliosis and oxidative mediator accumulation in APP/PS1 mice

As a marker for neuroinflammation we analyzed the expression of the glial fibrillar acidic protein (GFAP)

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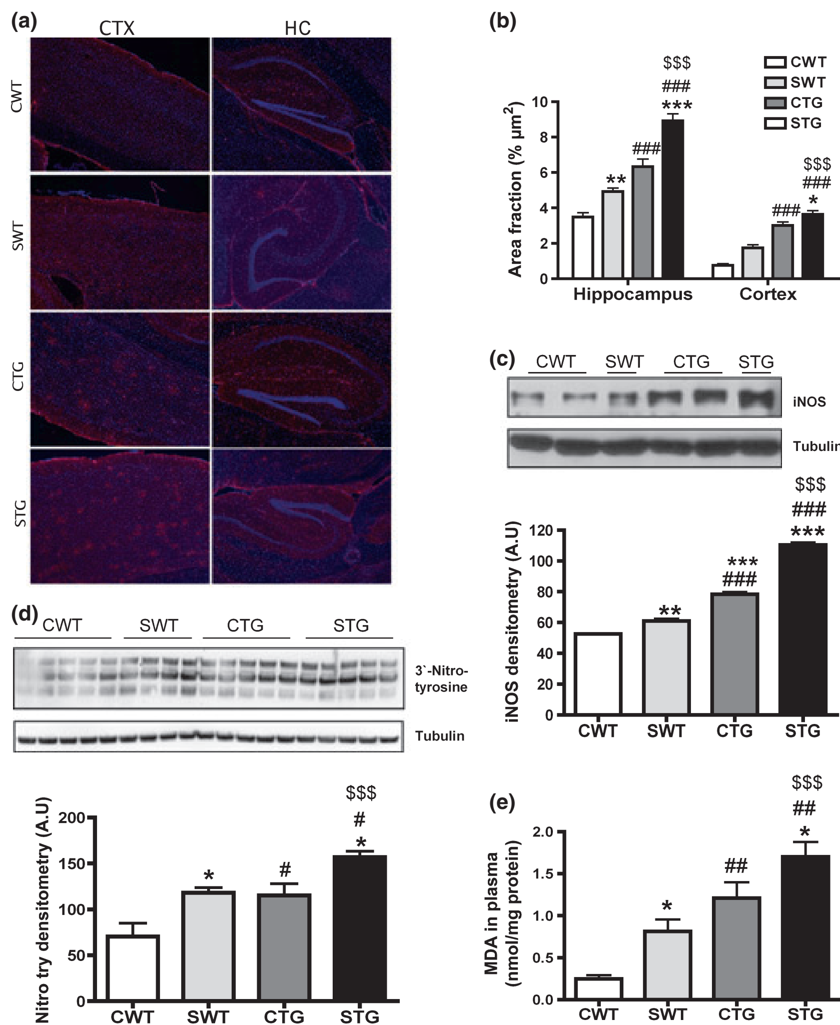

Fig. 3 Stress-induced astroglial activation and lipid peroxidation. (a) Immunohistochemistry for glial fibrillary acidic protein (GFAP) showed an increase in reactive astrocytes in prefrontal cortex (CTX) and hippocampus (HC) of WT and APP/PS1 transgenic mice subjected to the stress protocol (SWT, STG) compared with controls (CWT, CTG). 4′,6-Diamidino-2-phenylindole (DAPI) staining for nuclei was made in order to easily recognize hippocampus and prefrontal cortex. (b) Quantification of the GFAP-positive area ($n = 10$, mean ± SEM; one-way ANOVA, followed by Newman-Keuls: HC factor APP/PS1: CWT vs. CTG $^{###}p < 0.001$, SWT vs. STG $^{###}p < 0.001$; factor stress: CWT vs. SWT $^{**}p < 0.01$, CTG vs. STG $^{***}p < 0.001$; combination: CWT vs. STG $^{\$\$\$}p < 0.001$; Cx factor APP/PS1: CWT vs. CTG $^{###}p < 0.001$, SWT vs. STG $^{###}p < 0.001$; factor stress: CWT vs. SWT $^{***}p < 0.001$, CTG vs. STG $^{*}p < 0.05$; combination: CWT vs. STG $^{\$\$\$}p < 0.001$). (c) Densiometric analysis of NOS-2 expression by western blot in the forebrain of CWT, SWT, CTG, and STG mice ($n = 10$, mean ± SEM; one-way ANOVA, followed by Newman-Keuls *post hoc* test: factor APP/PS1: CWT vs. CTG $^{###}p < 0.001$, SWT vs. STG $^{###}p < 0.001$; factor stress: CWT vs. SWT $^{**}p < 0.01$, CTG vs. STG $^{***}p < 0.001$; combination: CWT vs. STG $^{\$\$\$}p < 0.001$). (d) Densiometric analysis of 3′-nitrotyrosine immunoreactivity by western blot in the forebrain of CWT, SWT, CTG, and STG mice ($n = 10$, mean ± SEM; one-way ANOVA, followed by Newman-Keuls *post hoc* test: factor APP/PS1: CWT vs. CTG $^{#}p < 0.05$, SWT vs. STG $^{#}p < 0.05$; factor stress: CWT vs. SWT $^{*}p < 0.05$, CTG vs. STG $^{*}p < 0.05$; combination: CWT vs. STG $^{\$\$\$}p < 0.001$). (e) Levels of malondialdehyde (MDA) were increased by stress in cortex in WT and APP/PS1 mice ($n = 5$, mean ± SEM; one-way ANOVA, followed by Newman-Keuls *post hoc* test: HC factor APP/PS1: CWT vs. CTG $^{##}p < 0.01$, SWT vs. STG $^{##}p < 0.01$; factor stress: CWT vs. SWT $^{*}p < 0.05$, CTG vs. STG $^{*}p < 0.05$; combination: CWT vs. STG $^{\$\$\$}p < 0.001$).

(Fig. 3a). GFAP was increased in cortex and hippocampus of animals submitted to stress in both wild type and APP/PS1 transgenic mice. The highest GFAP immunoreactivity, however, was detected in APP/PS1 mice submitted to stress. We also detected increased expression of NOS-2, a marker for nitrosative stress, in brain extracts (Fig. 3c). As a consequence of this, we observed increased protein nitration using a 3′-nitrotyrosine specific antibody (Fig. 3d), suggesting higher tissue levels of No and its reaction products like peroxynitrate. Keeping with this, the lipid peroxidation rate

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assessed by the thiobarbituric acid test for MDA, the final product derived from the membrane phospholipid attack, was higher in brains from stressed animals compared to their respective controls in both wild type and APP/PS1 transgenic mice (Fig. 3c).

Stress increases the expression of proinflammatory cytokine TNF α levels in APP/PS1 mice

As known from previous studies, several inflammation-related genes were increased in APP/PS1 mice including monocyte chemoattractant protein, macrophage inflammatory protein alpha, tumor necrosis factor α (TNFα), and COX-2, as measured by quantitative PCR (Fig. 4a).

There were only minor changes in between CWT and SWT animals; however, some of the targets including COX-2, monocyte chemoattractant protein and macrophage inflammatory protein alpha showed APP/PS1 trangene dependent up-regulation, while other such as IL-1β and TNFα were up-regulated by transgene expression but further enhanced by stress. Yet, IL10 was the only parameter determined that required the combined action of transgene expression and stress in order to be up-regulated. Together these data suggest that stress induces a strong inflammatory response, once tested on a murine AD model background.

Neuronal loss was only observed in the cortex of APP/PS1 mice and was not affected by stress

Staining for neuronal marker NeuN, we were not able to detect any neuronal loss in the hippocampus between the groups. However, a decrease in immunopositive area for

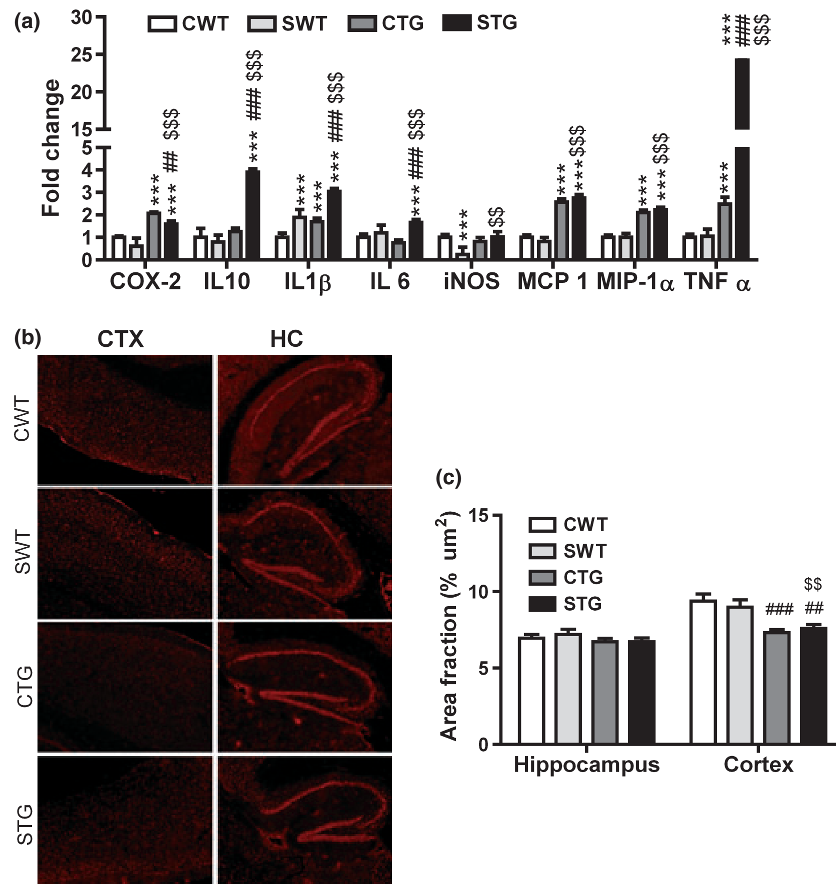

Fig. 4 Stress-dependent and -independent effects: inflammatory gene expression and neuronal loss in APP/PS1 mice. Expression of the proinflammatory cytokines tumor necrosis factor α (TNFα), macrophage chemoattractant protein (MCP-1), macrophage inflammatory protein-1α (MIP-1α), interleukin 1-β (IL1β) and interleukin 6 (IL6); enzyme cyclooxygenase-2 (COX2) and anti-inflammatory interleukin interleukin-10 (IL-10) was analyzed by quantitative real time PCR of mRNA from forebrain homogenates of in CWT, SWT, CTG, and STG mice. An effect of the APP/PS1 transgenes was observed for MIP-1α, MCP-1 and COX-2 mRNA levels, while stress increased TNFα expression in APP/PS1 mice ($n = 5$, mean ± SEM, two-way ANOVA followed by Bonferroni post-tests; CWT vs. SWT, CTG, STG; $^{***}p < 0{,}001$, CTG vs. STG; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, SWT vs. STG, $^{\$\$}{<}p = 0.01$, $^{\$\$\$}{<}p = 0.001$). (b) Cortex and hippocampus of CWT, SWT, CTG, and STG mice were stained for the neuronal marker NeuN. (c) Quantification of NeuN positive area revealed neuronal loss in APP/PS1 mice independent of stress ($n = 10$ ± SEM; one-way ANOVA, followed by Newman-Keuls *post hoc* test: Cortex factor APP/PS1: CWT vs. CTG $^{\#\#\#}p < 0.001$, SWT vs. STG $^{\#\#}p < 0.01$; combination APP/PS1 and stress: CWT vs. STG $^{\$\$}p < 0.01$).

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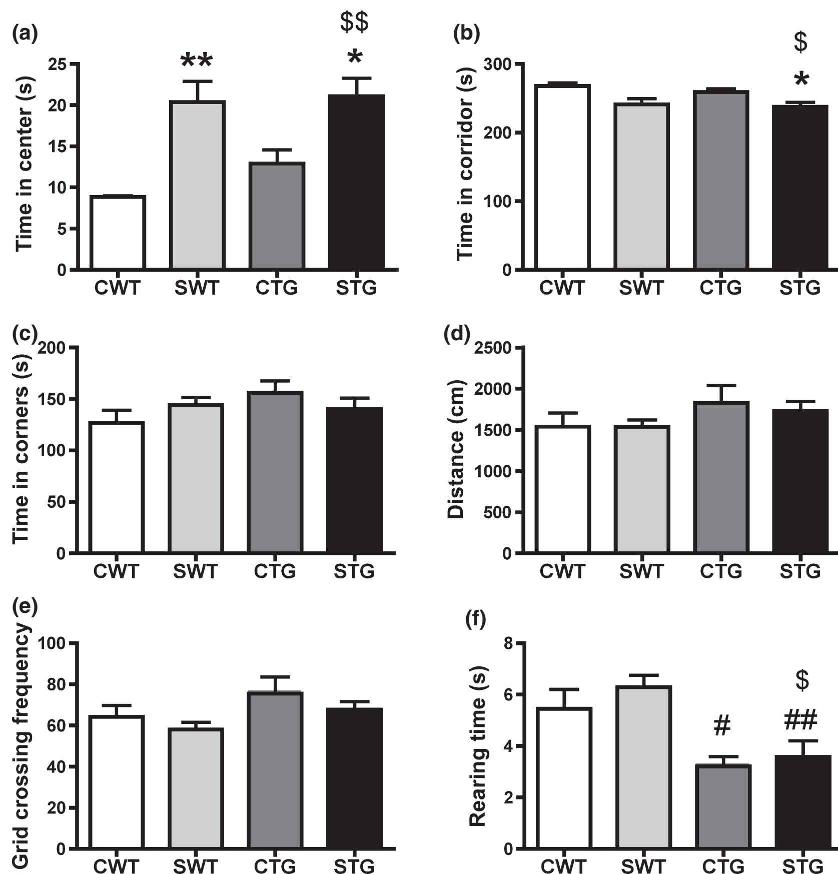

Fig. 5 Open-field behavior is affected by immobilization stress in wild-type and APP/PS1 mice. The activity of CWT, SWT, CTG and STG mice were evaluated in an open-field arena. The factor immobilization stress increased the time spend in the center (5A) whereas time in the corridor (5B) was decreased. Horizontal activity [distance travelled (5D) and grid crossing frequency (5E)] is slightly increased whereas rearing time was decreased by the APP/PS1 expression (5F) ($n = 10$; data are mean ± SEM; one-way ANOVA, followed by Newman-Keuls *post hoc* test: factor stress: CTG vs. STG $^{*}<p = 0.05$, CWT vs. SWT $^{**}p < 0.01$; factor APP/PS1: CWT vs. STG $^{\$\$}p < 0.01$, CWT vs. CTG $^{\#}p < 0.05$, SWT vs. STG $^{\#\#}p < 0.01$; combination APP/PS1 and stress: CWT vs. STG $^{\$}p < 0.05$.).

NeuN was observed in the cortex of APP/PS1 mice with no differences between stressed and non-stressed animals, indicating that neuronal loss at least at this age is not being influenced by increased levels of corticosterone and increased inflammation (Fig. 4b and c).

Stress decreased anxiety related behavior in the open-field arena

To assess the impact of chronic stress and the APP/PS1 transgene on exploration and anxiety related behavior, mice were analyzed on three consecutive days in the open-field arena (Fig. 5). The stress protocol was identical to the one initially described, but mice were tested after day 21 for three more days. The stress protocol did not impair locomotion in general, as the horizontal activity was not affected, whereas the APP/PS1 transgenes induced a slight hyperactivity (Fig. 5d and e). Consistently, rearing time was decreased by the APP/PS1 transgenes. The parameter time spent in the center and corridor reveals anxiety-related behavior to be significantly decreased by the factor stress. Habituation, a measure for non-declarative memory, was comparable in all groups over the three testing days (see Figure S1).

Discussion

This study indicates that exposure to chronic stress worsens neuroinflammation in a murine model of AD without altering levels of either Aβ levels or neuronal loss. This is important because it indicates that the inflammatory component of this neurodegenerative disease may be modulated and enhanced by exogenous factors at a very early stage and can increase independently from Aβ levels. The relatively young age of mice was chosen to study early stress induced effects, at a time, when Aβ deposition is about to start and has not mounted a robust inflammatory reaction yet.

Glucocorticoids have been shown to cause cognitive degeneration and brain aging (Pollerberg *et al.* 1986; Dellu *et al.* 1994) and several clinical studies have well-docu-

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mented elevated plasma cortisol levels in individuals suffering from dementia or AD (Hartmann *et al.* 1997; Weiner *et al.* 1997; Peskind *et al.* 2001; de Bruin *et al.* 2002; Csernansky *et al.* 2006). Furthermore, chronic stress has been shown to alter structure and function of the hippocampus (McEwen 1999; Kim and Diamond 2002). This may be of special relevance to AD, because the hippocampus is particularly vulnerable to various insults and belongs to those brain structures that are affected early during the course of AD (Arnold *et al.* 1991). Accordingly, depression and stress have been identified as potential risk factors for developing AD (Deshmukh and Deshmukh 1990; Sauro *et al.* 2003; Landfield *et al.* 2007). This hypothesis is further fueled by epidemiological findings showing that individuals prone to experience psychological distress are more likely to develop mild cognitive impairment or even AD compared to non-stressed individuals (Wilson *et al.* 2005).

However, the exact mechanisms by which stress accelerates AD pathogenesis are not yet fully understood. Here, we suggest that the first steps of stress mechanisms worsening AD may include an up-regulation of its neuroinflammatory component. In this study, transgenic mice showed higher levels of corticosterone in plasma than age-matched wild-type in the absence of stress, supporting the idea of a hyperactivated HPA axis in this murine AD model. In line with this assumption, the increase in corticosterone upon stress exposure was higher in APP/PS1 transgenic mice, as previously described (Dong *et al.* 2008).

Stress-induced corticosterone levels allowed for increased cortical and hippocampal neuroinflammation characterized by increased astrogliosis, cytokine mRNA levels and release of oxidative molecules. This hypothesis is strengthened by studies showing that physical and psychological stress elevates plasma levels of several cytokines (i.e. TNFα, IL-6, IFN) in animals and humans (Yamasu *et al.* 1992; Maes *et al.* 1998; Nukina *et al.* 1998; Dunn *et al.* 1999). In the present study, we have demonstrated that the mRNA level of one of these cytokines, TNFα, whose release is an essential requirement for the expression of the NOS-2 gene (Xie *et al.* 1994) is markedly enhanced by stress exposure. TNFα accounts for stress-induced activation of NOS-2 through the nuclear factor kappa B in brain (Madrigal *et al.* 2002) and is a key factor for the persistence of oxidative and nitrosative damage during repeated exposure to stress (Munhoz *et al.* 2004).

As a consequence, elevated NOS-2 expression and protein nitration was found in transgenic mice subjected to stress compared with all other groups, indicating that the APP/PS1 transgenes and stress act in concert and may synergistically increase nitrosative protein modification. Likewise, both chronic stress as well as APP/PS1 transgenes increased lipid peroxidation, nevertheless their combination revealed the highest levels arguing for at least an additive effect. An increased lipid peroxidation rate has been described in brain tissue from AD patients (Palmer and Burns 1994; Lovell *et al.* 1995; Marcus *et al.* 1998) and several studies have found increased levels of the final products of the lipid peroxidation process such as MDA (Yan *et al.* 1994), peroxynitrite (Good *et al.* 1996; Smith *et al.* 1997), carboniles (Smith *et al.* 1995) and advanced glycosylation end products (Smith *et al.* 1994), suggesting that lipid peroxidation contributes to AD pathogenesis (Christen 2000).

Stress increased neuroinflammation in the brain of APP/PS1 mice without changing levels of Aβ. In contrast, others have shown that chronic stress increases Aβ deposition (Dong *et al.* 2008; Lee *et al.* 2009) and that application of glucocorticoids has a similar effect (Green *et al.* 2006). One of the differences to our study is that the latter were conducted in the single transgenic Tg2576 and not in the APP/PS1 model. In addition, the contradictory outcomes may be explained by differences in the applied stress protocol. Dong *et al.* (2008) compared isolated-housed with group-housed mice, whereas Lee *et al.* (2009) used also a restrained stress protocol, but for only 2 h for 16 day compared with 6 h for 21 days in this study. In addition, we used complete forebrains for the determination of Aβ by ELISA, whereas Lee *et al.* (2009) used only the cortex. In the latter study, there was a rather mild increase in insoluble Aβ 1-40 and 1-42 was observed, that we might have missed by analyzing the forebrain.

However, even when of mild nature, the observed decrease of IDE expression, one of the enzymes playing a pivotal role for Aβ degradation, may have resulted in significant differences of Aβ over time. In keeping with this, it may be hypothesized that persistently decreased IDE may lead to decreased Aβ degradation and thus to increased Aβ levels at later ages which were not investigated in the present study. In young APP/PS1 transgenic mice analyzed here, the observed differences may not have been strong enough to result in Aβ changes or alternatively may have been compensated for by other mechanisms. In contrast to the above, there was no obvious interaction of both, chronic stress and APP/PS1 transgenes, on behavioral aspects. Although stress resulted in an increase of center time, which can be interpreted as anxiolytic effect, APP/PS1 transgene expression did decrease rearing activity. Future studies need to unravel whether either longer time of exposure of stress, older age or even a combination of both may affect behavioral parameters in a more fundamental additive or synergistic manner.

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Conflict of interest

The authors declare no conflict of interest.

Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1. Additional data were obtained from open-field behavioral analysis.

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