Consequences of early-life stress for microglia throughout life
Relevance for the hippocampus in aging and Alzheimer’s disease
Hoeijmakers, L.

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Early-life stress does not aggravate spatial memory or hippocampal neurogenesis in adult and middle-aged APP/PS1 mice

Lianne Hoeijmakers¹, Anna Amelianchik¹, Fleur Verhaag¹, Janssen Kotah¹, Paul J. Lucassen¹, A. Korosi¹

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Abstract

Early-life stress (ES) may enhance the risk to develop Alzheimer’s disease (AD) later in life. In AD mouse models, ES indeed advanced AD-related neuropathology and altered neuroinflammation. We here addressed whether chronic ES accelerates and/or aggravates AD-related cognitive decline in the classic APPswe/PS1dE9 mouse model, and whether this is associated with alterations in adult hippocampal neurogenesis (AHN), as a substrate for brain plasticity involved in cognition.

ES induced cognitive impairments in 3-month-old wild type, but not APP/PS1 mice, while it did not further modulate APP/PS1-induced changes in cellular proliferation or differentiation in the hippocampus. At 9 months of age, APP/PS1 mice showed spatial memory deficits and reduced AHN relative to wild type mice, which was not changed by the additional ES exposure.

In conclusion, while ES has been reported to modulate AD neuropathology and neuroinflammation, it failed to accelerate or aggravate the decline in cognition or AHN in APP/PS1 mice at the studied ages. Future studies are needed to unravel how ES affects the vulnerability to develop AD.

1. Introduction

Alzheimer’s disease (AD) is the most prevalent form of dementia in the elderly. It is characterized by an age-related accumulation of amyloid β (Aβ) neuropathology and tau-related neurofibrillary tangles in the brain (Querfurth and LaFerla, 2010; Scheltens et al., 2016). While mutations in the amyloid or tau genes induce familial AD in a few percent of the patients, gene-environment interactions likely play an important role in the majority of patients with sporadic AD (Mayeux and Stern, 2012). Indeed, multiple life-style factors have been described to modulate AD age-of-onset and progression (Külzow et al., 2016; Okonkwo et al., 2014). For example, stress experienced in elderly people was a potent accelerator of age-related cognitive decline (Aggarwal et al., 2014), while the total amount of lifetime distress was in addition associated with an aggravated age-related cognitive decline and AD development (Johansson et al., 2014; Sindi et al., 2016; Wilson et al., 2003; 2006; 2007).

Recent clinical studies have further suggested that exposure to stress or trauma early in life might enhance the vulnerability to develop dementia later in life (Seifan et al., 2015; Wang et al., 2016), although the study of this hypothesis has only recently begun. The early-life period is a sensitive time for brain development, during which the brain is particularly vulnerable to adversities, that can alter developmental trajectories and lead to life-long changes in brain function (Barker, 2004; Barker et al., 2013; Heim and Nemeroff, 2001). In fact, early-life stress (ES) has been shown to induce deficits in adult hippocampal structure and functioning, as well as cognitive impairments in both humans and rodent ES models (Calem et al., 2017; Naninck et al., 2015; Staff et al., 2012; Wang et al., 2016).

Interestingly, accumulating preclinical evidence indicates that ES might also modulate the progression of Aβ pathology. For example, processing of the amyloid precursor protein (APP) was increased in

Affiliations: 1, Brain plasticity group, Swammerdam Institute for Life Sciences, University of Amsterdam.
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Non-transgenic adult rats exposed to maternal separation stress (Martisova et al., 2012; 2013; Solas et al., 2010; 2013). In addition, ES aggravated Aβ pathology in BiAT and APPswe/PS1dE9 mice, which overexpress mutated AD genetic variants to drive neuropathology (Hoeijmakers et al., 2017; Hui et al., 2017; Lesuis et al., 2016). These ES-induced alterations in Aβ pathology have also been associated with altered Aβ-induced neuroinflammatory signaling and microglial activation in APP/PS1 mice (Hoeijmakers et al., 2017).

There is, furthermore, initial evidence that such alterations in AD-related neuropathology are also associated with a stronger impairment in spatial memory in 9-month-old, maternally stressed APP/PS1 mice, when compared to unstressed APP/PS1 mice (Hui et al., 2017). However, relative to the unstressed transgenic mice, 4-month-old APP/PS1 mice exposed to prenatal stress, or chronic ES-exposed 4-month-old biAT mice, showed reduced cognitive deficits or no differences in cognition, respectively (Lesuis et al., 2016; Sierksma et al., 2013), indicating that the consequences of ES for cognition might depend on the specific model or ages studied.

One structural substrate implicated in hippocampal plasticity and cognition is adult neurogenesis (AHN), which refers to the generation of new neurons in the adult hippocampus. Various APP-based mouse models have demonstrated reductions in the numbers of neurogenic cells in their hippocampus (Demars et al., 2010; Donovan et al., 2006; Rodríguez et al., 2008). As neurogenesis could respond to Aβ neuropathology as well as the related neuroinflammation (Biscaro et al., 2012; De Lucia et al., 2016; Varnum et al., 2015), one could hypothesize that an ES-induced aggravation of Aβ and the altered neuroinflammatory profile might also further affect AHN. Together, such ES-induced changes in the hippocampus might contribute to an acceleration or aggravation of cognitive decline.

We therefore here set out to study whether ES-induced changes in AD hallmarks, that we and others have characterized before, are also associated with accelerated and/or aggravated cognitive deficits and impaired neuronal plasticity. To this end, we exposed the well-characterized APPswe/PS1dE9 mouse model to chronic ES from postnatal day (P)2 to P9 to test if ES-exposed AD mice exhibit an earlier onset of cognitive decline (i.e. already at 2-3 months of age), or whether ES exposure might aggravate cognitive deficits at later ages. We further studied if ES affect the APP/PS1-related alterations in AHN. Finally, we tested if these measures correlate with one another as well as with the previously reported changes in AD-related hallmarks (including Aβ pathology and microglial markers Iba1 and CD68) in this cohort (Hoeijmakers et al., 2017).

2. Materials and methods

2.1 Mice and early-life stress paradigm

Bigenic APPswe/PS1dE9 hemizygous male mice on a C57BL/6J background and their wild type (WT) littermates were used in this study, as described previously (Hoeijmakers et al., 2017). All animals were bred in house and underwent the chronic early-life stress (ES) paradigm, consisting of limiting the nesting and bedding material for 1 week (Hoeijmakers et al., 2017; Naninck et al., 2015).
Briefly, the dams and pups were assigned to the ES or control (Ctrl) condition on P2. They were left undisturbed until P9 and moved to standard housing cages until weaning at P21. The standard housing consisted of cage enrichment, ad libitum water, standard chow, 20–22°C temperature, and 40–60% humidity. All mice were housed with 2–4 same-sex littersmates per cage. Experimental procedures were conducted according to the Dutch national law and European Union directives on animal experiments, and were approved by the animal welfare committee of the University of Amsterdam.

2.2 Experimental design

The mice were tested for cognitive functioning in various behavioral tests. Behavior was investigated in a first cohort of mice (cohort 1) to test if ES impairments were accelerated in APP/PS1 mice and therefore already present at 2 months of age. A second cohort of mice (cohort 2) was tested for accelerated decline in one behavioral task at 3 months, and sacrificed at 4 months for further analyses. A third cohort of mice (cohort 3) was used for behavioral testing at 8 months of age. Finally, cohorts 1 and 3 were used to investigate if ES aggravated cognitive impairments at 9 months of age, and they were sacrificed at 10 months of age for further analyses. All mice were injected with the cell-birth date marker 5-bromo-20-deoxyuridine (BrdU) prior to sacrifice (see section 2.4).

Cohort 1 included 45 mice (Ctrl WT n=9 of 7 litters, ES WT n=12 of 8 litter, Ctrl APP/PS1 n=13 of 7 litters, ES APP/PS1 n=16 of 7 litters). Cohort 2 included 38 mice (Ctrl WT n=10 of 5 litters, ES WT n=11 of 5 litter, Ctrl APP/PS1 n=9 of 5 litters, ES APP/PS1 n=8 of 4 litters). Cohort 3 included 20 mice (Ctrl WT n=6 of 3 litters, ES WT n=8 of 5 litter, Ctrl APP/PS1 n=4 of 3 litters, ES APP/PS1 n=4 of 4 litters). A total of 36 mice from cohort 1 underwent additional behavioral testing at 9 months (Ctrl WT n=9 of 7 litters, ES WT n=12 of 8 litter, Ctrl APP/PS1 n=8 of 5 litters, ES APP/PS1 n=7 of 5 litters).

2.3 Behavior

Behavioral testing was performed as previously described (Naninck et al., 2015). The object recognition task (ORT) is a non-spatial, emotionally neutral memory test that makes use of the inherent curiosity and novelty-seeking behavior of mice. The object location task (OLT) is a spatial, emotionally neutral memory test, which like the ORT, depends on novelty-seeking behavior. The elevated plus maze (EPM) is used to address basal exploration and anxiety-like behavior. The Morris Water Maze (MWM) is a spatial memory task in which mice can escape the water bath by locating a hidden platform using spatial cues.

The behavioral tasks were performed during the dark (lights-off) phase to accommodate to the natural, active period of mice, and they were therefore moved at least 1 month prior to testing, to a housing room with a reversed 12/12 day/night cycle (8 AM lights off). The testing room was lid by three red-light spots (25W), and mice were transferred daily to the room 1 hour prior to testing. Prior to behavioral testing, mice were handled for 2 days in the housing room and for 2 days in the testing room.
The behavioral performance of the mice was recorded and tracked for automated analysis of locomotion and position using Ethovision software (Noldus, Wageningen, Netherlands). ORT and OLT object exploration behavior and EPM arm exploration behavior were scored using Observer software (Noldus, Wageningen, Netherlands), by an investigator who was unaware of the experimental conditions.

2.3.1 ORT
The testing-box for the ORT was a rectangular blue-plastic box (l x w x h: 23.5 x 33.1 x 27 cm) with a sawdust covered bottom, cleaned in between trials with 25% ethanol. The ORT consisted of 4 consecutive days with on each day a 5-minute trial; 2 habituation trials during which the mice freely explored the testing-box, 1 training trial when the mice explored two identical objects in the testing-box, and 1 testing trial. One of the objects was replaced during this testing trial, and the mice were allowed to explore this novel object and the old, familiar object during the 5-minute trial.

Locomotion in the box was tested on all days as an indicator of basal exploration behavior. Mice that spent <10s exploring the objects during the training or testing trial were excluded from the analysis. The exploration time of the objects during the training phase and the testing phase was scored to test if this ratio was equal to 1, indicating no prior preference or bias. The novel object exploration time over familiar object exploration time was calculated after the testing phase. A ratio for novel/familiar exploration time that was >1 indicated a preference for the novel object and discrimination from the familiar object.

2.3.2 OLT
The OLT task was performed in the same box as the ORT, and likewise consisted of 4 days with sequentially 2 habituation trials, 1 training trial and 1 testing trial of each 5 minutes. During the training trial, the mice were exposed to two new identical objects placed in the middle of the box, of which one was replaced to a new location during the testing trial. If mice recognized this novelty on the testing day, they were expected to explore the object in the novel location more so than the familiar location.

Locomotion in the box was tested on all days as an indicator of basal exploration behavior. Mice that spent <10s exploring the objects at the different locations during the training or testing trial were excluded from the analysis. Similar to the ORT, the ratio of object exploration time during the training trials should be equal to 1, and a preference for the novel location was indicated by the novel/familiar location exploration time >1.

2.3.3 EPM
The EPM was a plus-shaped maze with a neutral 5 x 5 cm center and 35 x 5 cm long arms, raised 100 cm above the floor. Two opposed arms were enclosed with a 30-cm wall, and the two other arms without any enclosure were open arms (OAs). Of these OAs, the outer 17.5 cm of the arm is referred to as distal OA, and the remaining part of the OA area, in between the center and distal area, is referred to as proximal OA (see Fig. 2E for a schematic drawing of the EPM setup). Every mouse was placed in
the center facing one of the OA and allowed to freely explore the maze for 10 min. One animal fell of the maze and was therefore excluded from further analysis. Locomotion during the EPM was tested on all days as an indicator of anxiety and basal exploration behavior. OA exploration time was calculated as a percentage of total arm exploration time.

2.3.4 MWM

We addressed learning behavior during acquisition trials in the MWM and flexibility of this learning behavior during reversal training. We additionally adapted the MWM protocol to make the reversal training more difficult with the use of a smaller platform during reversal training because it requires more precise spatial navigation of the mice (Vorhees and Williams, 2006).

The MWM was a circular pool (110 cm diameter) filled with water (24 ± 1°C). For acquisition and reversal training, the mice underwent twice daily, 1-minute trials with a 30-min inter-trial time by placing them at different, random starting positions in this pool, to exclude egocentric learning strategies. After a trial, the mice were placed in a clean cage in front of an infrared (heating) lamp for ±1 min after the trial to prevent hypothermia.

The protocol started with 1 day of cued trials during which the pool was filled with clear water and a visible 12-cm diameter platform was placed in the center of the pool. When the mice did not locate the platform within the 1 minute trial, they were guided to and placed on the platform for 15 seconds. During the following 6 days of acquisition trials, the pool was surrounded by spatial cues to support allocentric spatial navigation, the water was adjusted to opaque by addition of non-toxic paint and the platform was submerged just below the water-surface in a fixed position within target quadrant 1 (Tq1) for acquisition training. For the probe trial on day 8, the platform was removed and the mice were placed in the pool for the full 1-minute trial. The protocol then continued with 5 days of reversal trials with a platform that was reduced to half the original size (6-cm diameter) and placed in a new location in the maze within Tq2. A second memory trial followed reversal training on day 14.

The latency to reach the platform (escape latency) was measured manually during acquisition and reversal training. The time spent in Tq1 and Tq2 was recorded during the respective probe trials on day 8 and day 14, as well as locomotion behavior during all trials.

2.4 Tissue collection and processing

All mice of cohorts 2 and 3, and several animals from cohort 1 (2 Ctrl WT, 1 ES WT and 2 Ctrl APP/PS1 mice) were sacrificed for the analyses of AHN at least 4 weeks after behavioral testing. Prior to sacrifice, the mice were injected intraperitoneally with the cell birth date marker 5-bromo-20-deoxyuridine (BrdU, Sigma-Aldrich), dissolved in 0.9% saline containing 0.007M NaOH. 4-month-old mice received 3 pulses of 100 mg/kg BrdU with a 2-hour interval on 2 consecutive days. 10-month-old mice received 3 pulses of 100 mg/kg BrdU with a 2-hour interval on 3 consecutive days. 2 hours after the last BrdU injection, the mice were sacrificed by transcardial perfusion with paraformaldehyde for
later immunohistochemical purposes as previously described (Hoeijmakers et al., 2017; Naninck et al., 2015). Tissue was processed to obtain 40 µm coronal sections in 6 parallel series as described before (Hoeijmakers et al., 2017; Naninck et al., 2015).

2.5 Immunolabeling for adult hippocampal neurogenesis markers

2.5.1 Antibodies

BrdU immunofluorescent labeling was obtained with a 1:500 dilution of the rat anti-BrdU antibody (Accurate Chemical and Scientific Corporation OBT0030, Westbury, NY, USA) and doublecortin (DCX) immunohistochemical labeling was obtained with a 1:800 dilution of the goat anti-DCX antibody (SantaCruz Biotechnology, Dallas, TX, USA). Immunolabeling for both antibodies followed previous descriptions (Hoeijmakers et al., 2018; Naninck et al., 2015).

2.5.2 Quantification

An even representation of the hippocampus over the rostral-caudal axis was obtained by selecting 6 coronal, bilateral sections of the hippocampus with 300 µm intersection distance, therewith including 3 sections rostral of bregma -2.30 mm and 3 sections caudal of bregma -2.30 mm. All quantifications were performed by a researcher blind to the experimental conditions.

BrdU+ cells in the sub granular zone (SGZ) of the dentate gyrus (DG) were counted manually on a Leica CTR5500 microscope (40x objective) using the Leica MM AF program (MetaMorph version 1.6.0, Nashville, TN, USA). DCX+ cells in the SGZ and granular cell layer (GCL) were counted manually on a Zeiss Axiophot light microscope (40x objective) with Microfire camera using StereoInvestigator software (MBF Bioscience, Williston, VT, USA). DCX+ cells were further classified for DCX+ cell maturation based on the morphological appearance; type I, horizontal cells without a process reflected immature, mitotic cells, type II cells with an apical process into the GCL reflected an intermediate stage and type III cells with a dendritic tree reaching to the molecular layer reflected the immature neuronal stage (Hoeijmakers et al., 2018). We further addressed sub-regional differences in DCX+ cell numbers (SGZ/GCL and rostral/caudal hippocampus).

2.6 Statistics

Statistical analysis was performed using SPSS 20.0 (IBM software) and Graphpad Prism 5 (Graphpad software). Data were considered statistically significant when p<0.05. All graphical representation of data shows mean + standard error of the mean (SEM).

Data was analyzed with condition (Ctrl-ES) and genotype (WT-APP/PS1) as independent factors in two-way ANOVA designs. Statistical models with litter included as a random factor were run to assess to whether litter effects influenced the dependent variables. Locomotion behavior over days in the ORT or OLT, and MWM escape latency was assessed using repeated measure ANOVAs, with “days” as the repeated factor. Exploration ratios in the ORT and OLT, and probe trial analyses were tested per experimental group using a one-sample T-test to assess the difference from “1” (equal ratio) or “25%” (chance level performance) respectively. Pearson’s correlation and linear regression analyses were employed for the inter-parameter correlations.
3. Results

3.1 ES does not accelerate the onset of cognitive impairments in young adult APP/PS1 mice

3.1.1 ES and APP/PS1 overexpression do not affect cognitive functioning at 2 months

Cognitive functioning was first addressed in 2-month-old mice (belonging to cohort 1) to test for an accelerated onset of cognitive impairments (Fig. 1A). The mice showed equal exploration of the objects or object locations during the training phases of the ORT and OLT at 2 months of age (data not shown; ORT object exploration ratio Ctrl WT $t(6)=2.114 \ p=0.079$, ES WT $t(6)=1.875 \ p=0.110$, Ctrl APP/PS1 $t(12)=0.135 \ p=0.895$, ES APP/PS1 $t(14)=0.1557 \ p=0.879$; OLT object exploration ratio Ctrl WT $t(8)=0.551 \ p=0.597$, ES WT $t(8)=1.769 \ p=0.115$, Ctrl APP/PS1 $t(12)=1.378 \ p=0.193$, ES APP/PS1 $t(14)=1.816 \ p=0.091$).

All mice were able to discriminate between the novel and familiar object in the ORT (Ctrl WT $t(6)=3.951 \ p<0.001$, ES WT $t(6)=4.981 \ p=0.003$, Ctrl APP/PS1 $t(12)=6.180 \ p<0.001$, ES APP/PS1 $t(14)=5.789 \ p<0.001$; Fig. 1B). During the OLT, Ctrl and ES mice of both genotypes explored the novel location of the object more than the familiar location (Ctrl WT $t(8)=2.697 \ p=0.027$, ES WT $t(8)=3.304 \ p=0.011$, Ctrl APP/PS1 $t(12)=3.811 \ p=0.003$, ES APP/PS1 $t(14)=2.243 \ p=0.042$; Fig. 1C).

3.1.2 Spatial memory is impaired by ES at 3 months of age in WT mice, but not in APP/PS1 mice with or without ES exposure

A second cohort (cohort 2) was tested for cognitive performance in the OLT at the age of 3 months (Fig. 1D). 3-month-old mice of both conditions and genotypes equally explored the two objects during the OLT training phase (data not shown Ctrl WT $t(9)=0.650 \ p=0.532$, ES WT $t(10)=0.572 \ p=0.580$, Ctrl APP/PS1 $t(8)=0.972 \ p=0.360$, ES APP/PS1 $t(7)=0.160 \ p=0.876$). ES exposure impaired discrimination of the novel location in WT mice only (ES WT $t(10)=0.311 \ p=0.762$), while all other experimental groups (Ctrl WT mice and Ctrl and ES APP/PS1 mice) explored the novel object location more than the familiar object location during the testing day (Ctrl WT $t(9)=8.421 \ p<0.001$, Ctrl APP/PS1 $t(8)=2.954 \ p=0.018$, ES APP/PS1 $t(7)=2.517 \ p=0.040$; Fig. 1E).

3.1.3 AHN is altered in APP/PS1 mice, but not ES mice, at 4 months of age

One month after the mice of cohort 2 underwent behavioral testing in the OLT, AHN was assessed at the age of 4 months. BrdU+ proliferating cells in the hippocampal SGZ were found to be reduced in APP/PS1 mice, and showed a similar trend after ES exposure, without any interaction between condition and genotype (genotype $F(1,33)=19.450$, $p<0.001$, condition $F(1,33)=3.471$, $p=0.071$, interaction $F(1,33)=0.413$, $p=0.525$; Fig. 1F). Representative images show the DCX+ cells in Ctrl WT (Fig. 1G) and Ctrl APP/PS1 (Fig. 1H) mice. The total number of DCX+ cells in the whole granular zone (i.e. the sub granular zone and granular cell layer) was not significantly affected by either the early-life condition or genotype, although DCX+ cell numbers tended to increase in APP/PS1 mice (genotype $F(1,32)=2.962$, $p=0.095$, condition $F(1,32)=0.043$, $p=0.840$, interaction $F(1,32)=0.219$, $p=0.643$; Fig. 1I).

Further classification of the different stages of DCX+ cell maturation, as based on their morphological appearance, indicated that type III DCX+ immature neurons were increased in APP/PS1 mice compared
to WT mice, without an effect of ES in either of the genotypes (type I genotype F(1,32)<0.001, p=0.985, condition F(1,32)=0.352, p=0.557, interaction F(1,32)=0.293, p=0.592; type II genotype F(1,32)=1.455, p=0.237, condition F(1,32)=0.006, p=0.941, interaction F(1,32)=0.292, p=0.593; type III genotype F(1,32)=5.897, p=0.021, condition F(1,32)=0.369, p=0.548, interaction F(1,32)=0.134, p=0.717; Fig. 1I). Both APP/PS1 and ES did further not induce differences in specific hippocampal sub-regions, i.e. GCL/SGZ or rostral/dorsal hippocampus (data not shown).

Furthermore, the changes in hippocampal neurogenesis did not correlate with the behavioral performance in the OLT at 3 months of age (data not shown).

### 3.1.4 Aβ and microglial markers correlate with AHN levels at 4 months

We had previously analyzed and reported on how ES affected β-amyloid pathology, Iba1 and CD68 in the hippocampus in APP/PS1 mice (Hoeijmakers et al., 2017), using the same 4-month-old mice as used in the current study. We therefore tested to what extent AHN and OLT behavior correlate with these measures. In 4-month-old mice, the number of neurogenic BrdU+ cells positively correlated with the extent of cell-associated amyloid in the DG of APP/PS1 mice (BrdU vs cell-associated amyloid: $r=0.593, p=0.015$). BrdU+ cell numbers in 4-month-old mice further correlated with microglial Iba1+ cell numbers in the DG and CD68 coverage in the DG (BrdU vs Iba1: $r=-0.432, p=0.008$; Fig. 1J; BrdU vs CD68: $r=0.468, p=0.012$). Controlling for genotype in these correlations showed that genotype was a strong contributor in both correlations ($p<0.01$) and that the significant correlation with Iba1 remained ($r=-0.335, p<0.05$), but not with CD68 ($p>0.05$), when genotype was included as a confounding factor. DCX+ cell numbers at 4 months did not correlate with any of the neuropathological hallmarks (data not shown).

### 3.2 APP/PS1 and ES-exposed mice showed hyperactive exploration in ORT, OLT and EPM at 8 months of age

#### 3.2.1 ES WT and APP/PS1 groups exhibit abnormal exploratory behavior during habituation and testing of the ORT and OLT

A third cohort of mice of all 4 experimental groups (cohort 3) was aged until 8 months with the aim to test if ES would have aggravated cognitive deficits in APP/PS1 mice (Fig. 2A). However, all ES and APP/PS1 mice showed abnormal exploration behavior during ORT and OLT habituation and testing phases when compared to Ctrl WT mice at this age, precluding any conclusions about cognitive functioning based on these tasks.

Ctrl WT mice reduce their mobility in the ORT-box over the 4 days of sequential habituation, training and testing, but locomotion was overall higher in ES WT mice and APP/PS1 mice showed a trend towards an increase in locomotion on the last day (days F(1,17)=82.758, $p<0.001$, genotype F(1,17)=3.271, $p=0.088$, condition F(1,17)=13.251, $p=0.002$, interaction F(1,17)=0.64, $p=0.317$; post-hoc: condition Day (D)Iik 1 $p=0.006$, condition D2 $p=0.003$, condition D3 $p=0.010$, genotype D4 $p=0.016$; Fig. 2B). In line with this observation, the average moving-speed during the ORT was increased in both ES-exposed groups, resulting in a trend towards an increase in the APP/PS1 groups (genotype F(1,17)=3.288, $p=0.088$, condition F(1,17)=13.108, $p=0.002$, interaction F(1,17)=1.032, $p=0.317$; Fig. 2C).
Early-life stress does not aggravate impairments in cognition or neurogenesis in APP/PS1 mice

3.2.2 Exploratory behavior of APP/PS1 mice in the EPM is atypical with increased open arm exploration, whereas ES mice fail to show any alteration

These findings on hyperactivity prompted us to test these same mice for anxiety-related exploration behavior in the EPM (Fig. 2F). Locomotion in the EPM was higher in the APP/PS1 mice compared to the WT groups, while ES did not affect this (genotype $F(1,15)=4.705$, $p=0.047$, condition $F(1,15)=2.668$, $p=0.123$, interaction $F(1,15)=0.089$, $p=0.769$; Fig. 2G). APP/PS1 mice showed a trend to spend more time in the open arms of the EPM, but this was not significant (genotype $F(1,16)=3.523$, $p=0.078$, condition $F(1,16)=2.8311$, $p=0.112$, interaction $F(1,16)=0.741$, $p=0.402$; Fig. 2H). They also spent more time exploring the distal part of the open arms in comparison to the proximal part of the open arm (genotype $F(1,17)=4.988$, $p=0.039$, condition $F(1,17)=2.397$, $p=0.140$, interaction $F(1,17)=0.1189$, $p=0.734$; Fig. 2I).

3.3 Cognition and AHN are impaired in 9-month-old APP/PS1 mice and not further modulated by previous ES exposure

3.3.1 MWM acquisition and reversal training is impaired in APP/PS1 mice and not affected by ES in either WT or APP/PS1 mice

Behavior of the mice from cohorts 1 and 3 was tested in the MWM at 9 months of age (Fig. 3A). APP/PS1 mice were significant slower to locate the platform than WT groups, even though all groups showed a reduced latency to escape during the course of the MWM acquisition (days $F(1,53)=28.714$, $p<0.001$, genotype $F(1,53)=32.566$, $p=0.001$, condition $F(1,53)=1.864$, $p=0.178$,
interaction $F(1,53)=0.520, p=0.474$; post-hoc for day$\times$genotype: Acq1 $p=0.008$, Acq3 $p<0.001$, Acq4 $p<0.001$, Acq5 $p<0.013$, Acq6 $p<0.001$; Fig. 3B). Even though all mice acquired the task, none of the groups spent above 25% (chance level) of the time in Tq1 during the probe trial (Ctrl WT $t(14)=0.738, p=0.473$; ES WT $t(19)=0.723, p=0.478$; Ctrl APP/PS1 $t(10)=1.106, p=0.295$; ES APP/PS1 $t(10)=2.520, p=0.030$; Fig. 3C).

During the more challenging reversal training, APP/PS1 mice were slower to locate the platform during all days of reversal training than WT mice (genotype $F(1,51)=29.985, p<0.001$, condition

![Figure 2: ES and APP/PS1 induces hyperactive exploratory behavior in multiple tasks](image)

A) After exposure of WT and APP/PS1 mice to the ES paradigm, the mice that belonged to cohort 3 were studied at 8 months of age in the object recognition task (ORT), object location task (OLT) and elevated plus maze (EPM). B) The distance moved during box exploration for the ORT was higher for ES mice on days 1 to 3, and higher for APP/PS1 mice on day 4. C) The average walking speed of both the ES and APP/PS1 mice was increased during these exploration days. D) Distance moved during box exploration for the OLT was higher for ES and APP/PS1 mice on days 1, 2 and 4. E) Average walking speed of ES and APP/PS1 was likewise increased. F) Schematic of the EPM setup, with 2 closed arms, 2 open arms (OA), and the division of the OAs in proximal and distal. G) Walking distance in the EPM was elevated in APP/PS1 mice. H) The time spent in the open arms was not significantly different between the 4 groups, but I) APP/PS1 mice spent more time in the distal part of the open arm than in the proximal part. Annotations: *, condition effect; #, genotype effect. Abbreviations: EPM, elevated plus maze; OA, open arm; ORT, object recognition task; OLT, object location task; P, postnatal day; SGZ, sub granular zone.
Early-life stress does not aggravate impairments in cognition or neurogenesis in APP/PS1 mice

F(1,51)=0.434, p=0.513, interaction F(1,51)=0.281, p=0.598; post-hoc for day*genotype: rev1 p=0.026, rev2 p=0.003, rev3 p=0.001, rev4 p<0.001, rev5 p=0.001; Fig. 3D). Only Ctrl WT mice showed a reduction in escape latency over the different training days, even though ES WT mice exhibited a trend to significance in acquiring the new location (rev days F(1,51)=5.294, p<0.001; Ctrl WT F(3.23,42.04)=4.809, p=0.005; ES WT F(3.10,58.90)=2.326, p=0.082; Ctrl APP/PS1 F(1.95,19.48)=0.684, p=0.513; ES APP/PS1 F(2.74,24.65)=0.586, p=0.615; Fig. 3D). During the probe trial that followed reversal training, none of groups displayed memory for the new location as all of the groups spent less than 25% of the time in the Tq2 (Ctrl WT t(13)=2.006, p=0.066; ES WT t(10)=3.689, p=0.004; Ctrl APP/PS1 t(19)=2.243, p=0.037; ES APP/PS1 t(9)=2.904, p=0.018; Fig. 3E).

3.3.2 AHN is reduced in APP/PS1 mice at 10 months

1 month after the end of the MWM paradigm, AHN was assessed in the 10-months-old mice. Proliferation, measured by BrdU+ cell numbers (Fig. 3F), was reduced in the SGZ of APP/PS1 mice at 10 months, but ES did not affect this in either genotype (genotype F(1,22)=11.41, p=0.003, condition F(1,22)=0.099, p=0.756, interaction F(1,22)=1.168, p=0.292; Fig. 3G). Representative images of DCX+ cells in 10-month-old WT (Fig. 3H) and APP/PS1 mice (Fig. 3I) and quantification showed that the total number of DCX+ cells was not affected in APP/PS1 mice at this age (genotype F(1,22)=1.746, p=0.200, condition F(1,22)=2.049, p=0.166, interaction F(1,22)=0.308, p=0.585; Fig. 3J). The morphological classification of DCX+ cells showed that the type I proliferative and type II intermediate maturation stages were reduced in APP/PS1 compared to WT mice, while ES did not further influence this reduction (Type I: genotype F(1,22)=5.853, p=0.024, condition F(1,22)=2.107, p=0.161, interaction F(1,22)=0.247, p=0.624; Type II: genotype F(1,22)=6.171, p=0.021, condition F(1,22)=1.671, p=0.210, interaction F(1,22)=0.025, p=0.876; Type III: genotype F(1,22)=0.001, p=0.976, condition F(1,22)=1.803, p=0.193, interaction F(1,22)=1.016, p=0.324; Fig. 3J).

Next to this, region-specific analysis showed that DCX+ cells that reside in the SGZ were reduced in ES groups and APP/PS1 groups, indicating that compared to Ctrl WT mice, the 3 other groups had fewer DCX+ cell numbers (genotype F(1,23)=21.299, p<0.001, condition F(1,23)=4.491, p=0.045, interaction F(1,23)=1.124, p=0.300; Fig. 3K). Furthermore, both BrdU+ cell numbers and DCX+ cell numbers did not correlate with the behavioral performance in the MWM at 9 months of age.

3.3.3 Microglial CD68 correlates with cognition and AHN at 10 months

Amyloid pathology and neuroinflammation parameters have previously been analyzed and reported for this cohort of 10-month-old mice (Hoeijmakers et al., 2017), and we now further analyzed inter-parameter correlations between these factors, and the new cognition and AHN data. At 10 months of age, AHN did not correlate with amyloid pathology while acquisition training in the MWM correlated with microglial CD68 coverage in the DG of 10-month-old mice (MWM acquisition vs CD68: r=0.454, p=0.030). Microglial CD68 coverage in the DG further showed a negative correlation with BrdU+ cell numbers and DCX+ cells numbers in the SGZ, but not with total DCX+ cells (DCX+ SGZ cells numbers vs CD68: r=-0.552, p=0.006). Interestingly, these correlations did not hold up after including genotype as a confounding factor (data not shown).
Figure 3: Spatial memory and AHN are impaired in middle-aged APP/PS1 mice

A) WT and APP/PS1 mice of cohort 1 and 3 were exposed to the ES paradigm or control condition and tested at 9 months of age in the Morris water maze (MWM). A schematic overview of the MWM protocol, which consisted of 1 day (D) with cued trials, 6 days of acquisition training followed by 1 probe trial, and sequentially 5 days of reversal training with a probe trial. The platform was placed in target quadrant 1 (Tq1) for the acquisition phase, and in the adjacent Tq2 for the reversal phase. B) WT mice reduced their latency to locate the platform and escape from the pool over the days of acquisition training, but APP/PS1 were slower in this. C) The percentage of time spent in Tq1 was not above chance level (25%) for any of the groups during probe trial 1. D) Escape latency during reversal training was slower in APP/PS1 mice compared to WT mice. E) Performance during the second probe trial was not affected by the early-life condition or genotype, and none of the groups performed above chance level. F) Example image of BrdU+ proliferative cells in the SGZ. G) The number of BrdU+ proliferating cells was reduced in APP/PS1 mice. Representative images of doublecortin (DCX)+ cells in H) a Ctrl WT mouse and I) a Ctrl APP/PS1 mouse. J) Doublecortin (DCX)+ cell numbers, and in particular subtypes I and II were reduced in APP/PS1 mice. K) In the sub granular zone (SGZ), the number of DCX+ cells was furthermore reduced by ES as well as by APP/PS1. Scale-bars are 100µm. Annotations: *, condition effect; #, genotype effect. Abbreviations: Ctrl, control; D, day; DCX, Doublecortin; ES, early-life stress; MWM, Morris water maze; P, postnatal day; SGZ, sub granular zone; Tq, target quadrant; WT, wild type.
4. Discussion

In this study, we addressed if chronic ES exposure accelerated or aggravated AD-related cognitive decline and alterations in adult neurogenesis in APP/PS1 mice, a classic model for aspects of AD. Cognitive deficits have previously been indicated in ES WT mice at 4 months of age, and we now showed that these deficits were not yet present when ES-exposed mice were studied at 2 months. OLT performance was impaired, however, in 3-month-old ES WT, but not ES APP/PS1 mice. The APP/PS1 mice further exhibited an increased number of differentiating DCX expressing cells, but decreased proliferating, BrdU+ cells at 4 months of age. AHN at this age did not correlate with OLT performance of these mice at 3 months, whereas BrdU+ cell numbers were associated with the APP/PS1-induced neuropathological and microglial changes at 4 months, that we reported before (Hoeijmakers et al., 2017). At 9 months of age, we confirm the learning impairments of APP/PS1 mice in the MWM and show that ES did not further modulate cognition of the APP/PS1 mice, nor of WT mice. AHN was reduced in APP/PS1 mice at 10 months of age, while the ES-exposed mice further showed a sub-regional decrease in DCX+ cells in the SGZ. Finally, DCX+ cell numbers and cognition in the 10-month-old mice correlated the previously reported with microglial CD68 coverage in the DG (Hoeijmakers et al., 2017). Together, these results show that ES does not accelerate or aggravate the AD-related cognitive decline or the alterations in AHN in APP/PS1 mice.

4.1 No accelerated cognitive and AHN decline in young adult (2-4 months) APP/PS1 mice exposed to ES

We show for the first time that ES mice were able to discriminate between novel and familiar objects at 2 months of age and that spatial memory impairments in ES WT mice had developed by 3 months. This observation is in line with, and extends on the previous descriptions of impairments in cognition in this ES model at ages 4-6 months (Naninck et al., 2015; Rice et al., 2008; Wang et al., 2011; 2013). Our results further indicate that ES-induced cognitive deficits in novelty-based learning tasks develop only after 2 months of age. Interestingly, exposure of rats to limited nesting and bedding material from P2 to P9 similarly induced deficits at 10, but not yet at 4 months of age (Brunson et al., 2005). Although these mouse and rat models are not identical, this does imply that chronic ES induced impairments in specific cognitive tasks arise not until later in adulthood.

Although ES WT mice were impaired in the OLT at 3 months of age, ES-exposed APP/PS1 mice were still able to learn the task at this time. ES thus does not seem to accelerate impairments in these transgenic mice and this observation even suggests a to some extent, protective effect of APP/PS1 overexpression at this age. We suggest that this difference in the phenotype after ES exposure in WT and APP/PS1 mice is potentially mediated by alterations in neurogenic processes. Although 4-month-old APP/PS1 mice exhibited reduced numbers of proliferating BrdU+ cells, the number of differentiating, DCX+ cells was enhanced in APP/PS1 mice, irrespective of ES. This might suggest that more of the BrdU+, proliferating cells in APP/PS1 mice ultimately differentiated to the neuronal lineage to express DCX and APP/PS1 mice might therefore altogether exhibit a larger neurogenic potential. An increase in among others DCX+ cell numbers has also been reported in 3-month-old APP/PS1 mice, as well as in
other AD-related transgenic lines, in particular during the earlier pathological stages when no or little plaque formation has developed yet (Krezymon et al., 2013; Unger et al., 2016; Wen et al., 2002). This phenotype has been proposed to be a potential compensatory mechanism of the brain to combat the degenerative effects of soluble Aβ peptides (Meneghini et al., 2013). However, the neurogenic cell numbers at 4 months of age did not correlate with OLT performance at 3 months, and an open question thus remains to what extent AHN alterations contribute to cognition in the ES-exposed and APP/PS1 mice.

We further addressed whether cognitive performance and AHN were associated with previously described changes in hippocampal Aβ levels and neuroinflammation in these mice (Hoeijmakers et al., 2017). Interestingly, BrdU+, but not DCX+, cell numbers correlated positively with cell-associated amyloid levels, and negatively with microglial Iba1 cell numbers in the DG. Clearly, APP/PS1 overexpression was a strong contributor in these correlations. Interestingly, the correlation of microglial Iba1 with BrdU+ cells remained significant after correction for the APP/PS1-induced variation, suggesting that AHN alterations might also be associated with ES-mediated alterations in neuroinflammatory signaling (Hoeijmakers et al., 2017). Indeed, hippocampal pro-inflammatory signaling per se can impact neuroplasticity, including AHN (Ekdahl et al., 2003; Jakubs et al., 2008; Maggio et al., 2013), which awaits further investigation in ES-exposed WT and APP/PS1 mice.

4.2 Cognitive decline in middle-aged, 9-month-old APP/PS1 mice is not aggravated by ES

The first cognitive deficits have been reported to arise in APP/PS1 mice after approximately 6 months of age (Edwards et al., 2014; Guo et al., 2015; Jankowsky et al., 2005; Zhang et al., 2012) and we therefore addressed whether ES would have aggravated cognitive performance at 9 months, when the deficits should have been firmly established. In comparison to WT mice, the APP/PS1 mice had indeed a slower learning curve during the acquisition and reversal phases. However, none of the groups fully acquired spatial memory for the platform location, as indicated by the poor probe trial performance of all 4 groups.

In order to better address more subtle differences between the groups, we used a smaller platform during reversal training that makes learning of the novel location more challenging and requires more precision (Vorhees and Williams, 2006). Now, APP/PS1 mice could indeed no longer locate this novel platform location, whereas Ctrl WT mice showed, and ES WT mice tended to show, learning during this reversal phase. However, this strong APP/PS1-induced impairment in reversal training did not allow any possible further impairment in ES-exposed APP/PS1 mice. It is overall evident that APP/PS1 mice are significantly impaired in MWM performance at 9 months and ES did not significantly alter, or worsen this performance any further.

It is interesting that the ES-exposed WT mice were not impaired in MWM acquisition relative to Ctrl WT mice either, as was reported for MWM learning in Ctrl and ES WT mice at 5 months of age (Naninck et al., 2015). A natural decline in the cognitive abilities of Ctrl WT mice might possibly have leveled out the (earlier) difference between Ctrl and ES mice (Koh et al., 2014; Lindner, 1997), resulting in a similar learning capacity around middle-age. Indeed, Ctrl WT mice were not able to locate the platform during the probe trial either.
Although chronic ES exposure in WT mice was reported to impair ORT learning at 8 months (Rice et al., 2008), the 8-month-old ES-exposed and APP/PS1 mice in this study showed hyperactive exploratory behavior in both the ORT and OLT, that was further confirmed by the atypical (distal) open arm exploration in the EPM. While others reported normal or reduced exploratory behavior of ES-exposed WT and APP/PS1 mice (Huang et al., 2016; Olesen et al., 2016; Rice et al., 2008), others have described similar (hyperactive) exploratory behaviors (Filali et al., 2011; Rodgers et al., 2012) that, however, hampered the proper assessment and evaluation of cognition with these tasks (Cohen and Stackman, 2015; Vogel-Ciernia and Wood, 2014).

Prior to our current study, a related report had shown a stronger impairment in MWM performance in APP/PS1 mice that were exposed to daily, 3-hour maternal separation from P2-P21 compared to unstressed APP/PS1 mice (Hui et al., 2017). Next to this, a more ‘positive’ early-life manipulation, i.e. early-life handling, was found to attenuate some cognitive impairments in 11-month-old APP/PS1 mice (Lesuis et al., 2017), and 4-month-old 3xTgAD mice (Cañete et al., 2015). Interestingly, we reported that 1-week of chronic ES enhanced plaque load in the DG (Hoeijmakers et al., 2017), whereas the APP/PS1 mice exposed to a 3-weeks-long maternal separation paradigm exhibited elevated Aβ plaque load in the total hippocampus as well as the cortex (Hui et al., 2017). Exposure to 3-week long maternal separation thus led to a more severe Aβ phenotype as well as more severe cognitive decline in APP/PS1 mice, relative to 1-week of chronic ES exposure in our current study. Considering that the level of Aβ has been implicate in the progression of cognitive decline in APP/PS1 mice, this might provide a possible explanation for the stronger impact of maternal separation stress on cognition decline (Hui et al., 2017).

Interestingly, MWM acquisition correlated with the previously studied microglial CD68 changes in the DG but not with the DG Aβ plaque load (Hoeijmakers et al., 2017), indicating that, similar to the phenotype at 4 months, the APP/PS1-induced neuroinflammatory changes might be associated with the emergence of the cognitive deficits (Czerniawski and Guzowski, 2014; Dinel et al., 2011; Fonken et al., 2016; Guo et al., 2015). Alternative behavioral protocols can be employed in future studies to address (subtler) ES-induced effects on cognition at different ages.

Next to cognitive deficits, APP/PS1 mice have been described to exhibit reduced AHN around the age of 9-10 months (Demars et al., 2010; Hamilton and Holscher, 2012; Hu et al., 2010; Niidome et al., 2008; Taniuchi et al., 2007; Verret et al., 2007), although there are expectations as well (Hamilton and Holscher, 2012; Taniuchi et al., 2007; Unger et al., 2016). The reduction in basal BrdU+ cell proliferation and DCX+ cell numbers in Ctrl and ES APP/PS1 mice at 9 months is therefore consistent with these previous studies. Furthermore, the reduction in DCX+ cell numbers correlated with the APP/PS1-induced elevation in microglial CD68 coverage in the DG (Hoeijmakers et al., 2017), whereas AHN did not correlated with MWM performance. Such a correlation suggests that the neuroinflammatory activation that follows APP/PS1 overexpression, and thus likely Aβ pathology, might be associated with a reduction of immature neurons as reflected by the changes in DCX+ cell numbers. Anti-inflammatory treatment has indeed been shown to induce pro-neurogenic effects on newborn cell survival in APP/PS1 mice (Biscaro et al., 2012), supporting such a relation between neuroinflammation and AHN.
In addition, exposure to chronic ES did not further aggravate the APP/PS1-induced reductions in AHN, although ES-exposed mice showed a sub-regional reduction in DCX+ cells in the SGZ, revealing a minor effect of ES on the DCX+ cells that have not yet migrated into the GCL. Interestingly, at 6 months, neurogenic cell proliferation and differentiation in the hippocampus were not affected by ES in WT mice either (Naninck et al., 2015). We now extended on these findings at other ages, and further show that even under a pathological condition of APP/PS1 overexpression, these neurogenic stages are not affected by ES exposure. Neurogenic cell survival, an additional measurements for AHN, were decreased in 6-month-old ES WT males (Naninck et al., 2015), while APP/PS1 mice have been reported to exhibit reduced cell survival levels as well (Verret et al., 2007). This process might therefore be more susceptible to ES exposure in APP/PS1 mice and remains to be studied in future experiments. Next to this, other markers for neuroplasticity in ES-exposed APP/PS1 could be addressed to elucidate if (chronic) ES-induced changes in WT mice are indeed differently manifested in the APP/PS1 model (Aisa et al., 2009; Sierksma et al., 2012; Wang et al., 2011).

4.3 Conclusions
To summarize, ES failed to accelerate or aggravate the decline in cognition and AHN in older APP/PS1 mice. The severe cognitive deficits and already low neurogenic numbers in the middle-aged APP/PS1 mice might have prevented a further reduction by ES at this late age (‘floor’ effect). Given the altered neuropathological Aβ levels and neuroinflammation in ES-exposed APP/PS1 mice, this raises the question whether ES exposure affects other forms of hippocampal neuroplasticity in APP/PS1 mice and aggravates the decline at other ages. Such knowledge can help to identify vulnerability for later-life cognitive decline in populations exposed to stress early in their lives, and may help to develop specified therapeutic interventions.

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