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Induced LC degeneration in APP/PS1 transgenic mice accelerates early cerebral amyloidosis and cognitive deficits

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ABSTRACT

Degeneration of locus ceruleus neurons and subsequent reduction of norepinephrine concentration in locus ceruleus projection areas represent an early pathological indicator of Alzheimer's disease. In order to model the pathology of the human disease and to study the effects of norepinephrine-depletion on amyloid precursor protein processing, behaviour, and neuroinflammation, locus ceruleus degeneration was induced in mice coexpressing the swedish mutant of the amyloid precursor protein and the presenilin 1 ΔExon 9 mutant (APP/PS1) using the neurotoxin N-(2-chloroethyl)-N-ethyl-bromobenzylamine (dsp4) starting treatment at 3 months of age. Norepinephrine transporter immunolabelling demonstrated severe loss of locus ceruleus neurons and loss of cortical norepinephrine transporter starting as early as 4.5 months of age and aggravating over time. Of note, dsp4-treated transgenic mice showed elevated amyloid β levels and impaired spatial memory performance at 6.5 months of age compared to control-treated APP/PS1 transgenic mice, indicating an accelerating effect on cerebral amyloidosis and cognitive deficits. Likewise, norepinephrine-depletion increased neuroinflammation compared to transgenic controls as verified by macrophage inflammatory protein-1α and -1β gene expression analysis. Exploratory activity and memory retention was compromised by age in APP/PS1 transgenic mice and further aggravated by induced noradrenergic deficiency. In contrast, novel object recognition was not influenced by norepinephrine deficiency, but by the APP/PS1 transgene at 12 months. Overall, our data indicate that early loss of noradrenergic innervation promotes amyloid deposition and modulates the activation state of inflammatory cells. This in turn could have had impact on the acceleration of cognitive deficits observed over time.

1. Introduction

The current prevalence of Alzheimer's disease (AD) comprises 18 million patients worldwide, and this number is assumed to double until 2025 (WHO). Due to the urgent nature of finding effective medication and specific diagnostic tools for AD several transgenic animal models have been developed which mimic the amyloid β (Aβ) and tau pathology as well as the neuroinflammatory component of the disease. Besides these main traits, human post-mortem studies have confirmed that an early and substantial degeneration of the locus ceruleus (LC), the main source of norepinephrine (NE) in the mammalian brain, strongly correlates with the progression and severity of dementia, with increase of Aβ plaque deposition as well as neurofibrillary tangle formation (Iversen et al., 1983; German et al., 1992; Marien et al., 2004; Bondareff et al., 1987). In order to model the human AD pathology in this study, APP/PS1 double transgenic mice were treated with dsp4. It has recently been suggested that dsp4's high-affinity uptake through the NET together with its completely irreversible mode of interaction with the NET contribute to its selectivity as noradrenergic neurotoxin (Wenge and Bönisch, 2009) leading subsequently to retrograde degeneration of the NE producing and containing neurons and axons of the LC while leaving other systems intact (Kalinin et al., 2007; Fritschy and Grzanna, 1989). Previously, NE-depletion has been shown to contribute to astroglial and microglial activation, upregulation of inflammatory markers such as macrophage inflammatory protein-1α and -1β (MIP-1α and MIP-1β), tumor necrosis factor alpha (TNFα), 'Regulated upon Activation, Normal T-cell Expressed, and presumably Secreted' (RANTES), glial fibrillary acidic protein (GFAP) and 'Cluster of Differentiation 11b' (CD11b) (Pugh et al., 2007), and elevated inducible nitric oxide synthase (iNOS) and nitric oxide levels in LC projection areas (Heneka et al., 2002, 2006). Secondary changes like the regulation of different adrenergic receptor

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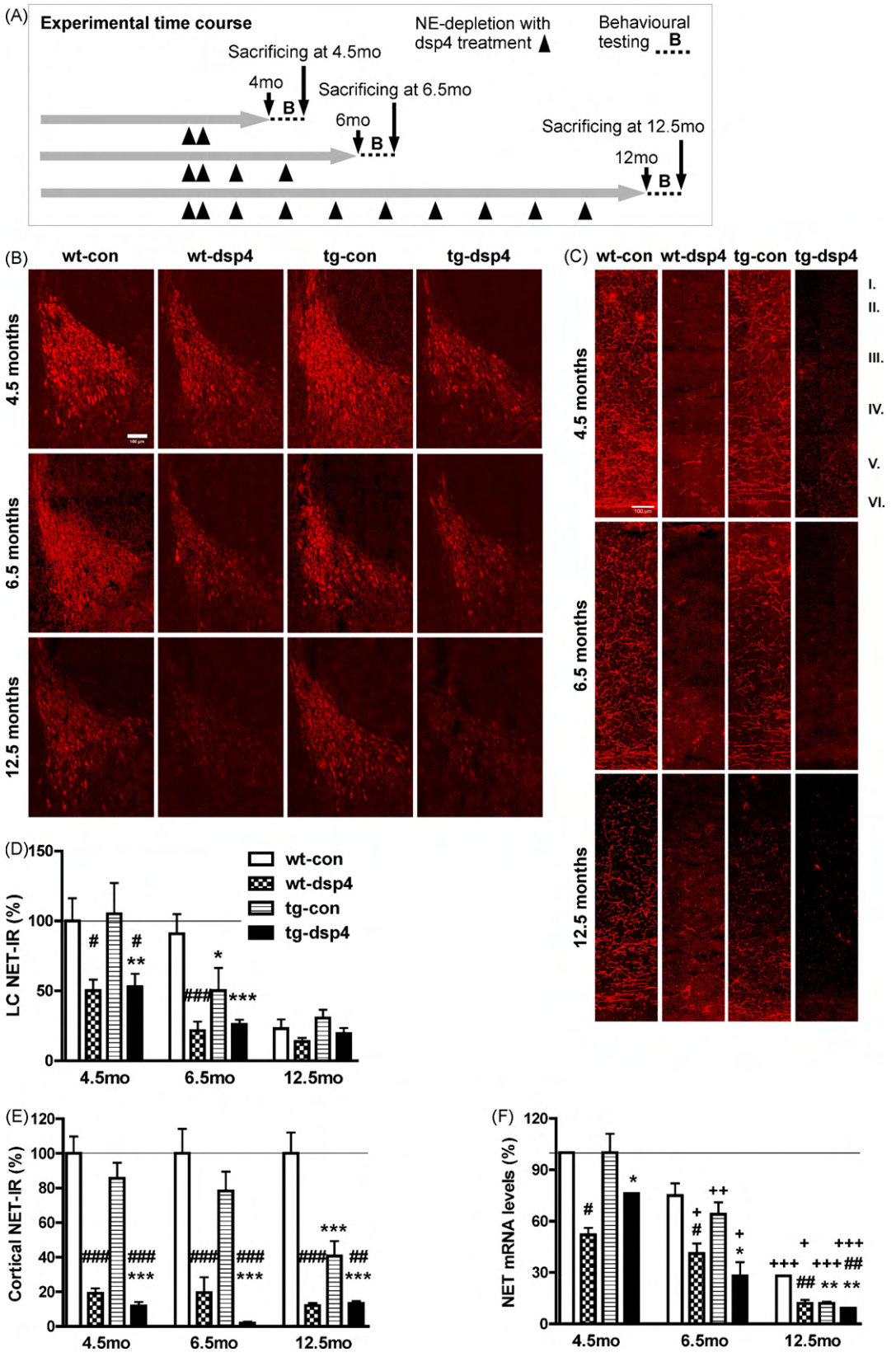

Fig. 1. Experimental time course. NE-depletion led to down-regulation of NET protein levels in the LC and the cortex as well as loss of NET mRNA expression. (A) Mice were treated (▲) with dsp4 to induce selectively degeneration of the locus ceruleus while control mice received saline treatment. Mice received first i.p. injections at 3 and 3.2 months of age followed by monthly administrations, which were stopped 1 month prior to behavioural testing at 4, 6 and 12 months of age. (B and C) NET-IR of 4.5, 6.5, 12.5 months old wild-type (wt) and APP/PS1 (tg) mice treated with dsp4 (dsp4) or saline (con). (B) Medial section of the LC at lat. 810 μm. (C) Retrosplenial granular and agranular cortices. Pictures depict a vertical segment of the retrosplenial granular and agranular cortices next to the hippocampus (Bregma: −1 to −3 mm). Roman numbers from I to VI point to the orientation of six cortical layers. Note the nearly absent NET-IR in the cortex of dsp4-treated mice. (D) Quantification of NET-IR in the LC. The NET-IR of dsp4-treated 4.5 months old mice was lower vs. wild-type and APP/PS1 controls ($p < 0.05$). By 6.5 month a further reduction of NET-positive areas were found in the LC due to transgenity ($p < 0.001$). Furthermore, 12.5-month-old mice of all groups demonstrated a severe LC NET loss as compared to 4.5 months old controls. (E) Quantification

subtypes and transporters have also been described using autoradiography as neurons attempt to compensate the loss of NE content in the CNS (Szot et al., 2006, 2007; Leverenz et al., 2001). However, incoherent data exist on the role of NE in learning and memory (Khakpour-Taleghani et al., 2009). Based on the above body of evidence we hypothesized that early LC degeneration and consecutive NE-depletion throughout the CNS over an extended investigation period exacerbates disease pathology in APP/PS1 mice and alters the brain noradrenergic system leading to learning and memory deficits via modulation of amyloid precursor protein (APP) processing as well as neuroinflammation.

The present study aimed at exploring alterations due to NE-depletion in norepinephrine transporter expression, amyloid precursor protein processing and Aβ pathology, as well as changes in the expression of inflammatory genes in mice. Furthermore, alterations in memory consolidation of APP/PS1 mice suffering from NE-depletion at 4, 6 and 12 months of age were evaluated.

2. Materials and methods

2.1. Materials

N-(2-Chloroethyl)-N-ethyl-2-bromo-benzylamine hydrochloride (dsp4; Sigma, C8417); isoflurane (Delta Select); Tris–HCl (Sigma, T3253-1KG); Tris–base (Roth, 5429.1); normal donkey serum (Chemicon, S30-100ML); paraformaldehyd (Fluka, 76240); ethanol (Merck, 1.00983); acetone (J.T. Baker, 8002); Triton X-100 (Fluka, 93426); donkey anti-mouse immunoglobulin G (IgG) (Jackson ImmunoResearch, 715-007-003); anti-norepinephrine transporter antibody (MAB Technologies, NET05-2); cyanine 3 (Cy3) fluorescent-labelled secondary antibody (Jackson ImmunoResearch, goat-anti-mouse, 115-167-003); Aqua Poly/Mount (Polysciences, 18606); phosphate buffered saline (PBS) (Biochrom, L182-10, 9.55 g/l); ethylenediaminetetraacetic acid (EDTA, Roth, 8043.1); ethylene glycol tetraacetic acid (EGTA, Roth, 3054.2); protease inhibitor mix (Sigma, P8340-1ML); nonyl phenoxylpolyethoxylethanol (NP40) (Igepal CA-630; Sigma, I8896-50ML); Na-deoxycholate (Na-DOC) (Sigma–Aldrich, D6750-25 g); bicinchoninic acid assay (BCA) Protein Assay Kit (Pierce, 23225); NuPAGE Novex 4–12% Bis–tris gels 26 wells (Invitrogen, WG1403BOX); 2-(N-morpholino)ethanesulfonic acid (MES) buffer (Invitrogen, NP0002); PageRuler Prestained Protein Ladder (Fermentas, SM 0671); nitrocellulose membrane (Invitrogen, LC2000); Tween 20 (Roth, 9127.1); skim milk (AppliChem, A0830,1000); 6E10 antibody (Signet, 9300-10); anti-presenilin 1-N-terminal (anti-PS1-NT) antibody (Merck, 529591); tubulin antibody (Merck, CP06); horseradish-peroxidase (HRP) conjugated secondary antibodies (Jackson ImmunoResearch); HRP substrate (Millipore, Immobilon Western, WBKLS0100); RNeasy Mini Kit (Qiagen, 74106); RNase Free DNase Set (Qiagen, 79254); advantage real-time for polymerase chain reaction-kit (Eurogentec, K1402-1); real-time quantitative polymerase chain reaction (qPCR) core kit (Eurogentec, RT-QP73-05).

2.2. Animals

APP/PS1 double transgenic mice (B6C3-Tg(APPswe,PSEN1dE9)85Dbo/J, The Jackson Laboratory) expressing a chimeric mouse/human amyloid precursor protein (Mo/HuAPP695swe; APP) and a mutant human presenilin 1 (PS1-dE9; PS1) protein were used. The animals were hemizygous or littermate control mice and had been backcrossed for at least eight generations. Animals were housed in groups of up to 5 in individually ventilated cages under standard conditions (22 °C, 12 h light–dark cycle) receiving food and water *ad libitum*. Immediately after finishing the behavioural studies at 4.5, 6.5 and 12.5 months of age mice were anesthetised with isoflurane and transcardially perfused with ice cold sodium chloride (0.9%). Brains were removed and the right hemispheres were snap frozen in liquid nitrogen. Left hemispheres were embedded in tissue-freezing medium (Tissue-Tek, Leica Instruments) and quick frozen above liquid nitrogen. The animal numbers were: 4.5 months old, $n = 9 \pm 1$; 6.5 months old, $n = 10 \pm 1$; 12.5 months old $n = 11 \pm 1$. Animal care and handling were performed according to the Declaration of Helsinki and approved by local ethical committees.

2.3. Study groups and dsp4 treatment

The study design contained four mice treatment groups: APP/PS1 transgenic mice treated with dsp4 or with saline and their non-transgenic littermate controls treated with dsp4 or with saline. Treatments were done intraperitoneally using 50 mg/kg dsp4 or saline at 3, 3.2 and a monthly schedule thereafter. Mice were last treated 1 month prior to behavioural testing (Fig. 1A).

2.4. Immunohistochemistry

Ten μm thin serial sagittal sections from the native right hemispheres were cut from median (−0.015 mm) to lateral (−2.015 mm) using a Leica cryostate (CM3050 S) at -23 ± 1 °C and mounted on positive loaded SuperFrostPlus slides (Menzel-Gläser). Slice series were lined up according to The Mouse Brain Atlas (Paxinos and Franklin, 2001) in order to match identical brain areas. Five sequential slices per mouse hemisphere 300 μm apart from each other were picked for serial triple stainings and quantifications. Immunohistochemistry was performed according to the slightly modified protocol of Lippoldt et al. (2000). Slices were dried for 1 h at ambient temperature and fixed for detection of NET-immunoreactivity (-IR) with 4% paraformaldehyd for 7 min followed by 100% ethanol for 5 min at −20 °C and acetone for 1 min at −20 °C. Slices were washed 3× 5 min in Tris–buffered saline (TBS; 100 mM Tris, 154 mM NaCl, pH 7.4). Non-specific binding sites were blocked for 1 h in a humidified chamber by TBST (TBS, 3% normal donkey serum, 0.1% Triton X-100). Donkey anti-mouse IgG (1:20) was added in the TBST blocking solution to block intrinsic mouse IgG. Sections were incubated in a humidified chamber overnight at 4 °C with anti-norepinephrine transporter (1:200) antibody. Slices were washed and incubated for 30 min in TBST with fluorescent-labelled secondary antibody (1:250). Slides were mounted with Aqua Poly/Mount. Negative control slices were incubated with TBST omitting primary antibodies. Fluorescence microscopy was done on an Olympus BX61 using identical exposure times and images were processed with Cell^P (Olympus). Quantification of NET-IR was performed by laying standard square-shaped ROIs over the LC. Cortical NET-IR was measured at four different cutting planes at 90, 390, 990, and 1290 μm. Intensity was detected by Cell^P using identical threshold values and the percent of stained area of the ROIs was generated as raw data.

2.5. Protein extraction

Hemispheres were homogenized in ice cold PBS, 1 mM EDTA, 1 mM EGTA, 3 μl/ml protease inhibitor mix. Homogenates were extracted in radio immunoprecipitation assay (RIPA) buffer (25 mM Tris–HCl pH 7.5, 150 mM NaCl, 1% NP40, 0.5% NaDOC, 0.1% SDS), centrifuged at 20,000 × *g* for 30 min and the remaining pellet containing insoluble Aβ was subsequently solubilized in 2% SDS, 25 mM Tris–HCl, pH 7.5.

2.6. APP processing and amyloid-β detection

Protein concentrations were determined using the BCA Protein Assay Kit. Proteins were separated by 4–12% NuPAGE gel using MES buffer at 150 V. PageRuler Prestained Protein Ladder and synthesized $A\beta_{1-40}$ peptide solutions were used as standard. Proteins were transferred to 0.2 μm nitrocellulose membranes. Membranes were boiled in water for 5 min and blocked for 30 min in TBS + 0.3% Tween 20 for Aβ detection, or in TBST containing 5% skim milk for other antibodies. Aβ was detected using antibody 6E10 (1:1000), APP using antibody 140 (1:500; generous gift of Jochen Walter; Wahle et al., 2006), presenilin using anti-PS1-NT antibody (1:1000), and α-tubulin using antibody CP06 (1:5000) followed by incubation with the appropriate horseradish-peroxidase (HRP) conjugated secondary antibodies. Immunoreactivity was detected by enhanced chemiluminescence reaction and luminescence intensities were analyzed using Chemidoc XRS imaging system (Biorad).

2.7. Quantification of messenger ribonucleic acid (mRNA) expression

qPCR investigation were carried out according to Livak and Schmittgen (2001). Briefly, serial sagittal slices (30 μm × 25 μm) of the left hemisphere were cut from median (−1.50 mm) to lateral (−2.25 mm) using a Leica cryostate (CM3050 S) from native, unfixed tissue. mRNA was isolated and purified from homogenized brain samples using the RNeasy Mini Kit and the RNase Free DNase Set according to the manufacturer's protocol. mRNA quantity was determined spectrophotometrically (NanoDrop). 2.5 μg mRNA samples were reversed transcribed using Advantage RT for PCR-Kit. qPCR was done using the qPCR Core Kit and ABI Prism 7000 (Applied Biosystems). Cycling conditions were 50 °C for 2 min, 95 °C for 10 min followed by 45 cycles of at 95 °C for 15 s and 60 °C for 1 min. Data acquisition and evaluation were done using Abi Prism 7000 SDS software 1.1 (Applied Biosystems). Original data [fold] was normalized to the 4.5 months old wild-type control group. Primer sequences used were: NET forward 5′-aaggagtgggctatgctgtga-3′, NET reverse 5′-ggcaggttcaaggtgaag-gat-3′, NET probe: 5′-catcgcctggtcactctactacctctttgc-3′; MIP-1α forward 5′-ccaagtcttctcagcgcca-3′, MIP-1α reverse 5′-gcaaaggctgctggtttca-3′, MIP-1α probe

of NET-IR in the parietal association cortex. NET immunoreactive innervations remained intact in all wild-type control mice groups, but were down-regulated in dsp4-treated mice of all ages ($p < 0.001$). APP/PS1 control mice showed a down-regulation which became by 12.5 months ($p < 0.001$). (F) Quantification of NET mRNA expression in the brain. Down-regulation of NET mRNA levels due to dsp4 treatment by 4.5 months was observed ($p < 0.01$). By 6.5 month a further decrease of mRNA level was found in all groups ($p < 0.01$). All 12.5 months old mice showed intensive NET mRNA loss, however, less pronounced in wild-type control mice ($p < 0.01$). Groups: wild-type control (wt-con, empty), wild-type dsp4-treated (wt-dsp4, striped), APP/PS1 control (tg-con, checked), APP/PS1-dsp4-treated (tg-dsp4, filled); bars represent mean ± SEM; Scale bars: (B) and (C) 100 μm; $n = 10 \pm 2$ animals per group; original data were normalized to the mean of the 4.5 months old wild-type control group (D and F) and the wild-type control group (E) [=100%; horizontal line], respectively; (hash mark) dsp4 treatment effect, (asterisk) transgenity effect, (plus sign) aging effect; (#, *, +) $p < 0.05$, (##, **, ++) $p < 0.01$, (###, ***, +++) $p < 0.001$.

5′-agctgacaccccgactgcctgc-3′; MIP-1β forward 5′-tcagcaccaatgggctctg-3′, MIP-1β reverse 5′-ctgtgaagctgccgggag-3′, MIP-1β probe 5′-ccctcccacttcctgctgtttctcttaca-3′.

2.8. Behavioural studies

Two weeks prior to the tests mice were housed individually in a reversed 12 h dark–light cycle. All tests were performed during the active phase of the animals in dim light by the same blinded experimenter. All tests were done at the ages of 4, 6 and 12 months, respectively. Animal movements were tracked by an automated monitoring system (EthoVision, Noldus Information).

2.8.1. Morris water-maze (MWM) test

The MWM was performed as described by Lord et al. (2009) with some modifications as briefly follows. MWM test was carried out on 7 consecutive days: mice were placed in the water with their heads towards the tank wall. After each run mice were taken out of the water, quickly dried, carefully spun two times left and two times right and put back into the water-maze at different starting points in order to disturb established visual orientation from the previous run. The temperature of the water was 22 ± 1 °C. Pre-training: on day 1 and 2 all mice were first tested according to a straight-swim pre-training protocol in a tank of 35 cm × 45 cm × 30 cm filled with opaque water on 2 consecutive days to screen out mice with motor or swimming deficits. A platform located 1 cm below the water was located opposite to the start location. *Hidden platform testing:* On the following 4 days each mouse was given eight trials per day in two blocks in a 1 m diameter white circular tank surrounded by a white plastic curtain, where three extra-maze visual cues of different colours and forms were hung from the tank side. The water was made opaque by addition of non-toxic paint and a 15 cm × 15 cm quadrant escape platform was located 1 cm below water surface. Mouse movement data, location, total distance moved, and time spent travelling to the platform during each of the trials were recorded. Evaluation of platform finding speed pointed to the degree of spatial memory retention. Therefore mice finding the platform within 15 s and/or mice not finding the platform at all were also statistically analyzed. *Probe trial*: After the last hidden platform test on day 6, each animal was given an extra 1 min probe trial in the water-maze without the platform. The times entering and spent in each quadrant of the tank were measured. *Visual cued testing:* on day 7 the platform was placed to a different quadrant of the water tank with a visible cue 7 cm above the platform. Mice were given eight trials in two blocks, and the latency to find the platform was recorded.

2.8.2. Open field exploration

Open field exploration was performed as previously published (Weberpals et al., 2009). Briefly, mice were placed into the center of a plastic open field arena (60 cm × 60 cm × 60 cm) with white bottom and black walls and were allowed to explore the arena over 3 consecutive days for 10 min each day. The arena was thoroughly cleaned with 70% ethanol solution after each trial. A 5 cm wide border margin was defined as corridor, and the inner square of 40 cm × 40 cm included the center area. Locomotor activity (distance moved), center and corridor times, horizontal activity (rears), grooming, defecation, and urination were assessed.

2.8.3. Novel object recognition test

Novel object recognition test was carried out according to previously established protocol with minor changes (Heneka et al., 2006). Briefly, the test procedure consisted of three sessions: habituation, training, and retention. Each mouse was individually habituated to a plastic open field arena (60 cm × 60 cm × 60 cm) during the open field exploration test for 3 days. During the training session, two identical objects (object A) were placed into the two opposing corners of the center area of the arena 30 cm apart from each other, and mice were allowed to explore the area and the objects for 15 min. The total time spent exploring both identical objects was recorded to examine possible but not desired place or object preference. Exactly 1 h later, during the retention session, mice were put back into the same arena in which one familiar (object A) and one novel object (object B) replacing the second object A were placed. Mice were then allowed to explore freely for 15 min and the time spent exploring each object was recorded. Exploration of the object was considered when the head of the animal was at least facing the object from a minimum distance of 1–2 cm or closer, but recording was cut as soon as mice turned their heads away from the previously investigated object. Time spent exploring the objects during trials was determined. The arena and all objects were thoroughly cleaned with 70% ethanol solution after each trial.

2.9. Statistical analysis

All data were analyzed by one-way ANOVA followed by Newman–Keuls post-test using Prism (GraphPad).

3. Results

3.1. NE-depletion results in down-regulation of NET in the LC and the cortex

NET immunohistochemistry was in correlation with previously reported results (Schroeter et al., 2000). Stainings were performed in the medial region of the LC (lat. 810 μm). NET-IR was reduced by 50% at 4.5 months due to two consecutive dsp4 treatments as compared to saline-treated wild-type and APP/PS1 controls (Fig. 1B and D). By 6.5 months of age a further reduction up to 70% of NET-positive area, was found in the LC of dsp4-treated mice. Further aging and dsp4 treatments resulted in 70–80% loss of NET-immunoreactivity within the LC as compared to 4.5 months old saline-treated controls. NET immunoreactive innervations in the cortical areas were down-regulated in dsp4-treated mice at all investigated ages when compared to age-matched wild-type controls (Fig. 1C and E).

In support of the above described immunohistochemical findings, analysis of NET mRNA expression (Fig. 1F) revealed a down-regulation by 50% in dsp4-treated wild-type and by 25% in dsp4-treated APP/PS1 (APP/PS1-dsp4) groups at 4.5 months. Aging and dsp4 treatment led to further reductions by 6.5 and 12.5 months in parallel to the findings observed for the NET-IR within the LC.

3.2. Altered APP processing in dsp4-treated APP/PS1 mice

Western blot analysis showed that Aβ-levels of 6.5-month-old APP/PS1-dsp4 mice were 5-fold higher than in 4.5-month-old APP/PS1 littermates and were found elevated (2-fold, $p < 0.05$) when compared to age-matched saline-treated APP/PS1 controls. A further increase of Aβ-levels by 5- and 3-fold was found at 12.5 months in saline- and dsp4-treated APP/PS1 mice groups, respectively. However, there was no statistically significant difference at this age between saline- and dsp4-treated APP/PS1 groups (Fig. 2A and B). Of note, β-C-terminal fragment (β-CTF) levels were found elevated by 1.5-fold at 12.5 months compared to 4.5-month-old dsp4-treated animals (Fig. 2A and D). The levels of human and murine holo APP as well as α-C-terminal fragment (α-CTF) remained unchanged in all groups (Fig. 2A).

3.3. NE-depletion exacerbates neuroinflammation in the brain

The expression of the inflammatory genes MIP-1α and MIP-1β was analyzed using qPCR. Both, MIP-1α and MIP-1β mRNA levels doubled in 4.5 and 6.5 months old APP/PS1 mice compared to wild-type littermates (Fig. 3A and B). At 12.5 months of age both, MIP-1α and MIP-1β mRNA levels were strongly increased in APP/PS1 mice by 12- and 6-fold, respectively, in response to dsp4 treatment. An increase was also observed in wild-type mice of both groups as well as in APP/PS1 control mice but to a lesser degree.

3.4. NE loss increased behavioural and cognitive deficits of APP/PS1 mice

Deploying Morris water-maze test, we detected spatial memory deficits of both APP/PS1 and APP/PS1-dsp4 groups by 4 months of age compared to wild-type littermates. At 6 months, dsp4-treated mice, especially the APP/PS1 transgenic group, performed worse compared to the respective saline-treated groups with respect to distance and time travelled to platform (Fig. 4A and B). Interestingly, noradrenergic depletion and transgene expression caused learning and memory impairments of a similar degree, which was additive in dsp4-treated APP/PS1 transgenic mice. Both transgenic and dsp4-treated groups were seriously impaired in spatial memory by 12 months of age (Fig. 4A and B). A similar patter of spatial memory loss could be found when mice were analyzed for excellent memory retention (platform found within 15 s), and no retention (unable to find platform) (Fig. 4C). During the probe trial post-test 12-month-old APP/PS1 as well as both dsp4-treated groups performed equally worse (Fig. 4D). The visual

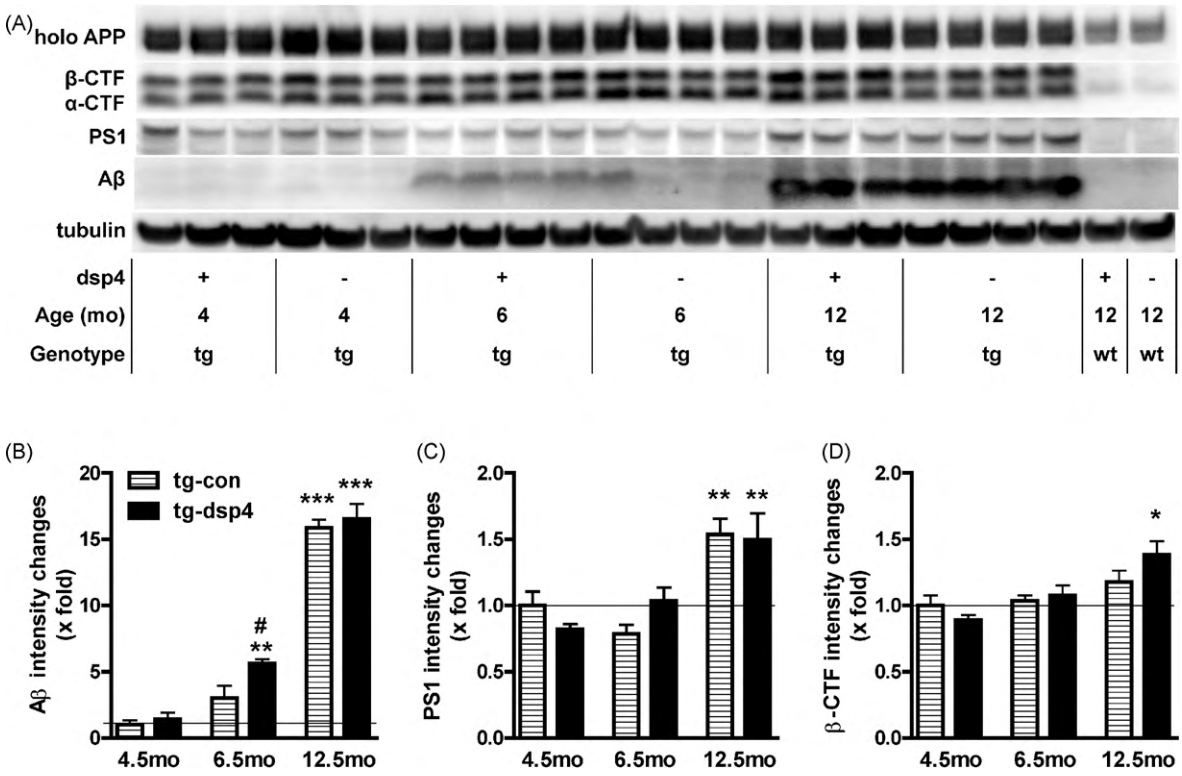

Fig. 2. NE-depletion altered APP processing in APP/PS1 mice. (A) Western blot analysis of holo APP, β- and α-CTF, presenilin 1 (PS1), amyloid-β levels. The levels of human and murine holo APP as well as α-CTF were not altered in APP/PS1 (tg) mice upon dsp4 treatment or aging. (B) Amyloid-β levels were elevated by 6.5 months as compared to 4.5 months old APP/PS1 mice ($p < 0.01$). Importantly, at the age of 6.5 months NE-depletion led to higher Aβ-level as compared to the age-matched saline-treated (con) APP/PS1 control group ($p < 0.05$). This difference disappeared by 12.5 months of age as Aβ-level was further elevated in both APP/PS1 groups ($p < 0.001$). (C) Presenilin 1 levels were first elevated by 12.5 months in both APP/PS1 groups ($p < 0.01$) and (D) β-CTF level was slightly elevated by 12.5 months and exclusively in the APP/PS1-dsp4 group ($p < 0.05$). Groups: APP/PS1 control (tg-con, checked), APP/PS1 dsp4-treated (tg-dsp4, filled); bars represent mean ± SEM; n = 3–4 female animals per group; After background subtraction original data [intensity × mm] was normalized to the mean of the 4 months old APP/PS1 control group [=1; horizontal line]; (hash mark) dsp4 treatment effect, (asterisk) transgenity effect; (#, *) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$.

cued post-test testing confirmed that none of the mice were blind and they could all orient themselves (Fig. 4E).

To test the non-declarative memory, we carried out habituation in the open field arena. At 4 months of age vertical locomotor activity (distance moved) as well as horizontal activity (number of rearings) of both dsp4-treated wild-type and APP/PS1 mice were suppressed by 20 and 50%, respectively, worsening over time in APP/PS1-dsp4 mice (Fig. 4F and G). Time spent in the center zone of the open field was strongly reduced by APP/PS1 mice at 12 months of age (Fig. 4H).

APP/PS1 mice showed some working memory impairment at 6 and 12 months of age exclusively as a result of transgenity and independent from dsp4 treatment in the object recognition test (Fig. 4I).

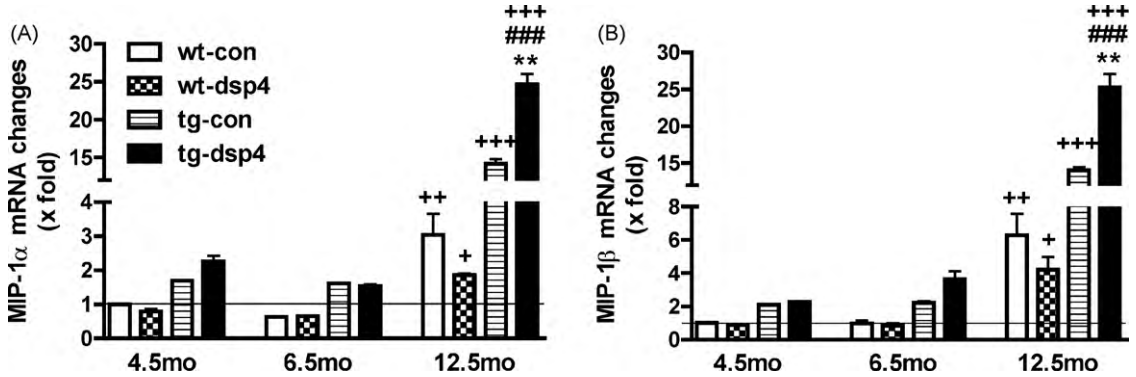

Fig. 3. Dsp4 treatment led to elevated neuroinflammation in the brain. Quantification of (A) MIP-1α and (B) MIP-1β mRNA expressions in the brain. MIP-1α and MIP-1β mRNA levels in APP/PS1 (tg) mice groups were twice as high when compared to controls (con) by 4.5 and 6.5 months of age. By 12.5 months of age both wild-type (wt) groups showed a slight increase in both mRNA levels ($p < 0.05$, $p < 0.01$). Note the elevation at 12.5 months in APP/PS1 groups ($p < 0.001$). MIP-1α and MIP-1β mRNA levels in dsp4-treated mice was excessively elevated over controls ($p < 0.001$). Groups: wild-type control (wt-con, empty), wild-type dsp4-treated (wt-dsp4, striped), APP/PS1 control (tg-con, checked), APP/PS1-dsp4-treated (tg-dsp4, filled); bars represent mean ± SEM; n = 7 animals per group; original data were normalized to the mean of the 4.5 months old wild-type control group [=1; horizontal line]; (hash mark) dsp4 treatment effect, (asterisk) transgenity effect, (plus sign) aging effect; (+) $p < 0.05$, (**, ++) $p < 0.01$, (###, +++) $p < 0.001$.

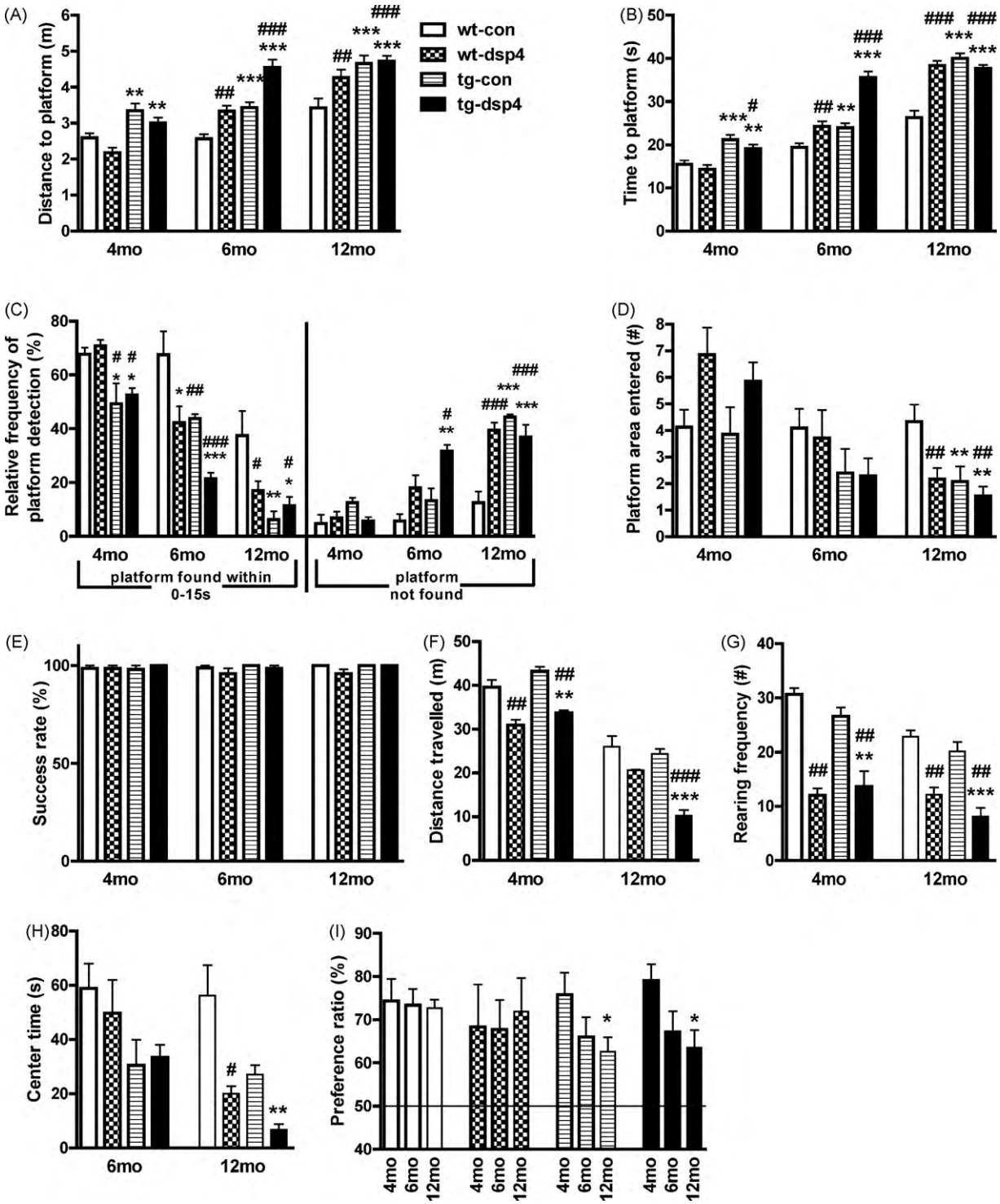

Fig. 4. NE-depletion increased behavioural and cognitive deficits in both APP/PS1 and wild-type mice. (A–E) Morris water-maze. Transgenic mice showed impairment in platform finding already by 4 months of age compared to wild-type (wt) littermates ($p < 0.01$). At 6-month dsp4-treated (dsp4) mice, especially the APP/PS1-dsp4 group, performed worse compared to the wild-type control group with respect to the (A) distance travelled (distance to platform) and (B) time needed to reach the platform (time to platform, $p < 0.001$). Both transgenic and dsp4-treated groups were seriously impaired in spatial memory by 12 months of age ($p < 0.001$). (C) A similar pattern of spatial memory loss was detected when mice were stratified in two groups: mice who found the platform within 15 s and who did not find the platform within 1 min. (D) Platform area entered during probe trial. Six months old transgenic mice of both saline and dsp4 treatments were performing worse as compared to their wild-type littermates. Differences were at 12 months of age as worsening continued in NE-depleted and APP/PS1 (tg) groups ($p < 0.01$). (E) Success rate of finding visually cued platform. All mice were able to find the new place of the visually cued platform. No of the mice were blind. (F–H) Open field test. (F) Vertical locomotor activity (distance travelled) as well as (G) horizontal activity (rearing frequency) of dsp4-treated mice were suppressed at 4 months ($p < 0.01$) and declined further over time ($p < 0.001$). (H) Time spent in the center zone of the open field arena (center time) was reduced in transgenic mice at 6 months vs. wild-type littermates. (I) Novel object recognition test. All groups were performing equally at 4 and 6 months and NE-depletion showed no effect at any age, however, object recognition was negatively influenced by APP/PS1 transgene expression at 12 months ($p < 0.05$). Groups: wild-type control (wt-con, empty), wild-type dsp4-treated (wt-dsp4, striped), APP/PS1 control (tg-con, checked), APP/PS1-dsp4-treated (tg-dsp4, filled); bars represent mean ± SEM; $n = 10 \pm 2$ animals per group; (hash mark) dsp4 treatment effect, (asterisk) transgenity effect; (#, *) $p < 0.05$, (##, **) $p < 0.01$, (###, ***) $p < 0.001$.

4. Discussion

Locus ceruleus (LC) degeneration is a well-proven hallmark of Alzheimer's disease (AD) (German et al., 1992; Marien et al., 2004; Zarow et al., 2003), which shows a close correlation with the pathological Aβ aggregate formation (Mann et al., 1985; Bondareff et al., 1987; Förstl et al., 1994). It has to been emphasized, that post-mortem studies found only modest or absent LC cell loss in non-demented age-matched controls while AD patients revealed a substantial loss of LC neurons (Mann, 1983; Mann et al., 1983; Iversen et al., 1983). While the exact start of dysfunction and degeneration in this brainstem nuclei is not known, LC loss can be already observed in patients suffering from mild cognitive impairment (Grudzien et al., 2007). APP/PS1 mice resemble some characteristics of human AD, but are almost devoid of LC degeneration. Next to Aβ accumulation, additional factors like long-lasting norepinephrine-depletion may influence cognition and behaviour and thus account for clinical AD symptoms, in particular at early stages. We therefore used the chemotoxin dsp4 to study the effects of induced LC degeneration on LC pathology in comparison to Aβ levels and behavioural changes in APP/PS1 transgenics.

The current study demonstrated severe loss of murine NET innervations in the LC and in the respective projection areas in the cortex. Similarly, Jaim-Etcheverry and Zieher (1980) showed a marked, rapid, and persistent depletion of NE in rat CNS as a result of dsp4 treatment. Cheetham et al. (1996) depleted NE in rodent brain using different concentrations of dsp4 (10–100 mg/kg) and found 100% depletion of NE content in the frontal cortex already at the concentration of 50 mg/kg dsp4, while dopamine and serotonin concentrations remained unaffected. Furthermore, they analyzed NET binding in the frontal cortex using [^{3}H]-nisoxetine and the number of binding sites were decreased by 86% in response to the administration of 50 mg/kg dsp4, which was not associated with an alteration in binding affinity. Underlining the relevance of this finding for human AD, Tejani-Butt et al. (1993) have performed binding studies on LC sections of elderly post-mortem AD and non-demented brain controls with [^{3}H]-nisoxetine demonstrating similar results in humans. The above findings indicate that chemically or AD-caused loss of LC neurons are responsible for the observed reduction of NET-immunoreactivity within the LC and its projection areas. However, further mechanisms, including a negative feedback regulation of NET transcription and expression in response to dsp4 treatment, cannot be excluded. Dsp4 caused severe loss of tyrosine-hydroxylase immunoreactive LC neurons could also have contributed to the observed decrease of NET binding sites within the LC and has been previously demonstrated (Heneka et al., 2006). However, it was not responsible for the reduction of cortical NET expression found in the present study.

APP/PS1 mice show an age-dependent increase of Aβ deposition within the cortex and hippocampus starting at around 6 months and revealing an already widespread Aβ plaque load at 12 months of age. Interestingly, NE-depletion accelerated Aβ accumulation evaluated at 6.5 months. This difference, however, was not longer detectable at 12.5 months, most likely due to a ceiling effect caused by the longstanding and extensive amyloid production and aggregation in this model. Combined, these data suggest that the noradrenergic deficiency affects cerebral amyloidosis at a relatively early time point in this murine model and therefore, LC degeneration, found already in mild cognitive impairment patients (Grudzien et al., 2007), may also act as an accelerating factor for Aβ deposition in the early phases of human AD.

Importantly, our data on decreased NET-IR in the CNS consorts with the gradual loss of spatial learning and memory found in the Morris water-maze test as mice scored gradually worse due to dsp4 treatment, transgenity or aging. As shown previously, young wild-type mice performed better as compared to age-matched APP/PS1 littermates (Trinchese et al., 2004; Arendash et al., 2001). In our study LC degeneration and subsequent NE-depletion affected spatial memory first at 6 months of age. In contrast, 12-month-old mice were generally performing weaker and either transgene expression or dsp4 treatment alone did worsen memory performance in comparison to age-matched controls. However, no further deterioration of memory performance was observed by the combined action of dsp4 treatment and APP/PS1 transgene expression, suggesting a ceiling effect similar to the observation for Aβ accumulation. Together, these data suggest that in parallel to the enhancing effect on Aβ aggregation, norepinephrine-depletion aggravates behavioural changes and cognitive decline in relatively young mice, again suggesting a role for the pre-phases or early human AD. Nevertheless, extensive cerebral amyloid deposition at later ages play a more important role in the progression of the disease and overrides the effect of NE-depletion in this model.

Free exploratory capability of APP/PS1 and dsp4-lesioned mice was inhibited as a result of the intense neuronal histopathological changes in the LC, while saline-treated mice performed equally at younger ages as similarly shown by others (Arendash et al., 2001; Lalonde et al., 2004). In addition, NE-depletion partially and gradually inhibited the vertical and horizontal locomotor activities of wild-type and APP/PS1 mice in the open field exploration test. Our single finding during the novel object recognition test was, that both transgenic mouse groups, independent from NE-depletion, were performing slightly worse at 12 months, which correlates to the results of previous studies (Lapiz et al., 2000; Chen et al., 2000; Howlett et al., 2004). In summary, the above behavioural analysis suggests that NE-depletion compromises spatial learning and memory, suppresses exploratory activity and possibly elevates anxiety in mice already at an early age, while leaving object recognition abilities unchanged.

A further hallmark of AD is the vicious cycle of Aβ plaque formation, neuronal death, activation of microglia and astroglia cells, overproduction of a wide range of cytokines, interleukins and other inflammatory markers all leading to the progression of the disease (Heneka and O'Banion, 2007). As previously demonstrated norepinephrine exerts substantial anti-inflammatory and anti-oxidative effects within the CNS (Heneka et al., 2002, 2006; Feinstein et al., 2002). In keeping with this, NE-depletion in the present mouse model influenced inflammatory processes. Aβ plaque-bearing mice showed strongly increased mRNA expression levels of MIP-1α and MIP-1β at 12.5 months unlike their littermates controls. NE-depletion of APP/PS1 mice, however, led to an even more robust elevation compared to saline-treated littermates, most likely as a result of the missing anti-inflammatory effect of NE. The above findings quantitatively correlate to the results of Pugh et al. (2007) on elevated neuroinflammation using 8 and 11 months old transgenic mice treated with dsp4.

In conclusion, the results are in accordance with and support the body of evidence that LC degeneration contributes to the pathogenesis of AD and the progression of the cognitive decline especially at early phases of the disease as shown in a mouse model of AD. Furthermore, we have shown that the NE-deficit in the CNS contributes to a rapid and severe down-regulation of the NE reuptake transporter whose expression is possibly sensitive to the absence of its ligand. Finally, we provided evidence for the influence of NE-depletion on APP processing, on inflammatory processes, e.g. the activation state of the inflammatory cells, in NE-depleted mouse brains. Taken together, these data suggest that NE could act as an indispensable endogenous suppressor of inflammation in the brain. In turn, decreasing NE levels could give rise to a vicious cycle between LC degeneration, neuroinflammation, APP

processing and neuronal cell death fueling the early phase of disease.

Future studies will evaluate the expression of adrenergic receptor subtypes in the brain in this specific animal model of AD.

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