Consequences of early-life stress for microglia throughout life
Relevance for the hippocampus in aging and Alzheimer’s disease
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Early-life stress increases inter-individual variability in spatial memory performance, but does not alter hippocampal neurogenesis and neuroinflammation in aged mice

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Abstract

Individuals exposed to early-life stress (ES) have an increased vulnerability to develop cognitive deficits and age-related diseases. Yet, the specific manner in which ES affects the brain is affected with aging is not well understood. As age-related cognitive decline may involve disruptions in neuroplasticity or inflammatory signaling, we here tested the hypothesis that ES would aggravate the age-induced decline in later cognition, hippocampal neuronal plasticity and inflammatory signaling in mice in C57BL/6J (wild type) mice.

C57BL/6J mice were exposed to chronic ES from postnatal day (P)2 to P9 and tested at 19 months of age, for general exploratory behavior in the open field (OF) and for cognitive performance in the Morris water maze (MWM). Various additional physiological, hippocampal neuroplasticity and neuroinflammatory measures were determined in control (Ctrl) and ES mice at 20 months of age. In addition, we compared several of these parameters to young adult (4 months) and middle-aged (10 months) Ctrl and ES mice to address the trajectory of the age-related alterations for these factors.

Exploratory behavior in the OF was not affected by ES. Neither did ES alter the average escape latency or probe trial performance in the MWM, although ES mice did exhibit a higher inter-individual variability in performance. We classified the Ctrl and ES mice as aged unimpaired (AU) and aged impaired (AI) learners with a faster or slower escape latency, respectively. This classification revealed that ES AU mice had a shorter escape latency when compared to ES AI mice, while the average escape latency of Ctrl AU mice was similar to Ctrl AI mice. Body weight, organ weights and corticosterone levels of the mice were not affected by ES. In the brain, ES led to a lasting reduction in hippocampal volume. 20-month-old mice further showed a decline in neurogenesis in the hippocampus when compared to younger mice (4-10 months), but this was not altered by ES at either age. Also, mRNA expression of various synaptic markers at 20 months was not affected by ES exposure. Similarly, ES did not affect age-related alterations in microglial priming factors and other neuroinflammatory markers.

To conclude, ES did not aggravate the decline in cognition, hippocampal neuroplasticity and inflammatory profile with aging in C57BL/6J mice. Interestingly, inter-individual variability in cognitive performance was enhanced by ES, possibly suggesting that ES exposure results in an aggravated age-related phenotype in some mice, while others seem to be more resilient. Further investigation is needed to understand the neurobiological substrates of this ES-induced inter-individual variance, as it might be at the basis of the vulnerability to age-related cognitive decline.

Introduction

Individuals with a history of childhood adversities have a higher probability to develop diseases during aging (Ferraro et al., 2016; Schafer and Ferraro, 2011). A few recent clinical studies indicated that childhood adversities were associated with higher prevalence and severity of mild cognitive impairment.

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in old age (Kang et al., 2017; Wang et al., 2016). Similarly, a stronger age-related decline in cognition has been observed in various rodent models of ES, for example in rats exposed to prenatal maternal restraint stress during the last week of gestation (Vallee et al., 1999) or to maternal separation (MS) stress from postnatal day (P1) to P21 (Solas et al., 2010), but exceptions exist as well (Jauregui-Huerta et al., 2015). Interestingly, maternal deprivation (MD) at P3 was further shown to alter the individual variability in cognitive performance within the aged MD group, which resulted in more impaired and unimpaired learners (Oitzl et al., 2000). However, it remains to be elucidated which neurobiological substrates may underlie such differences in cognitive decline after ES exposure.

Aging is accompanied by many structural and functional changes in the brain. For example, age-related cognitive decline in rodents has been associated with reduced hippocampal volume and neuroplasticity, including reductions in neurogenesis and synaptic plasticity measures (for reviews on this topic see Foster, 1999; Kempermann, 2015; Lynch et al., 2006; Rosenzweig and Barnes, 2003). Brain aging is further characterized by cellular aging or senescence, a concept that refers to the phenotypic changes in cellular morphology and in the RNA expression pattern of both neurons and glia. This phenotype has been collectively termed as the senescence-associated secretory phenotype, and includes, but is not limited to, a persistent upregulation of pro-inflammatory markers, alterations in oxidative stress and in growth factor expression (Coppé et al., 2010; Tan et al., 2014).

ES-induced cognitive impairments are further often accompanied by specific changes in the hippocampus. Preclinical rodent models of ES exposure have demonstrated a decrease in hippocampal volume early in life which lasts into adulthood (Hoeijmakers et al., 2017; Naninck et al., 2015). Furthermore, ES exposure in rodents reduced dendritic complexity, spine numbers and hippocampal neurogenesis in adulthood (Ivy et al., 2008; 2010; Naninck et al., 2015; Rice et al., 2008; Solas et al., 2010; Wang et al., 2013). In addition, ES exposure also affected hippocampal microglia in adult mice and altered their response to inflammatory insults (Hoeijmakers et al., 2017; Ślusarczyk et al., 2015), suggesting that ES might sensitize or prime microglia such that they respond differently to later challenges.

Many of the above measures are also affected by aging and these ES-induced deficits might therefore be aggravated in aged individuals. Indeed, pre-clinical studies have shown that long-term potentiation of CA1-CA3 synapses was stronger impaired in 16-month-old MS-exposed rats in comparison to unstressed rats (Sousa et al., 2014). In addition, MS reduced mRNA expression of the activity-regulated cytoskeleton-associated protein (ARC) in the adult hippocampus and, importantly, this specific decrease became stronger with age in MS-exposed rats, relative to aged unstressed rats (Solas et al., 2010). Finally, ES has been described to decrease stress-induced corticosterone levels and to alter expression of corticosterone receptors in the hippocampus in MD-exposed aged (31-33 months) rats (Workel et al., 2001). While these examples show that ES can affect various measures during aging, a comprehensive study on how ES impacts cognitive impairments and age-related changes in the brain of aged mice is lacking.
In this study, we therefore tested if aging would differentially regulate: 1) cognitive functioning in 19-month-old ES mice compared to control (Ctrl) mice. In addition, we studied 2) if ES altered body weight, adrenal and thymus weight and corticosterone levels in the aged mice. In the hippocampus, we further investigated if 3) neuroplasticity and 4) the expression of neuroinflammatory factors were differently regulated by ES in mice at 20 months of age, as well as in comparison to younger ES-exposed mouse groups of ages 4 and 10 months.

2. Materials and methods

2.1 Early-life stress paradigm and housing

C57BL/6J male mice were bred in house to standardize their perinatal environmental conditions. Breeding of the experimental animals and the early-life stress (ES) paradigm were performed as previously described (Hoeijmakers et al., 2017; Naninck et al., 2015). Briefly, dams and pups were weighted on P2 and randomly placed in a control (Ctrl) cage with standard bedding and nesting material (one square cotton) or in the early-life stress condition (ES) with half the amount of nesting material (1/2 square cotton), no bedding material, and a fine-gauge stainless steel mesh raised 1 cm above the cage floor. Dams and pups were left undisturbed until P9, when they were weighted and placed in standard cages in the housing room until weaning at P21.

A total of 15 Ctrl males out of 6 litters and 18 ES males out of 8 litters were included in the study. Next to this, Ctrl and ES mice of 4 and 10 months of age (n=4 per group) were included in order to address age-related gene expression patterns. Mice were housed with two to four littermates per cage under standard housing conditions, defined as a temperature of 20–22°C, 40–60% humidity, cage enrichment and ad libitum standard chow and water. The mice were kept on a standard 12/12h light/dark schedule (lights on at 8 AM) until 16 months of age and then transferred to a reversed 12/12h light/dark schedule (lights on at 8 PM) to allow a long period of acclimatization prior to behavioral analysis at 19 months. Body weight of the males was monitored at 13, 18, 19 and 20 months of age.

2 out of the 33 males were excluded before the end of the study; one ES male was sacrificed at 18 months with a severe back lesion, one ES male died prematurely of an unknown cause. Experimental procedures were conducted according to the Dutch national law and European Union directives on animal experiments, and approved by the animal welfare committee of the University of Amsterdam.

2.2 Behavioral analysis

19-month-old mice were tested for basal locomotion and anxiety-related behavior in the open field (OF) and 7 days later in the Morris water maze (MWM) for cognitive performance (see Fig. 1A for an experimental design). Behavioral testing was conducted during the dark phase to accommodate to their natural, active period and the testing room was lid by three red-light spots (25W). To habituate, the mice were transferred to the room either 4 hours (OF) or 1 hour (MWM) prior to testing (Naninck et al., 2015). Behavioral testing was recorded and mice were tracked for automated analysis of locomotion, velocity and position using Ethovision software (Noldus, The Netherlands).
Effects of early-life stress on cognition, neuroplasticity and neuroinflammation in aged mice

2.2.1. Open field
The OF consisted of a rectangular arena (54 x 37 x 33 cm) filled with sawdust bedding. The arena was cleaned in-between trials with 25% ethanol and half the amount of sawdust was replaced. The middle part of the arena (27 x 18.5 cm) was assigned as the center zone. Mice were allowed to explore the arena for 10 minutes. Distance moved, locomotion, velocity and time in the center zone were analyzed.

2.2.2. Morris water Maze
Mice were handled for 4 consecutive days in the testing room prior to MWM start. The MWM protocol included 3 cued trials on day 1 (60 sec trials; 15 min inter-trial time), followed by 3 daily acquisition trials (60 sec trials; 15 min inter-trial time) for 6 consecutive days, and finally a single 60 sec-probe trial 4 hours after the last acquisition trial (Kohman et al., 2013; van Praag et al., 2005). For all trials, the mice were placed in the MWM pool at different, random starting positions to exclude ego-centric learning strategies and were placed in front of an infrared (heating) lamp for ±1 min after the trial to prevent hypothermia.

For the cued trials, the mice were place in the circular MWM pool (110 cm diameter), filled with clear water (24±1°C), and a visible 12 cm diameter, circular platform in the middle of the maze. Mice needed to reach the platform within 60 sec, and were otherwise guided to the platform and placed on top for 15 sec. For the acquisition trials, the pool was surrounded by spatial cues to support allo-centric spatial navigation, 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 the target quadrant (Tq). During the probe trial, the platform was removed from the Tq to record to time in Tq. The escape latency, swim path and speed were analyzed for each of the trials.

2.2.3. Classification of aged unimpaired/impaired mice for analysis of inter-individual variance
We further investigated the individual differences in MWM performance within the Ctrl and ES group by a classification of impaired and unimpaired performers (Gallagher et al., 1993; Oitzl et al., 2000). Classification of the MWM performance was adapted from these studies and was based on MWM data of 17 young 4-month-old unimpaired mice from in our group (Naninck et al., 2015; 2017). The young mice improve their escape latency during MWM acquisition training after the first two acquisition days. They successfully locate the platform on the last days of acquisition training within 15 seconds in >50% of the trials, and generally don’t show “unsucessful” trials with an escape latency over 50 seconds. We used this information to calculate a learning index (LI) defined as the percentage of successful trails (<15s) subtracted by the percentage of unsuccessful trials (>50s) for both young mice and aged mice. The calculated learning index was used to classify the aged mice as aged unimpaired (AU) when the LI > average of young mice, and as aged impaired (AI) when or LI < average of young mice.

2.3 Corticosterone measurements
Two days prior to sacrifice, tail blood was collected in EDTA tubes with a tail cut at beginning of the light-phase (08:00-08:15 PM) and at the beginning of the dark-phase (08:00-08:15 AM) for analysis of
the respective circulating basal and peak corticosterone levels. Blood samples were centrifuged for 15 min at 14,000 RPM, 4°C to isolate the blood plasma. Corticosterone concentrations were further measured with a high-sensitive corticosterone enzyme immunoassay (IDS Ltd, Boldon Colliery, UK).

2.4 Tissue collection and processing

8 Ctrl males and 11 ES males were sacrificed for later immunohistochemical purposes by transcardial perfusion as previously described, using the same procedures to obtain 40 µm coronal sections in 6 parallel series (Hoeijmakers et al., 2017; Naninck et al., 2015).

7 Ctrl and 5 ES 20-month-old males, as well as all mice of 4 and 10 months of age were sacrificed by rapid decapitation, and the hippocampi were rapidly dissected, frozen and processed for gene expression analysis with qPCR as previously described (Hoeijmakers et al., 2017). The adrenals and thymus were furthermore dissected and weighted and the skin and organs were inspected for age-related (macroscopic) abnormalities as an indicator of general health.

2.5 Immunostaining for DCX and quantification

2.5.1 DCX immunostaining

Immunohistochemistry for DCX, which is expressed by newborn cells from a few days after their birth until their early adult neuronal stage, is generally used as a marker for adult neurogenesis. Free-floating sections were washed in 0.05 M TB, pH 7.6 prior to staining and in-between all incubation steps. After initial washing, sections were incubated for 15 min in 0.3% H₂O₂ in TBS and then they were incubated for 30 min in 2% milk in TBS, and subsequently with 1:1600 goat anti-DCX (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in supermix (0.1% Triton X-100, 0.25% gelatin in TBS) for 1 hour at room temperature followed by overnight incubation at 4°C. The secondary biotinylated donkey anti-goat antibody (1:500, Jackson Laboratories, Bar harbor, ME, USA) was incubated for 2 hours in supermix, followed by 90 min incubation with of avidin-biotin complex (ABC) 1:800 in TBS (Vecstain elite ABC- peroxidase kit, Brunswig Chemie, Amsterdam, Netherlands). Sections were incubated for 30 min with 1:500 tyramide in 0.01% H₂O₂ in TBS to amplify the immunoreactive signal, followed by a second 1:800 ABC in TBS incubation for 90 min. Finally, sections were thoroughly washed in 0.05 M TB (pH 7.6) and incubated in 0.2 mg/1 ml diaminobenzidine, 0.01% H₂O₂ in 0.05 M TB for chromogen development. Afterwards, free-floating sections were thoroughly washed in TBS and mounted from 0.01M PB (pH 7.4) on pre-coated glass slides (Superfrost Plus slides, Menzel – Gläser, Braunschweig, Germany). Sections were counterstained with hematoxylin and cover-slipped.

2.5.2 DCX quantification

8 coronal, bilateral sections of the hippocampus were selected for quantification, with 4 sections rostral of bregma -2.30 mm and 4 sections caudal of bregma -2.30 mm and a ± 300 µm intersection distance, to obtain an even representation of the hippocampus over the rostral-caudal axis. All quantification procedures were performed by a researcher unaware of the experimental conditions.

DCX immunoreactive cells in the granular cell layer (GCL) and subgranular zone (SGZ) were quantified manually on a Nikon Eclipse Ni-E light microscope with a Nikon DS-Ri2 Camera (Nikon, Tokio, Japan).
DCX immunoreactive cells were further distinguished based on their morphological appearance to reflect their relative maturity or developmental stage; (I) horizontal cells without a process reflected immature mitotic cells, (II) cells with an apical process into the GCL reflected an intermediate stage and (III) cells with a dendritic tree reaching to the molecular layer reflected the immature neuronal stage (Hoeijmakers et al., 2018). The Cavalieri principle was employed in order to estimate the volume of the GCL and DG as previously described (Hoeijmakers et al., 2017; 2018).

In order to analyze how ES affects DCX+ cell numbers throughout life, we included the data from one of our previous studies on DCX+ cells in Ctrl and ES mice at 4 and 10 months of age (see Chapter 4). A total of 9 Ctrl and 11 ES 4-month-old mice and 8 Ctrl and 9 ES 10-month-old mice were used for the analysis. The DCX+ immunostaining and quantification in this study followed the same protocols as described for the 20-month-old mice.

### 2.6 Gene expression measurement with RT-PCR

Gene expression analysis was performed as previously described (Hoeijmakers et al., 2017). Briefly, relative gene expression of microglial genes was assessed by PCR amplification of cDNA using Hot FirePol Evagreen qPCR supermix (Solis Biodyne, Tartu, Estonia), and cytokine gene expression was measured by the use of Taqman* probes (Applied Biosystems, Foster City, CA, USA). All primer pairs were tested for efficient amplification of 90-110%. Relative gene expression was quantified using the 2$^\Delta\Delta$ct method. Reference genes not affected by the experimental conditions and satisfied the requirements for reference target stability as calculated using qBASE software (Biogazelle, Gent, Belgium).

The primers were: *Rpl13a* forward primer (fw) 5’CCCTCCACCCCTATGACAAGA3’, reverse primer (rev) 5’TGGCCTGTTCGTAACCCTC3’; *Rpl0* fw 5’GCTTCATTGTGGAGCAGACA3’, rev 5’CATGGTGTTCTTGGCCCATACG3’ and *Sdha* GTTGCTGTGTGGCGTACTG3’, rev 5’GCGCAATGGAATACGGACAC3’ for the reference genes. Target genes: *Iba1* Fw 5’AACAAGACACAGAGGGCCAACT3’, rev 5’TGTGACATCTCACCTCAATCAG3’; *Cdh6* Fw 5’TGGACAAGGGACACCTGGG3’, rev 5’GGAGGACAGCGACGATG3’; *Cd11b* Fw 5’GGGTACCTTGACTACGTAATTGG3’, rev 5’CGTGTACCAGCTGGCTTA3’; *Ccr2* Fw 5’AGGGAGACAGCATCGAGT3’, rev 5’ACAACCCAACCGAGACCTT3’; *Ccl2* Fw 5’AGCTGTAGTTTTTGTACCAACG3’, rev 5’GTGCTGAAACCTAGGGA3’; *Cx3cr1* Fw 5’ACCGGTACCTTGCCATCTTAG3’, rev 5’AGTACCCCGACACTCGTGG3’; *Cx3cl1* Fw 5’GGAGAAGAGTAGAGACCTACAG3’, rev 5’GTGTGCCATCAAGGAG3’; *Nlrp3* Fw 5’CAGGCCAGCTGGDGAGACACAG3’, rev 5’GCGGCTTTCTTGCTCCTGATA3’; *Hmgb1* Fw 5’CGGAGAAGCTTCAGACCG3’, rev 5’AGAGGCGGATGTCCCTCCAAG3’; *Axl* Fw 5’AGACAGATGGGCTTGGGTACTG3’, rev 5’GAAAGGAGCTTTCCAGCGCA3’; *Dectin* Fw 5’AAAGCAGCAATTCGCTCTCC3’, rev 5’GGGCTTCCACTGATGCG3’; *Spp1* Fw 5’TTCAATGAAAGGCGATGACCA3’, rev 5’CGAGCTGAGGGCGATGAGG3’; *Cd11c* Fw 5’GTAGGTCTATTGTTGGGCTTCCA3’, rev 5’CAGGGGTAGACAGAGATG3’; *Psd95* Fw 5’TACCTAAAGCTGGCAGAC3’, rev 5’TCATGGTACCGAGTCTGAG3’ and *Synapsin1* Fw 5’CAGCAACATACCTGTGGG3’, rev 5’GGTTCTCCAGTACCCGCA3’. Used qtaqman probes were: Mm00446973_m1 and Mm00725448_s1 for the respective reference genes TBP and RPL0, and for the target genes: interleukin (IL)-1β Mm00432428_m1, IL-6 Mm00446190_m1, and TNFα Mm00443258_m1.
2.7 Statistical analysis

Data are expressed as the mean ± standard error of the mean (SEM) and were analyzed using Graphpad Prism 6 (Graphpad, San Diego, CA, USA) or SPSS 20.0 (IBM, Armonk, NY, USA). Significant outliers were tested by means of a grubb’s test; one Ctrl mouse was excluded from all behavioral data analyses. Data were considered statistically significant when p < 0.05.

In this study, multiple mice from the same litter were included in the experimental groups. Therefore, the degree to which litter influenced the dependent variables was statistically addressed and “litter” was introduced in further analyses as a random factor when it significantly contributed. All single-factor parameters were analyzed with “condition” as independent factor using the unpaired Student’s t-test. Probe-trial performance was analyzed with the one-sample t-test (hypothetical value 25%). The repeated-measures ANOVA was used to analyze MWM data with “acquisition day” as a repeated factor and “condition” as independent factor, and Bonferroni post-hoc testing. Pearson correlations and linear regression analyses were employed to address the contribution of body weight to OF exploration and to MWM escape latency and path length. The distribution of Ctrl and ES MWM performance over the AU and AI groups was analyzed with the Pearson’s chi-squared ($\chi^2$) test.

3. Results

3.1 ES leads to an amplification of individual differences in MWM performance

The mice were tested for basal exploratory behavior in the OF and for cognitive performance in the MWM at 19 months of age (Fig. 1A). Locomotion behavior was addressed in the OF to examine exploratory behavior, anxiety and possible movement abnormalities. The total distanced moved in the OF as well as the walking speed in the OF were not different between Ctrl and ES mice ($t(28)=0.009, p=0.993$; walking velocity data not shown: $t(28)=0.001, p=0.991$; Fig. 1B). Locomotion behavior can be influenced by body weight of the mice, and, while ES did not affect the average body weight at 19 months of age ($t(25.24)=0.750, p=0.460$), a correlation analysis indicated that the variation in locomotion was indeed associated with body weight (pearson correlation: $r=-0.403, p=0.025$; Fig. 1C). The latency to reach the center of the OF, measured as an indicator for general anxiety, was not affected by ES ($t(27)=0.114, p=0.910$; Fig. 1D).

The learning pattern of both aged Ctrl and ES mice showed a typical saw-tooth pattern (Fig. 1E). The average latency to the platform during acquisition training did not differ between Ctrl and ES mice, and was reduced in both groups over the 6 training days (condition $F(28)=0.820, p=0.373$, acq days $F(28)=8.370, p<0.001$; post-hoc: Acq1 vs Acq3, $p=0.007$, Acq1 vs Acq4, $p=0.005$, Acq1 vs Acq5, $p<0.001$, Acq1 vs Acq6, $p<0.001$; Fig. 1F). The average swimming speed in the MWM was not different between Ctrl and ES groups ($t(28)=0.05, p=0.957$). In addition, escape latency was strongly correlated with the average path length at each acquisition day (correlations latency vs path length per acquisition day: $R^2$ ranges from 0.723 to 0.865, all correlations $p<0.001$). Controlling for body weight or swimming speed indicated that these associations were not further influenced by either factor (effect of body weight or swimming speed, $p>0.05$ for all correlations).
Effects of early-life stress on cognition, neuroplasticity and neuroinflammation in aged mice

Figure 1: MWM performance is not further impaired by previous ES exposure in 19-month-old mice
A) Locomotor activity and anxiety related behavior were assessed in the open field (OF), followed 7 days later by the Morris water maze (MWM) task in order to assess cognitive performance. The MWM protocol consisted of 1 day with cued training and 6 days of acquisition training with each 3 daily, 1 minute trials at a 15 minute-interval, followed by a probe trial 4 hours after the last acquisition trial on day (D) 7. B) Locomotion in the OF is not affected by ES. C) The locomotion in the OF is negatively correlated with the body weight of both Ctrl and ES mice. D) Latency to the center in the OF is not different between Ctrl and ES mice. E) Example traces of a Ctrl mouse and an ES mouse show the saw-tooth pattern in learning behavior which is typical for aged rodents during acquisition training. F) Both Ctrl and ES mice show a significant decrease in the time to locate the platform over the days of acquisition training. G) The average escape latency over all trials is not different between Ctrl and ES mice, although the variation in ES mice is significantly larger. H) A learning index (LI) of young adult (4 months) Ctrl mice is calculated to use the average LI (i.e. a value of 15) as a threshold for the classification of learning behavior in aged mice as aged unimpaired (AU) from aged impaired (AI). J) ES AI mice have a slower average escape latency than Ctrl AU and ES AU groups. K) The time spent in the target quadrant (Tq) during the probe trial is not significant from chance level (25%, dashed line) in both the Ctrl and ES group. Annotations: #: sig effect of acquisition day; ^: sig from Ctrl AI, @: sig from Ctrl AU; %: sig from ES AU.
The average latency over all trials was not different between the Ctrl and ES groups ($t(28)=0.906, p=0.373$). However, the overall average latency to escape over all 18 trials revealed that the variation in MWM performance tended to be higher in the ES group compared to Ctrl group ($F(13;15)=2.631, p=0.087$; Fig. 1G).

A classification of the groups based on individual performance as aged unimpaired (AU) and aged impaired (AI) allowed to further analyze this within-group variation. We based the classification of AU and AI mice on the learning index (LI) of young adult mice, i.e. an average value of 15 (Fig. 1H), and we classified aged mice with a LI>15 as AU and LI<15 as AI (Fig. 1I). The distribution of mice over the AU and AI groups was found to be independent of the early-life condition (Ctrl AU $n=9$, Ctrl AI $n=5$, ES AU $n=12$, ES AI $n=4$; $\chi^2(1)=0.408, p=0.694$), while the analysis of the average MWM escape latency over all trials indicated that Ctrl AU and Ctrl AI mice performed similar, but the ES AU group had a faster escape latency than ES AI mice ($\text{condition } F(26)=0.119, p=0.732, \text{classification } F(26)=24.319, p<0.001, \text{interaction } F(26)=6.256, p=0.019; \text{post-hoc: Ctrl AU vs ES AI } p=0.016, \text{Ctrl AI vs ES AU } p=0.014, \text{ES AU vs ES AI } p<0.001$; Fig. 1J). Further analysis of the escape latency per day showed that AI mice, independent of the early-life condition, were indeed overall slower to escape, in particular on acquisition days 3, 4 and 5 (interaction acq days with classification $F(26)=2.736, p=0.022$; post-hoc: acq3 $p=0.004$, acq4 $p<0.001$, acq5 $p=0.034$; data not shown). During the probe trial, neither Ctrl nor ES mice performed above chance-level (difference from chance-level (25%) Ctrl: $t(13)=0.169, p=0.868$; ES: $t(15)=0.284, p=0.780$; Fig. 1K).

### 3.2 Physiological parameters in 20-month-old mice are not affected by ES.

The body weight of 20-month-old Ctrl and ES mice did not differ ($t(29)=0.252, p=0.800$; Fig. 2A). Interestingly, the body weight of all groups was similar at 13 months of age, and increased in AI classified mice to a significantly higher body weight at 19 and 20 months relative to AU classified mice, irrespective of condition (condition $F(1,26)=0.504, p=0.484$, classification $F(1,26)=1.811, p=0.190$, interaction $F(1,26)=0.166, p=0.687$, body weight*classification $F(1,26)=6.958, p=0.011$; post-hoc: impairment 18 months $F(1,26)=3.984, p=0.057$; impairment 19 months $F(1,26)=6.369, p=0.018$; impairment 20 months $F(1, 26)=7.609, p=0.011$; Fig. 2B).

ES did not modulate the relative weights of the thymus and adrenal glands (Thymus weight $t(27)=1.104, p=0.279$; Adrenal glands weight $t(29)=0.752, p=0.458$; Fig. 2C-D). The basal level of circulating corticosterone at the beginning of the light phase, and at peak levels at the onset of the dark phase, were not affected by ES either (Corticosterone basal $t(16.50)=1.757, p=0.097$; Corticosterone peak $t(25)=0.038, p=0.970$; Fig. 2E). The ES group had a lower variance in their basal corticosterone levels ($p=0.048$) and a trend to a higher variance in the peak levels ($p=0.058$) compared to Ctrl mice.

Next to this, we observed macroscopic tissue abnormalities in 9 out of 31 mice. These abnormalities included an enlarged liver (1x), tissue/tumor growth on the intestines (4x), on fat tissue (2x), on the liver (1x) and on the thymus (1x). The prevalence of these abnormalities did not differ between conditions (Ctrl 5/15, ES 4/16; $\chi^2(1)=0.261, p=0.704$).
3.3 Aged-induced changes in neuroplasticity are not aggravated by ES exposure in 20-month-old mice.

Hippocampal neurogenesis was addressed in the 20-month-old mice by immunohistochemistry for DCX+ cells in the GCL and SGZ of the DG (Fig. 3A). DCX+ cells were further classified based on their morphological appearance in a proliferative, intermediate and post-mitotic stage (Fig. 3B). The volume of the granular zone as well as of the total DG was significantly smaller in ES mice than Ctrl mice \( t(15)=2.663, p=0.018 \); Fig. 3C; DG data not shown Ctrl(8)=3.67±0.09, ES(9)=3.42±0.06, \( t(15)=2.304, p=0.036 \). The total number of DCX+ cells per hippocampus was not altered by previous ES exposure \( t(15)=0.489, p=0.632 \); Fig. 3D). ES did not affect the DCX+ cells in specific maturation stages (proliferative \( t(15)=0.769, p=0.454 \); intermediate \( t(15)=1.310 p=0.210 \); post-mitotic \( t(15)=0.004 p=0.991 \)). No regional differences in DCX+ cell numbers were observed between the two groups either (data not shown).

Comparing DCX+ cell numbers in the 20-month-old mice with numbers in 4 or 10-month-old Ctrl and ES mice showed a significant reduction with age, of approximately 1500, 250 and 40 cells at 4, 10 or 20 months respectively \( age F(2,49)=553.02, p<0.001 \). ES did not further affect these DCX+ cell numbers at any of these ages \( condition F(1,49)=0.005, p=0.945, interaction F(2,49)=0.299, p=0.743; Fig. 3E \). Interestingly, the more immature DCX+ cells residing in the SGZ decreased significantly in the 10-month-old ES group compared to age-matched controls, but not in the other groups (data not shown \( t(18)=1.021, p=0.321, t(15)=3.497, p=0.003, t(15)=1.152, p=0.267 \). In addition, hippocampal mRNA expression of the synaptic markers SYNAPSIN1 and PSD95 in the aged mice was not affected by ES (SYNAPSIN1 \( t(10)=0.903 p=0.388 \); PSD95 \( t(10)=1.387 p=0.195 \); Fig. 3F).
3.4 Exposure to ES does not affect the hippocampal expression profile of neuro-inflamatory markers in aged mice.

Expression of the pro-inflammatory cytokines IL-1β, IL-6 and TNFα in the hippocampus was not different in 20-month-old ES mice when compared to Ctrl mice (IL-1β $t(10)=0.001$, $p=0.999$; IL-6 $t(10)=-0.212$, $p=0.836$; TNFα $t(10)=-0.374$, $p=0.716$; Fig. 4A). CD11b, CD68 and Iba1 (general microglial markers) mRNA expression patterns were not affected by previous ES exposure either (CD11b $t(10)=0.368$, $p=0.721$; CD68 $t(10)=-0.877$, $p=0.401$; Iba1 $t(10)=-0.275$, $p=0.789$; Fig. 4B). HMGB1 and NLRP3 are damage-associated molecular patterns (DAMPs) and were not differently expressed in aged Ctrl and ES mice (Hmgb1 $t(10)=-0.315$, $p=0.759$; Nlrp3 $t(10)=-1.089$, $p=0.302$; Fig. 4C). Hippocampal mRNA expression of the chemokines CX3CL1 and its respective receptor CX3CR1 (CX3CL1 $t(10)=-1.209$, $p=0.254$; CX3CR1 $t(10)=-0.826$, $p=0.428$; Fig. 4D) or the chemokine CCL2 and its respective receptor CCR2 (CCL2 $t(10)=-0.288$, $p=0.779$; CCR2 $t(10)=-0.761$, $p=0.464$; Fig. 4E) did not differ between Ctrl and ES-exposed mice at 20 months.
The expression profile of several microglial priming-related factors revealed typical age-related alterations in 20-month-old mice compared to younger (4 and 10 months) groups. AXL was expressed significantly less in the hippocampus of 10-month-old mice than in 20-month-old mice, irrespective of the early-life condition (age F(21)=4,497, p=0.024; condition F(21)=0.278, p=0.604; interaction F(21)=1,522, p=0.241; post-hoc: 10 vs 20 p=0.015; Fig. 5A). Hippocampal mRNA expression of CD11c, DECTIN and SPP1 in 20-month-old mice was enhanced with aging, but not modified by ES exposure (CD11c age F(17)=13.223, p<0.001; condition F(17)=0.348, p=0.348; interaction F(17)=0.952, p=0.952; post-hoc: 4 vs 20 p=0.004, 10 vs 20 p<0.001; Fig. 5B; DECTIN: age F(20)=6.131, p=0.008; condition F(20)=3.629, p=0.071; interaction F(20)=0.651, p=0.532; post-hoc: 4 vs 20 p=0.018, 10 vs 20 p=0.023; Fig. 5C; SPP1: age F(21)=10.023, p=0.001; condition F(21)=3.939, p=0.060; interaction F(21)=0.651, p=0.532; post-hoc: 4 vs 20 p=0.002, 10 vs 20 p=0.004; Fig. 5D).

4. Discussion

We show that 20-month-old C57BL6/J mice exposed to chronic ES from P2 to P9, 1) did not exhibit an aggravation in the age-related cognitive deficits, although an increase in the individual variation in MWM performance was apparent in the ES group. 2) ES-exposed mice further showed no differences in physiological measures compared to Ctrl mice at 20 months. 3) Hippocampal DG and granular zone volume were reduced in ES mice and, while hippocampal neurogenesis declined with age, this and other neuroplasticity measures were not different between Ctrl and ES groups. Finally, 4) aging altered the neuroinflammatory profile at 4, 10 and 20 months without an additional effect of ES exposure. Overall, these observations lead to the conclusion that chronic ES exposure from P2 to P9 does not aggravate age-related cognitive decline, neuroplasticity deficits and neuroinflammation in old mice.
4.1 Cognitive performance in aged rodents and individual variation with aging

This study is to our knowledge the first to address the consequences of ES for cognition in aged mice, while previous studies on this topic were conducted in rats (Jauregui-Huerta et al., 2015; Oitzl et al., 2000; Schaaf et al., 2001; Solas et al., 2010; Vallee et al., 1999). We used the MWM to address cognitive functioning in the 19-month-old Ctrl and ES mice and showed that exposure to ES did not exacerbate the cognitive decline. This is in contrast to several previous studies that described an aggravation of the decline with aging in ES-exposed rats (Solas et al., 2010; Vallee et al., 1999).

Interestingly, other studies had shown MWM performance to be highly variable in old rodents (Bizon et al., 2009; Oitzl et al., 2000) and we showed that this variance in individual MWM performance was enhanced after ES exposure. As previously shown in a MWM study on MD-exposed rats, the classification of performance as aged unimpaired, partially impaired or impaired learners revealed that a larger percentage of the MD-exposed rats was labeled as either AU or AI compared to the Ctrl group, of which most animals were partially impaired at 30-32 months of age. Although the average values of both the control and MD groups were similar, such relevant ES effects only became apparent when the individual distribution was taken into account (Oitzl et al., 2000). These data are in line with our current results in that individual extremes seem to be amplified in old age in mice subjected to ES as shown by the larger average escape latency between ES AU and AI classified ES mice, than between Ctrl AU and AI mice when we use an adapted classification approach based on an arbitrary value (Gallagher et al., 1993; Oitzl et al., 2000). However, others have indicated that aging per se does not lead to a clear-cut bimodal population of ‘good’ and ‘bad’ learners, but rather a broad learning range in performance of aged rats (Barnes et al., 1997) and this spread of learning capabilities is therefore important to consider for future interpretations.

Overall, our observations support previous data (Oitzl et al., 2000) and raise further questions. Young adult mice exposed to chronic ES have repeatedly been shown to exhibit later deficits in the MWM
and other cognitive tasks (Naninck et al., 2015; Wang et al., 2011), while the aged ES-exposed mice performed on average similar to Ctrl mice. One hypothesis that can explain this change in phenotype with age is that the ES-induced impairments at a young age might not aggravate further at later ages. This will result in a comparable level of impairment, when the performance of Ctrl mice declines with increasing age (Koh et al., 2014; Lindner, 1997). It will therefore be very informative to clarify how the trajectory of cognitive performance of ES mice changes over time in comparison to Ctrl mice, studying mice at ages younger and older than 20 months. Such an experimental design would reveal whether chronic ES in mice indeed accelerates the curve of age-related cognitive decline and whether it is a suitable model to mimic and study the cognitive decline in aging that is indicated by clinical studies on aged individuals with a history of ES.

4.2 Chronic ES does not induce lasting consequences for hippocampal neuroplasticity markers

ES exposure has been shown to reduced synaptic protein levels, spine numbers and neurogenic cell survival in young adult mice (Liu et al., 2016; Naninck et al., 2015, Wang et al., 2013). We studied DCX+ cell numbers and SYNAPSIN1 and PSD95 mRNA expression and show that these were not differentially affected in the aged Ctrl and ES groups. We confirm that DCX+ cell numbers, like the process of neurogenesis in general, show a strong age-related decline in the hippocampus (Heine et al., 2004; Hoeijmakers et al., 2018; Kuhn et al., 1996; Seki and Arai, 1995). Even though absolute DCX+ cell numbers were not affected by ES exposure in mice at 6 months (Naninck et al., 2015), or, as shown in this study, at 4 or 10 months (see Chapter 4), we had expected that ES might have further aggravated the age-related decline in cell numbers.

Although the total numbers of DCX+ cells were equal between Ctrl and ES groups at all ages, 10-month-old ES mice exhibited a reduction of immature DCX+ cells in the SGZ (see Chapter 4). This sub-regional observation was no longer present in the 20-month-old ES mice, and might therefore indicate that, although minor, the age-related decline between 10 and 20 months might be accelerated to some extent in ES mice. In addition, chronic ES in young adults specifically reduced the survival of newborn cells, and not DCX+ cell numbers, in the hippocampus at ages 4-6 months (Kanatsou et al., 2017; Naninck et al., 2015). We were, however, not able to reliably address cell survival in 20-month-old ES mice, because at this age too few mature, newborn cells will be present per animal for such a quantification. The 2-week long expression window of DCX (Brown et al., 2003; Kempermann et al., 2004), allows a reliable quantification of a substantial number of DCX+ cells and an additional analysis of different DCX+ maturation stages in both conditions.

Besides neurogenesis, we observed no difference in mRNA expression of SYNAPSIN1 and PSD95 in the hippocampus of the aged Ctrl and ES mice. These markers were reported to be reduced by ES during development (Liu et al., 2016) and to our knowledge, no previous studies have investigated these specific markers in adult mice exposed to this ES paradigm. Interestingly, spine density in adult chronic ES-exposed mice was found to be specifically reduced in the CA3 sub-region (Wang et al., 2013) and, since we measured mRNA expression levels in the total hippocampus, it is possible that our measurements were not sensitive enough to pick up on such region-specific differences within the
entire hippocampus. To further elaborate on neuroplasticity changes in ES-exposed aged mice and to exclude the possibility that more subtle ES effects remain undetected, it would therefore be useful to also study other measures of synaptic (protein) levels in a region-specific manner, using for example immunochemistry methods.

4.3 The age-induce inflammatory profile is not altered by ES exposure

We showed that aging altered the mRNA expression of several microglial priming-related genes in the hippocampus. Aging is indeed consistently reported to induce a pro-inflammatory phenotype that reflects microglial priming in the brain (Von Bernhardi et al., 2015; Holtman et al., 2015). Our data confirms that this phenotype includes, but is not limited to age-related changes in expression of Axl, Dectin, Spp1 and Cd11c (Raj et al., 2015). Aging has in addition been reported to alter cytokine expression levels (i.e. IL-1β, IL-6 and TNFα) and other inflammatory markers such as fractalkine (Bachstetter et al., 2011; Frank et al., 2010; Njie et al., 2012). Such an abnormal expression is in fact part of and to some extent reflecting on the senescence-associated secretory phenotype, seen with cellular aging (Coppé et al., 2010) and ES does thus not seem to aggravate this phenotype.

Early-life experiences, such as infection, can lead to a heightened age-related pro-inflammatory profile in aged rats, as a result from microglia that have become primed by the early-life infection (Bilbo, 2010). Priming of the neuroinflammatory response will indeed elicit an enhanced reaction, and in this case, aging itself is thought to serve as the secondary inflammatory ‘hit’. ES has been shown to affect neuroinflammation earlier in life too (Delpech et al., 2016; Hoeijmakers et al., 2017; Ślusarczyk et al., 2015), and it seems to prime or sensitize the microglial response in later life (Elwenspoek et al., 2017; Hoeijmakers et al., 2015). ES modulation of neuroinflammatory regulation might therefore require a secondary insult, before programmed responses become apparent (Hoeijmakers et al., 2017). Apparently, in our study, aging per se did not act as a secondary ‘hit’ for the neuroinflammatory expression profile in chronic ES-exposed mice. Although ES affected inflammation in younger mice (see Chapter 3 and Hoeijmakers et al., 2017), early-life infection is likely a much more potent, and longer lasting, inflammatory regulator than ES, and therefore ES might not induce additional consequences in aging.

4.4 ES as an accelerator of (cellular) aging, where does this research leave us?

Our findings of age-related changes in the brain do not support the hypothesis that ES accelerates brain aging per se and several new directions and questions have emerged that remain to be answered. While the expression of the microglial priming associated markers specifically changed between 10 and 20 months, several inflammatory factors were enhanced by ES exposure at 10 months, while this was normalized again by 20 months of age (see Chapter 3 and Hoeijmakers et al., 2017). This indicates that these expression profiles in Ctrl and ES mice particularly changed between 10 and 20 months. In addition, 20 months of age marks the beginning of age-related deficits and mortality (Flurkey et al., 2007; Forster et al., 2003) and the severe dysregulation of the brain that occurs at later ages might therefore still be differentially regulated by the early-life condition. In conclusion, the timing when age-related hallmarks are studied will be crucial to determine if the aging process is affected by early-life and ES might therefore impact the currently studied factors at other ages.
In addition, we observed an enhanced inter-individual variance in MWM acquisition within the ES mice, but we were not able to reliably address such within-group variance in relation to cognitive performance in the brain parameters. However, previous studies have shown that the altered within-group variance for cognitive performance with aging is also found in the deficits in, for example Reelin or brain derived neurotrophic factor expression, in the brain (Kennard, 2011; Stranahan et al., 2011). As an example, cognitively impaired aged rats that were exposed to MD had no increase in learning-induced brain derived neurotrophic factor (BDNF) mRNA expression, when compared to MD-exposed, unimpaired aged rats (Schaaf et al., 2001), illustrating how the within-group variance can be reflected in neuroplasticity measurements. Therefore, analysis of age-related changes on the group as well as individual level will contribute to our understanding of ES-induced alterations in aged rodents.

4.5 Conclusions
To conclude, we addressed age-related consequences of ES including changes in behavior and various brain and neuroinflammatory parameters. This is the first study to describe the consequences of chronic ES in aged mice and shows an enhanced variation in cognitive performance with aging, confirming a previously described phenotype for aged rats, whereas age-related alterations in neuroplasticity and inflammatory factors were not exacerbated by ES exposure. New questions arise from these findings and we highlight the remaining gaps in our knowledge on aging of ES-exposed individuals. This study therewith highlights the need to address age-related consequences for brain functioning in future studies, in order to better understand and identify the potential vulnerability for age-related deficits during life.

5. Acknowledgements
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6. References


Effects of early-life stress on cognition, neuroplasticity and neuroinflammation in aged mice


