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 mediated sensitization of the neuroinflammatory response to acute and chronic challenges

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- a preliminary report -
Abstract

Stress exposure during early-life (ES) is associated with later cognitive impairments and is hypothesized to enhance the progression of Alzheimer’s disease (AD)-related β-amyloid (Aβ) neuropathology. In addition, neuroinflammation can play a role in the progression of Aβ neuropathology and there is recent evidence that ES altered the inflammatory profile in the hippocampus of wild type and APP/PS1 mice, an AD mouse model that develops Aβ neuropathology and exhibits (chronic) neuroinflammatory activation as the result of this. How ES affects microglial functioning is not well understood to date. We investigated if ES alters microglial functioning in vitro and questioned if ES modulates microglia and the expression of microglial regulatory factors in vivo, under basal conditions in wild type mice and in the APP/PS1 mouse model.

Mice were exposed to ES consisting of limited bedding and nesting material from postnatal day (P)2 to P9. Primary hippocampal glial cultures were prepared from P9 Ctrl and ES pups and used to study in vitro functional changes in the (micro)glial inflammatory response to lipopolysaccharide (LPS), and the phagocytic responses to either exposure with beads or with Aβ1-42. The expression of microglial regulatory factors was further studied in the hippocampus of ES-exposed pups and adult wild type and APPswe/PS1dE9 mice at ages 4 and 10 months. In addition, the proliferation and clustering of microglia was studied in the hippocampus of Ctrl and ES APP/PS1 mice.

LPS exposure elicited an inflammatory response in primary glial cultures, a response that was exacerbated in cultures derived from ES mice. Next to this, preliminary data on the in vitro phagocytosis assays indicated that microglia from ES mice tended to internalize more beads than Ctrl microglia, while exposure to Aβ1-42 failed to elicit such an increase in internalization. The hippocampal mRNA expression profile showed that ES reduced the mRNA expression of the inflammasome component NLRP3, and of fractalkine CX3CL1 at P9. In contrast, expression of the microglial receptor CX3CR1 was enhanced in ES-exposed mice at 10 months of age. In addition, 10-month-old APP/PS1 mice had an elevated expression of NLRP3 and CCR2, whereas ES exposure in these mice lowered expression of these factors. ES further reduced the number of dividing microglia in the hippocampus of APP/PS1 mice, but not the numbers of clustered microglia at Aβ plaques.

Our findings support the concept that ES can lead to a sensitized response of microglia. The in vitro inflammatory and phagocytic response were enhanced in ES-derived cultures. In addition, ES induced a dysregulation in microglia at P9 and the chronic inflammatory stimulation induced by Aβ accumulation in the brains of APP/PS1 mice led to an abnormal, dampened inflammatory response in 10-month-old ES APP/PS1 mice. These data highlight the lasting nature of ES effects on inflammatory mediators and microglial programming. They raise the question whether an early modulation of the neuroinflammatory signaling changes in ES-exposed individuals can at least in part prevent the later-life risks associated with such exposure, for example in relation to AD.

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1. Introduction

Adverse events that occur during development, like childhood neglect, abuse or maltreatment, have been shown to induce long-lasting effects on general health and cognition (Ferraro et al., 2016; Kaplan et al., 2001; Mueller et al., 2010; Schafer and Ferraro, 2011). Although the enhanced risks of such stressful events in early-life are well-recognized to modulate brain structure and function, our understanding of the underlying systems that modulate the latent consequences for later brain functioning is less well developed. During the last few years, various clinical studies have indicated that children exposed to childhood trauma often exhibit a dysregulated immune system (Baumeister et al., 2015; Coelho et al., 2014; Dube et al., 2009). Studies of early-life stress (ES) in rodents have elaborated on these findings and have shown that, next to peripheral immune regulation, also the central immune response in the brain was altered by ES (Diz-Chaves et al., 2012; 2013; Hoeijmakers et al., 2017; Szczesny et al., 2014; Ślusarczyk et al., 2015). How ES exactly affects different microglial functions in the brain has so far barely been studied.

Microglia are the main immune cells of the brain and they mediate inflammatory regulation as well as other processes in both the developing and adult brain. In the healthy brain, microglia for instance communicate with neurons and astrocytes through the release of, and response to, cytokines and chemokines, that for example regulate phagocytosis of synapses and apoptotic cells (Ekdahl, 2012; Schafer et al., 2012; Tremblay et al., 2011). In addition, microglia have the capacity to sense pathological events that include not only activation by bacterial pathogens, but also neuropathological events, like amyloid β (Aβ) peptide accumulation. Indeed, accumulation of Aβ peptides and the formation of Aβ plaques in the brain of Alzheimer’s disease (AD) patients elicits a strong inflammatory response from microglia (Babcock et al., 2015; Baron et al., 2014). In addition, microglia are able to clear these Aβ peptides from the brain by phagocytosis, and this role of microglia is thought to mediate the progression of Aβ pathology in AD (Heneka et al., 2015; Heppner et al., 2015). Thus, these cells play a crucial role in the healthy as well as the AD brain.

We recently showed that ES exposure altered AD-related Aβ hallmarks in APPswe/PS1dE9 mice, a transgenic model of Aβ neuropathology. These alterations were associated with altered neuroinflammation. Specifically, 10-month-old ES APP/PS1 mice exhibited higher levels of Aβ neuropathology in the hippocampus with a concomitant reduction in microglial activation markers (Hoeijmakers et al., 2017). This indicated that ES might indeed enhance the risk for AD through an altered neuroinflammatory response of microglia. However, it is not clear to date which neuroinflammatory factors regulate the ES mediated changes, under both pathological, and non-pathological conditions. In addition, APP/PS1 mice exhibit Aβ-induced neuroinflammation and it remained to be answered whether ES also modulates the response to acute challenges.

We therefore set out to determine whether ES modulates microglial functioning using in vitro inflammatory and phagocytic stimuli. We then addressed how the expression of specific microglial regulating factors is affected by ES exposure in the developing brain, as well as in brains of adult wild...
type and APP/PS1 mice, an AD mouse mode that accumulates Aβ in the brain. In addition, we studied how ES affected microglial proliferation and clustering of microglial cells at Aβ deposits in these AD mice.

2. Methods

2.1 Experimental setup

All mice in this study underwent early-life stress (ES) from postnatal day (P)2 to P9, as previously described (Hoeijmakers et al., 2017; Naninck et al., 2015). Briefly, dam and pups were placed in a control (Ctrl) condition with normal bedding material and 1 square cotton nestlet (5 cm x 5 cm), or in the limited environment, with half the amount of nesting material on a stainless-steel mesh with only minimal bedding material. On the morning of P9, the mice were either sacrificed or moved to standard housing cages. This standard housing consisted of cage enrichment, ad libitum water, standard chow, 20–22°C temperature, and 40–60% humidity. Mice were weaned at P21 and thereafter housed with 2-4 same-sex littermates per cage.

Hippocampal primary glial cultures were used to study microglial functioning after ES-exposure and these cultures were derived from C57BL/6J P9 pups. The cultures were treated with lipopolysaccharide (LPS) to stimulate the inflammatory response or with phagocytic stimuli (i.e. beads or Aβ1-42) to test the phagocytic response of microglia.

The expression of hippocampal microglial regulatory factors was studied in C57BL/6J Ctrl and ES P9 pups, as well as in wild type (WT) and APPswe/PS1dE9 mice of ages 4 and 10 months. The APPswe/PS1dE9 hemizygous male mice on a C57BL/6J background and their WT littermates were subjected to the ES paradigm or Ctrl environment as previously described (Hoeijmakers et al., 2017). A number of 6 Ctrl pups from 2 litters and 6 ES pups from 3 litters were included. The number of 4-month-old mice in this study was per group: 7 Ctrl WT mice of 4 litters, 7 ES WT of 3 litters, 6 Ctrl APP/PS1 of 4 litters and 4 ES APP/PS1 of 3 litters. The number of 10-month-old mice in this study was per group: 6 Ctrl WT mice of 4 litters, 6 ES WT of 5 litters, 6 Ctrl APP/PS1 of 3 litters and 6 ES APP/PS1 of 4 litters.

We further studied microglial proliferation and microglial clustering in the close vicinity of amyloid plaques in 6 Ctrl and 4 ES APP/PS1 mice at 10 months of age. The cell birth date marker 5-bromo-20-deoxyuridine (BrdU, Sigma-Aldrich, Saint Louis, MO, USA) was injected 3 times a day with a 2-hour interval on 3 consecutive days, at a concentration of 100 mg/kg BrdU dissolved in 0.9% saline containing 0.007 M NaOH. 2 hours after the last BrdU injection, the mice were transcardially perfused with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffered saline (PBS) as previously described (Hoeijmakers et al., 2017).

2.2 Hippocampal primary glial cultures of Ctrl and ES litters

The protocol for glial cultures from male Ctrl and ES P9 hippocampi was adapted from previously described protocols for P2 cultures (Ledeboer et al., 2000; Vincent et al., 1997). Pups were rapidly
decapitated on the morning of P9 and the brains were dissected and placed in cold 0.6% glucose-containing HBSS for the isolation of the hippocampi, after removal of the meninges and choroid plexus. The hippocampi were dissociated to a single-cell suspension by titration in culture medium, which consisted of 1% Pen/Strep (15140 Gibco), 1% l-Glutamine (31550 Gibco) 10% Fetal Calf Serum (batch 42G9551K, 10270 Gibco), 44% DMEM (11965 Gibco), 44% HAM’s F10 (31550 Gibco). 24-wells plates (LPS experiment) or coverslips (phagocytosis assays) were coated with 15 µg/ml poly-l-lysine (P6282 Sigma-Aldrich, Saint Louis, MO, USA) for 30 min and air-dried for 2 hours prior to use. 500 µl of the single-cell suspension was plated to obtain 250,000 cells in each well. Cells were kept for 10 days in a 37°C, 5% CO$_2$ incubator and the medium was refreshed on days in vitro (DIV)1, DIV4 and DIV7.

2.2.1 LPS-induced inflammatory response in Ctrl and ES primary glial cultures

The cells were treated with 0 ng/ml, 1 ng/ml, 10 ng/ml or 100 ng/ml LPS (E. Coli, clone 055:B5, BD Difco) on DIV10 to elicit an inflammatory response. After 4 hours, part of the wells were fixed with 4% PFA in culture medium at room temperature for later immunocytochemistry (ICC) analysis and stored at 4°C. The remaining cells were lysed in 200 µl TRizol (Invitrogen, Carlsbad, CA, USA) for qPCR expression analysis and stored at -80°C. 3 independent Ctrl and 3 independent ES cultures were used and each LPS concentration was repeated in 2 wells per culture for both the ICC and qPCR analysis.

2.2.2 Phagocytosis assays in Ctrl and ES primary glial cultures

The cells were treated with either Alexa488-conjugated beads (L4655, Sigma-Aldrich, Saint Louis, MO, USA) or with Hilite™Fluor 647-labeled human β-amyloid1-42 (AS-64161, Anaspect, Fremont, CA, USA). The particle size of the Alexa488-conjugated beads was 1.0 µm and the beads were coated with carboxylate-modified polystyrene latex. The cells were treated with 1:400 beads in medium for 30 min. Hilite™Fluor 647-conjugated human Aβ1-42 peptides were dissolved in 0.1 M PBS to obtain a 125 uM concentration, and were incubated at 37°C for 24 hours to allow to formation of fibril aggregation, while shaken every few hours, and stored at -80°C (Chakrabarty et al., 2010; Jones et al., 2013; Michelucci et al., 2009). The Hilite™Fluor 647-conjugated Aβ was vortexed for 10 seconds prior to use to brake-down large aggregates, and then administrated to the cells in a 0.5 µM concentration for a duration of 30 min. After treatment, the cells were fixed with 4% PFA in culture medium at room temperature for later ICC analysis. The bead assay was analyzed in 5 Ctrl and 4 ES cultures (3 wells per culture) and the Aβ1-42 assay analysis was analyzed in 3 Ctrl and 3 ES cultures, using 3 wells per culture.

2.3 Immunocytochemistry of mixed glial cultures

The PFA-fixed cells treated with LPS were stained to control for possible differences in the ratio of astrocytes and microglia in the cultures. The cells treated with either beads or Aβ1-42 were stained to analyze internalization of the respective treatment-agent by microglia and astrocytes.

A-specific antibody binding was blocked by incubating the cells with 1% bovine serum albumin for 30 min. This was followed by primary antibody incubation with 1:1000 rabbit anti-Iba1 (190-19741 Wako, Osaka, Japan) and 1:1000 mouse anti-GFAP (MAB3402 clone GAS Merck-Millipore, Billerica, MA, USA) in 0.3% triton X-100, 0.1 M PBS at room temperature for 1 hour, followed by overnight incubation at
4°C. Cells were washed with 0.1 M PBS and incubated with the following 1:1000 secondary antibodies in 0.1 M PBS (Invitrogen, Carlsbad, CA, USA): donkey anti-rabbit Alexa488 plus goat anti-mouse Alexa568 for the LPS cultures and Aβ1-42 cultures and goat anti-rabbit Alexa568 plus goat anti-mouse Alexa647 for the bead cultures. After washing, the nuclei were stained with 1:10,000 Hoechst for 30 seconds (Sigma-Aldrich, Saint Louis, MO, USA) and cover-slipped with Vectashield (Vector Laboratories, Burlingame, CA, USA).

2.3.1 Analysis of LPS-treated glial culture ICC
LPS-treated cells were imaged with a Leica DM IL inverted microscope setup (20x objective). A number of 4 semi-random images were taken per well, avoiding the rim or center of the field, to obtain a representative overview of the culture. The number of Iba1+ microglia, GFAP+ astrocytes and Iba1-/GFAP-/Hoechst+ nuclei were counted manually within the images.

2.3.2 Analysis of phagocytosis assays
The beads and Aβ1-42 treated cells were imaged with a Nikon Ti confocal setup (60x objective). 4 z-stacks (1 µm inter-slice distance) were taken per coverslip to obtain a representative overview of the culture. Iba1+ microglial and GFAP+ astrocytes were outlined in these confocal images using FIJI/Image J software and the presence of fluorescent signal from beads or Aβ1-42 was measured within these cells. The average number of internalized beads and Aβ1-42 was calculated per cell. The average internalization of beads or Aβ1-42 was calculated per coverslip and taken as an individual measurement for the statistical analysis.

2.4 Immunofluorescent staining of brain sections
2.4.1 Immunostaining protocol
PFA-fixed brain tissue was processed in preparation for immunostaining to obtain 40 µm coronal section as previously described (Hoeijmakers et al., 2017). The sections were stained for BrdU to label proliferating cells (1:500 rat anti-BrdU, Accurate Chemical and Scientific Corporation OBT0030, Westbury, NY, USA), Iba1 as a marker for microglia/ macrophages (1:5000 rabbit anti-Iba1, 019-19741, Wako Chemicals, Neuss, Germany) and for β-amyloid using the monoclonal antibody 6E10 (1:1500 mouse anti-human Aβ 1-16, SIG-3932-1000, BioLegend, San Diego, CA, USA). The sections were mounted on pre-coated glass slides (Superfrost Plus slides, Menzel – Gläser, Braunschweig, Germany) and dried overnight. Sections were washed between all steps with 0.05 M tris buffered saline (TBS) pH 7.6. The tissue was pretreated with 0.01 M citrate buffer pH 6.0 for 15 minutes in a microwave to reach and maintain a temperature of ±95°C for ±10 minutes. After cool-down to room temperature, all sections were incubated for 30 min in blocking mix containing 1% bovine albumin serum, 0.1% triton X-100, in 0.05 M TBS. Primary antibodies were diluted in the blocking mix and incubated for 2 hours at room temperature, followed by an overnight incubation at 4°C. Alexa fluor-conjugated secondary antibodies were diluted 1:1000 in blocking mix and incubated for 2 hours (donkey anti-rabbit Alexa488, goat anti-rabbit Alexa568 and goat anti-mouse Alexa647, Invitrogen, Carlsbad, CA, USA) and then cover-slipped with Vectashield (Vector Laboratories, Burlingame, CA, USA).

2.4.2 Analysis of proliferating BrdU+ cells and microglial clustering at plaques
3 bilateral sections in the caudal part of the hippocampus (bregma >2.30 mm) were selected with a
±300 µm intersection distance. A 750 µm by 400 µm z-stack (5 µm inter-slice distance) was imaged in the dentate gyrus (DG) molecular layer (ML) with a Nikon Ti confocal setup (60x objective), resulting in 6 large-field images per animal that were used for the analysis of BrdU+ cells. The plaques that were visible in the middle slice of the z-stack were further imaged with a 30 µm Z-stack (1 µm steps) for the analysis of iba1+ cells at plaques.

Images were processed with FIJI/Image J software. The number of BrdU+/iba1+/DAPI+ cells and BrdU+/iba1-/DAPI+ cells were quantified as a measure for microglial and other cell type proliferation. The volume of the imaged plaques was measured using the ImageJ 3DViewer plugin after setting a threshold to specifically select the plaque. The iba1+/DAPI+ cells, as well as BrdU+ cells, were then counted around each plaque as an indicator of microglial clustering.

2.5 RT-qPCR for mRNA expression analysis

The mice were sacrificed by rapid decapitation at P9, 4 months or 10 months of age, after which the hippocampus was rapidly dissected and stored at -80°C for the analysis of mRNA expression of different microglial regulators. The conversion of extracted RNA to cDNA from frozen hippocampal unilateral tissue and TRIzol-lysed cells was performed as previously described (Hoeijmakers et al., 2017). Relative gene expression of factors regulating microglial functioning in the hippocampus and LPS-induced cytokine release were assessed by PCR amplification of cDNA using Hot FirePol Eva-green qPCR supermix (Solis Biodyne, Tartu, Estland), and measured by the 7500 Real-time PCR system (Applied Biosystems, Foster City, CA, USA). All primer pairs had an amplification efficiency of 90-110%. Multiple reference genes were used for normalization. These reference genes were not differentially expressed between experimental groups and they met the requirements for reference target stability quality control, M < 0.5, CV < 0.25 (Derveaux et al., 2010; Hellemans et al., 2007), as calculated using qBASE software (Biogazelle, Gent, Belgium). The fold change in relative gene expression of the target genes was finally calculated using the 2^∆∆CT method, after normalization for 2 to 3 reference genes. Selected reference genes: Rpl0, Rpl13, α-tub for LPS-induced cytokine measurements; Rpl0, Rpl13, Sdha for the P9 hippocampal measurements; Rpl0, Rpl13, Sdha for measurements in tissue of 4-month-old mice; Rpl0, Rpl13 for measurements in tissue of 10-month-old mice.

Primer sequences: Rpl13a forward (fw) 5’-CCCTCCACCTATGACAAGA-3’, reverse (rev) 5’-TCGCCCT-GTTCGCGTAACCTC-3’; Rpl0, (fw) 5’-GGTTGTGGAGGACAGACA-3’, rev 5’-CATGGTGTTGTCGCCATCAG-3’; Sdha, fw 5’-GGTTGTGGAGGACAGACA-3’, rev 5’-GCACAGTGCAATGACACC-3’; A-tub, fw 5’-GGTTGTGGAGGACAGACA-3’, rev 5’-GCACAGTGCAATGACACC-3’; Hmgb1, fw 5’-GGTTGTGGAGGACAGACA-3’, rev 5’-GCACAGTGCAATGACACC-3’; Ccl2, fw 5’-AGGGAGACAGCAGATCGAGTG-3’, rev 5’-TGGTGTGGAGGACAGACA-3’; Ccr2, fw 5’-AGGGAGACAGCAGATCGAGTG-3’, rev 5’-TGGTGTGGAGGACAGACA-3’; Cx3cl1, fw 5’-GGTTGTGGAGGACAGACA-3’, rev 5’-TGGTGTGGAGGACAGACA-3’; Cx3cl1, fw 5’-GGTTGTGGAGGACAGACA-3’, rev 5’-TGGTGTGGAGGACAGACA-3’; Cx3cr1, fw 5’-AGGGAGACAGCAGATCGAGTG-3’, rev 5’-TGGTGTGGAGGACAGACA-3’, IL-1β, fw 5’-GGTTGTGGAGGACAGACA-3’, rev 5’-TGGTGTGGAGGACAGACA-3’.
### 2.6 Statistics

All quantification procedures were performed by a researcher blind to the experimental conditions. Data are expressed as the mean ± standard error of the mean (SEM) and were analyzed using Graphpad Prism 5 (Graphpad, San Diego, CA, USA) or SPSS 20.0 (IBM, Armonk, NY, USA). Significant outliers were tested by means of a Grubb’s test (Graphpad, San Diego, CA, USA) and data were considered statistically significant when p < 0.05. Multiple males (2-4) from one litter were included in this study, resulting in nested data. The effect of the nested factor “litter” was tested with a multilevel approach with the inclusion of litter as a random factor. The mRNA expression was analyzed using two-way ANOVA designs with either the factors “condition” and “genotype” (hippocampus) or “condition” and “treatment” (LPS cultures). Bonferroni post-hoc testing was employed when required. The association between plaque volume and number of clustered Iba1+ cells was addressed by Pearson’s correlation. Unpaired Student’s t-tests were used for all single-factor dependent variables.

### 3. Results

#### 3.1 LPS-induced inflammatory signaling is exacerbated in ES-derived mixed glial cultures

The inflammatory response of Ctrl and ES glial cells was assessed in vitro with a mixed glial culture containing both astrocytes and microglial cells after treatment with the LPS at DIV10 (Fig. 1A). The culture consisted of Iba1+ microglial cells and twice the number of GFAP+ astrocytes at DIV10 (Fig. 1B). The proportion of microglial Iba1+ cells and astrocytic GFAP+ cells was not different between Ctrl and ES cultures (data not shown; ratio astrocytes/microglia Ctrl: 2.1 ± 0.4, ES: 2.7 ± 0.7, t(6)=0.736, ns). Next to this, the number of cells at DIV10 and ICC phenotyping of microglia and astrocytes did not correlate with the mRNA expression profile (data not shown).

Although the mRNA expression of HMGB1 was not induced by LPS exposure or ES per se (condition F(1,30)=0.609, ns, treatment F(3,30)=1.646, ns, interaction F(3,30)=1.459, ns; Fig. 1C), LPS treatment elicited a very potent inflammatory response in both ES and Ctrl cultures (NLRP3: condition F(1,33)=29.291, p<0.001, treatment F(3,33)=22.317, p<0.001, interaction F(3,33)=0.460, ns; IL-1β: condition F(1,39)=26.576, p<0.001, treatment F(3,39)=66.530, p<0.001, interaction F(3,39)=0.760, ns; IL-6: condition F(1,39)=30.062, p<0.001; treatment F(3,39)=47.926, p<0.001; interaction F(3,39)=5.609, p=0.003; Fig. 1D-F). Post-hoc analysis indicated that NLRP3, IL-1β and IL-6 mRNA expression were significantly upregulated by 1 ng/ml or higher concentrations of LPS in Ctrl and ES cultures (for both Ctrl and ES cultures: 0 ng/ml treatment group was sig from 1 ng/ml, 10 ng/ml and 100 ng/ml treatment groups for Nlrp3, IL-18 expression and IL-6 with p<0.01; and 1ng/ml was significant from 100 ng/ml treatment as well for IL-6 expression with p<0.01). Next to this, the LPS-induced increase in NLRP3, IL-1β and IL-6 was more potentiated in ES cultures than Ctrl cultures, in particular for IL-6 mRNA expression (NLRP3: post-hoc Ctrl 1 ng/ml vs ES 1 ng/ml p=0.021, Ctrl 10 ng/ml vs ES 10 ng/ml p=0.002, Ctrl 100 ng/ml vs ES 100 ng/ml p=0.002; IL-1β: post-hoc Ctrl 1 ng/ml vs ES 1 ng/ml p=0.010, Ctrl 10 ng/ml vs ES 10 ng/ml p=0.002, Ctrl 100 ng/ml vs ES 100 ng/ml p=0.008; IL-6: post-hoc Ctrl 1 ng/ml vs ES 1 ng/ml p<0.001, Ctrl 10 ng/ml vs ES 10 ng/ml p<0.001, Ctrl 100 ng/ml vs ES 100 ng/ml p<0.001).
3.2 Preliminary indications for an ES-induced increase of the in vitro phagocytic response to beads but not to Aβ1-42

3.2.1 Phagocytic assay with beads

The phagocytic response of glial cells was tested by in vitro exposure to beads (Fig. 2A). Microglia internalize such beads (Fig. 2B), as indicated by the presence of Alexa488 beads within the Iba1+ microglial cell (Fig. 2C). Microglia showed a high number of internalized beads, with an average of 19.8 beads in Ctrl microglia and 36.9 beads in ES microglia, thus showing a very strong trend to enhanced internalization of beads after ES ($t(24)=1.999, p=0.057$; Fig. 2D). Both Ctrl and ES astrocytes internalized few beads, with an average of 4.6 and 5.0 beads per astrocyte respectively ($t(15)=0.143, ns$; Fig. 2E).
Figure 2: Internalization of beads, but not Aβ1-42, seems to be increased in ES microglia
A) Mixed glial cultures prepared from P9 hippocampi of Ctrl and ES mice were exposed to A488 beads on DIV10. B) Image of microglia cells with co-localized A488 beads and C) orthogonal view indicating the internalized beads within the Iba1+ microglial cell. D) ES-exposed microglia show a strong trend (p=0.057) to internalization of more beads relative to Ctrl cultures. E) Astrocytes internalize few beads, without a difference between Ctrl and ES cultures. F) Mixed glial cultures prepared from P9 hippocampi of Ctrl and ES mice were exposed to A647 Aβ1-42 on DIV10. G) Image of microglia cells with co-localized Aβ1-42 and H) orthogonal view indicating the internalized Aβ1-42 within the Iba1+ microglial cell. I) Aβ1-42 was primarily internalized by microglia cells and J) to a lesser extent by astrocytes, without an effect of ES. Scale bars: 10 µm.
3.2.2 Phagocytic assay with Aβ1-42
The phagocytic response was similarly addressed by in vitro exposure to HiLyte™Fluor 647 Aβ1-42 at DIV10 (Fig. 2F). Aβ1-42 was internalized by the glial cells as indicated by co-localization of Aβ1-42 within the Iba1+ microglia (Fig. 2G-H). Although the internalized Aβ1-42 co-localized on average with 16.1% of the Ctrl Iba1+ microglia, and with 9.3% of the ES Iba1+ microglia, this difference was not statistically different (t(15)=1.350, ns; Fig. 2I). The internalization of Aβ1-42 was not significantly different between Ctrl astrocytes (2.0%) and ES astrocytes (0.5%) either (t(15)=1.389, ns; Fig. 2J).

3.3 Fractalkine and NLRP3 expression are reduced by ES in the hippocampus of P9 pups
Hippocampal mRNA expression of the danger associated molecular pattern (DAMP) HMGB1 was not affected and NLRP3 was reduced by ES (HMGB1: t(10)=-0.138, ns; NLRP3: t(10)=2.762, p=0.020; Fig. 3A). Expression of CCL2 and CCR2 mRNA was not affected by ES at P9 (CCL2: t(10)=1.283, ns; CCR2: t(10)=0.146, ns; Fig. 3B). Fractalkine (CX3CL1) mRNA expression was reduced after ES in the P9 pups, but expression of the receptor CX3CR1 was not significantly altered (CX3CL1: t(10)=3.068, p=0.012; CX3CR1: t(10)=1.747, ns; Fig. 3C).

3.4 Neuroinflammation mediating factors are dysregulated in 10-month-old ES APP/PS1 mice
Hippocampal mRNA expression of the DAMPs HMGB1 and NLRP3 was not affected by either ES exposure or APP/PS1 overexpression at 4 months of age (HMGB1: genotype F(1,17)=0.859, ns, condition F(1,17)=1.730, ns, interaction F(1,17)=0.107, ns; NLRP3: genotype F(1,17)=0.829, ns, condition F(1,17)=0.254, ns, interaction F(1,17)=1.017, ns; Fig. 4A). Similarly, CCL2 and CCR2 mRNA expression were not altered either by condition or genotype in 4-month-old mice (CCL2: genotype F(1,16)=0.087, ns, condition F(1,16)=0.861, ns, interaction F(1,16)=0.290, ns; CCR2: genotype F(1,16)=0.785, ns, condition F(1,16)=2.045, ns, interaction F(1,16)=0.881, ns; Fig. 4B). The neuron-derived chemokine CX3CL1 increased in 4-month-old APP/PS1 mice, without an effect of early-life condition (CX3CL1: genotype F(1,17)=8.726, p=0.009, condition F(1,17)=0.656, ns, interaction F(1,17)=1.876, ns; Fig. 4C), whereas the microglial receptor CX3CR1 was not differentially expressed at this age (CX3CR1: genotype F(1,17)=0.521, ns, condition F(1,17)=0.142, ns, interaction F(1,17)=1.473, ns; Fig. 4C).
At 10 months of age, both DAMPs were differentially expressed. HMGB1 mRNA expression revealed an interaction effect, with a trend to increased expression in Ctrl APP/PS1 mice compared to Ctrl WT mice (HMGB1: genotype $F(1,20)=1.545$, ns, condition $F(1,20)=0.001$, ns, interaction $F(1,20)=6.294$, $p=0.021$, post-hoc trend for Ctrl WT vs Ctrl APP/PS1 $p=0.090$; Fig. 4D). NLRP3 mRNA expression was furthermore enhanced in all groups when compared to Ctrl WT mice (NLRP3: genotype $F(1,20)=22.295$, $p<0.001$, condition $F(1,20)=1.039$, ns, interaction $F(1,20)=18.224$, $p<0.001$, post-hoc Ctrl WT vs ES WT $p=0.008$, Ctrl WT vs Ctrl APP/PS1 $p<0.001$, Ctrl WT vs ES APP/PS1 $p=0.004$; Fig. 4D). Although the early-life condition did not affect CCL2 chemokine expression, the APP/PS1 induced expression of CCR2 in Ctrl APP/PS1 mice was less potent in the ES APP/PS1 mice (CCL2: genotype $F(1,18)=2.020$, ns, condition $F(1,18)=0.610$, ns, interaction $F(1,18)=0.031$, ns; CCR2: genotype $F(1,18)=1.093$, ns, condition $F(1,18)=0.709$, ns, interaction $F(1,18)=13.379$, $p=0.002$, post-hoc Ctrl WT vs Ctrl APP/PS1 $p=0.015$, Ctrl APP/PS1 vs ES APP/PS1 $p=0.044$; Fig. 4E). Expression of the chemokine CX3CL1 was not affected by either condition or genotype (CX3CL1: genotype $F(1,20)=0.008$, ns, condition $F(1,20)=0.797$, ns, interaction $F(1,20)=1.090$, ns; Fig. 4F). ES enhanced the expression of the fractalkine receptor CX3CR1 and APP/PS1 overexpression had a tendency to a similar upregulation in CX3CR1 expression (CX3CR1: genotype $F(1,20)=3.847$, $p=0.064$, condition $F(1,20)=5.805$, $p=0.026$, interaction $F(1,20)=1.635$, ns; Fig. 4F).

Figure 4: Neuroinflammatory mediators is altered by ES in 10-month-old APP/PS1 mice
At 4 months of age, hippocampal expression of A) the DAMPs HMGB1 and NLRP3 was not altered by genotype or early-life condition. Similarly, B) expression of chemokine CCL2 and its receptor CCR2 was not different between groups. C) APP/PS1 mice had an increased expression of the chemokine CX3CL1, but not its receptor CX3CR1. In the hippocampus of 10-month-old mice, D) the expression of the inflammasome component NLRP3 was increased in Ctrl APP/PS1 mice, but to a lesser extent in ES APP/PS1 mice, whereas the inflammasome activator HMGB1 was not affected. E) CCL2 expression was not different between groups, but CCR2 expression was increased in only the Ctrl APP/PS1 mice. F) Next to this, CX3CL1 expression is not different between groups at 10 months, but CX3CR1 expression is increased in ES mice. Annotations: *, condition effect; #, genotype effect; &, interaction effect. Post-hoc annotations: @, sig from Ctrl WT; %, sig from Ctrl APP/PS1.
3.5 Microglial proliferation, but not microglial clustering, is reduced in ES APP/PS1 at 10 months

3.5.1. Percentage of BrdU+/Iba1+ cells is reduced in 10-month old ES APP/PS1 mice

BrdU expressing, dividing cells were quantified in the ML of the DG to study the proliferative response of microglia in APP/PS1 mice exposed to ES. 4-month-old APP/PS1 showed very few BrdU+ cells (data not shown), while the ML contained multiple BrdU+/Iba1+ and BrdU+/Iba1- cells at 10 months of age (Fig. 5A-B). The total number of BrdU+ cells was equal between Ctrl and ES mice, with approximately 3 to 4 BrdU+ cells per 750 x 400 µm image (t(8)=0.771, ns; data not shown). The percentage of BrdU+/Iba1+ cells was decreased in ES APP/PS1 mice (t(8)=2.341, p=0.047; Fig. 5C).

3.5.2. The number of Iba1+ clustered cells is not affected by ES in 10-month-old mice.

The number of 6E10+ plaques at 4 months of age was too low for quantification analysis of microglial clustering, while 6E10+ plaques at 10 months showed abundant clustering of Iba1+ cells (Fig. 6A). The clustering of Iba1+ cells was strongly correlated with 6E10+ plaque volume in both Ctrl APP/PS1 and ES APP/PS1 mice and these regression slopes were not different from one-another (Ctrl $R^2=0.704$, $p<0.001$; ES $R^2=0.516$, $p<0.001$; difference regression slopes $F(1,150)=0.046$, ns; Fig. 6B-C). In addition, the average plaque volume was not affected by ES (t(8)=1.031, ns; Fig. 6D), nor was the clustering of Iba1+ cells different after normalization for the plaque volume (t(8)=0.177, ns; Fig. 6E). In addition, the number of BrdU+/Iba1+ cells at the 6E10+ plaques were not affected by ES (BrdU+/Iba1+ cells t(8)=0.392, ns; data not shown).

Figure 5: ES reduced microglial proliferation in 10-month-old APP/PS1 mice
A) Proliferating BrdU+ cells (yellow arrow and arrowhead) in the the molecular layer (ML) of the dentate gyrus. One cell is in close proximity of the 6E10+ plaque (arrow). B) The overlay and orthogonal view of these images reveals that one of the BrdU+ cells is Iba1+ (arrow), while the other cell is Iba1- (arrowhead). C) BrdU+ cell numbers in 10-month old APP/PS1 mice shows that ES reduced the percentage of BrdU+/Iba1+ cells. Scale bars: 50 µm. Annotation: *, sig from Ctrl APP/PS1.

4. Discussion

In this ongoing study, our preliminary results point to a sensitization of the neuroinflammatory response after ES. An acute inflammatory challenge with LPS to cultured cells collected after ES, showed that ES exposed hippocampal glial cells respond in an exacerbated fashion, with a higher expression of pro-inflammatory factors. Also, the preliminary data on the phagocytic response of ES microglia revealed a trend to a higher number of internalized beads than Ctrl microglia, while the average internalized Aβ1-42 in ES microglia was lower, although not significant. The mRNA expression of NLRP3 and CX3CL1 was
reduced directly after stress exposure at P9. At 10 months of age, ES reduced the normally enhanced expression of microglial regulatory factors in APP/PS1 mice. We furthermore showed that proliferation of microglia was reduced in ES APP/PS1 mice at 10 months, while the numbers of clustered microglia surrounding Aβ plaques in the ML of the DG was not altered by ES exposure. Altogether, these data point to an enhanced inflammatory response to acute challenges in P9 cultured (micro)glia and to a dampened reaction to the chronic in vivo inflammatory stimulation by Aβ neuropathology in APP/PS1 mice.

4.1 Evidence for an exacerbated response to acute inflammatory challenges
We exposed cultured glial cells to an inflammatory challenge with LPS in order to test the inflammatory response of ES-exposed glial cells, and showed that the pro-inflammatory response was exacerbated in ES cultures. These observations are in line with evidence from earlier studies on the sensitization of the inflammatory response by stress (Frank et al., 2010; Ślusarczyk et al., 2015; Veenema et al., 2008). One of these studies, furthermore, showed that, without the addition of LPS, prenatal stress already enhanced pro-inflammatory signaling in cortical primary microglial cultures (Ślusarczyk et al., 2015). In contrast to this observation, there was no difference between Ctrl and ES cultures in the pro-inflammatory expression profile without LPS exposure in the current study. In addition, our ES cultures showed no difference in glial cell numbers and the pro-inflammatory response after LPS was not associated with either the number of cells or specific cell-types at the time of exposure either. This indicates that the ES phenotype in our cultures does not relate to a differential starting point at the time of LPS exposure. These observations thus suggest that ES indeed sensitized glia to a subsequent inflammatory challenge in vitro, although such sensitization cannot be attributed to specifically microglia or astrocytes at this point.
To further test the functional microglial response after ES, we used a phagocytosis assay with beads or Aβ1-42 to further stimulate the cells in a different setting. It is important to note that the observations of these experiments are still preliminary because of the low number of analyzed cultures. Currently, the data seems to point to an enhanced response of the ES-exposed microglia to beads, and possibly an opposite reduced internalization of Aβ1-42 aggregates. There is nevertheless insufficient statistical power to support these observations at this point, but it remains interesting to interpret these preliminary observations. Pro- and anti-inflammatory cytokines are well-described to determine the phagocytic capacity of microglia (Chakrabarty et al., 2010; Michelucci et al., 2009; Zahn et al., 1997). A differential inflammatory response of glia cells to beads and Aβ1-42 stimulation can explain the seemingly opposite phenotypes of these two assays. Indeed, beads generally exert an inherent phagocytic response of microglia without severe inflammatory regulation, whereas both microglia and astrocytes react to oligomer and fibril Aβ1-42 with a phagocytic response, as well as the release of pro-inflammatory cytokines (Chakrabarty et al., 2010; Jones et al., 2013).

In the current study, we prepared primary glial cultures that contain microglia as well as astrocytes. The altered inflammatory signaling and bead internalization after ES exposure might therefore by partially mediated by astrocytes or by the interplay between astrocytes and microglia that may have altered specific functionality of either cell type, or both. Astrocytes have for instance been shown to influence microglia phagocytosis through the secretion of factors such as GM-CSF (Zahn et al., 1997) and they react by the exposure to LPS as well as Aβ1-42 (Hamby et al., 2012; Norden et al., 2016; Pomilio et al., 2015). In contrast to a previous report on astrocytic phagocytic activity (Jones et al., 2013), we observed little internalization of beads and Aβ1-42 by the astrocytes in both Ctrl and ES cultures. To date, very little evidence is available on the consequences of ES for astrocytes per se, and it will be interesting to further study the contribution of these individual cell types, as well as their interaction after ES exposure.

With these in vitro experiments, we showed that the response of microglia in culture was altered by the exposure to ES and this suggests that ES sensitizes the response of (micro)glia to different stimuli. Although these in vitro experiments are a useful tool to study an ES-modulated phenotype, others have shown that microglia rapidly change their expression profile in culture (Gosselin et al., 2017) and one should therefore be cautious to draw conclusions about the (direction of) in vivo ES responses based on these results. We further addressed how ES affects the expression of different factors that regulate microglial functions in the hippocampus of WT mice and in mice that exhibit chronic inflammatory activation as the result of accumulated Aβ in the brain.

4.2 Loss of fractalkine expression after ES might drive the microglial phenotype at P9

We studied the expression of several factors in the hippocampus of WT P9 mice and showed that microglial functioning in the P9 hippocampus was dysregulated after ES exposure, since the expression of NLRP3 mRNA and CX3CL1 mRNA was reduced by ES. These observations extend on our previous descriptions of elevated IL-1β mRNA expression and reduced microglial morphology in the ES hippocampus of P9 mice (Hoeijmakers et al., 2017).
The decreased expression of CX3CL1 in ES P9 pups was in line with the decreased CX3CL1 protein levels in prenatal stress exposed P7 rats (Ślusarczyk et al., 2016). The neuron-derived expression of CX3CL1 is an important off-signal for inflammatory activation of microglia (Biber et al., 2007), and loss of CX3CL1 is linked with increased pro-inflammatory signaling, including IL-1β (Cardona et al., 2006). The reduced expression of CX3CL1 mRNA seems therefore in line with the previously reported increased IL-1β expression levels and reduced microglial complexity after ES (Hoeijmakers et al., 2017).

This phenotype might be instrumental in the contribution of microglia to the ES phenotype during development. For instance, neuronal-microglial fractalkine signaling has been shown to play an essential role in phagocytosis during developmental neurogenesis and synaptic pruning (Paolicelli et al., 2011; Tremblay and Majewska, 2011). Interestingly, the development of dendrites and spines of pyramidal neurons was suppressed in the P9 brain, when the mice were subjected to the same chronic ES model as studied here (Liu et al., 2016). It will therefore be very interesting to study if loss in fractalkine signaling contributes to such an aberrant developmental phenotype in the ES hippocampus.

Interestingly, the mRNA expression of NLRP3 has not been addressed in earlier ES studies, whereas later-life stress upregulated NLRP3 mRNA expression (Frank et al., 2014) and was interpreted as a priming mechanism of microglia (Frank et al., 2010). Such enhanced NLRP3 mRNA expression can be mediated by elevated HMGB1, which is a signal for damage or danger (Frank et al., 2016) or by activation of the glucocorticoid receptor (Busillo et al., 2011; Frank et al., 2012; 2014). The employed ES model in this study was previously reported to increase the level of corticosterone in the ES P9 male pups (Naninck et al., 2015; 2017) and we had therefore expected a similar priming effect of ES on NLRP3 mRNA expression as reported for chronic stress. However, the level of circulating corticosterone at P9 is much lower than in (stress-exposed) adult rodents, and might therefore not have the same modulatory effect.

In contrast to the phenotype at P9, NLRP3 mRNA expression was enhanced in 10-month-old ES WT mice, but not in 4-month-old mice. In addition, mRNA expression of the fractalkine receptor CX3CR1, was also enhanced by ES at 10 months of age. Previously, we showed that the complexity of hippocampal microglia, microglial cell numbers and expression of various other factors did not differ between Ctrl and ES WT mice at 10 months of age (Hoeijmakers et al., 2017). It is therefore interesting that this phenotype changes by 10 months, however, at this point no clear explanation can be provided for this phenomenon in ES WT mice.

4.3 Chronic Aβ exposure dampens the neuroinflammatory response
APP/PS1 mice exhibit an ongoing accumulation of Aβ peptides, with little Aβ neuropathology at 4 months and abundant pathology with subsequent inflammatory stimulation at 10 months (Guo et al., 2015; Hoeijmakers et al., 2017; Jankowsky et al., 2004). We previously showed that Aβ neuropathology in the hippocampus of ES APP/PS1 mice was reduced at 4 months and aggravated at 10 months of age, similar to various neuroinflammatory factors (Hoeijmakers et al., 2017). We now showed that 10-month-old APP/PS1 mice exhibited a strong increase in NLRP3 and CCR2 expression, which was less profound in the ES-exposed APP/PS1 mice, while the expression of CX3CR1 mRNA was enhanced by ES. This confirms our previous observations.
Fractalkine signaling between neurons and microglia has been shown to alter the phagocytic capacity of microglia (Lee et al., 2010), and a deficiency in microglial CX3CR1 was shown to alter the neuroinflammatory profile and to reduced Aβ deposits (Lee et al., 2010, Liu et al., 2010). An intrinsic increase in CX3CR1 mRNA expression in ES-exposed mice and neuron-microglia interaction could therefore mediate an altered neuroinflammatory profile and progression of Aβ neuropathology.

In addition to this, ES APP/PS1 mice exhibited reduced CCR2 expression, when compared to Ctrl APP/PS1 mice. This receptor for the CCL2 chemokine is only expressed by mononuclear phagocytes and is essential for the recruitment of these cells to insults (Mack et al., 2001; Mizutani et al., 2012). A difference in CCR2 mRNA can thus indicate a change in monocyte infiltration. On the other hand, the hippocampal tissue was not perfused with saline prior to (mRNA) isolation, and our CCR2 expression can thus also reflect the presence of perivascular monocytes instead of infiltrated monocytes in the hippocampus. To our knowledge, stress exposure (in early-life) has this far not been reported to mediate monocyte infiltration (Delpech et al., 2016). However, monocyte infiltration under a pathological condition, like AD, might be differentially affected by ES and further characterization of the microglial and monocyte profiles in ES-exposed APP/PS1 mice are therefore needed to clarify if this plays a role.

In addition to these alterations in the hippocampal expression profile of APP/PS1 mice, we showed that ES reduced the percentage of dividing BrdU+/Iba1+ microglia in the ML of the DG in APP/PS1 mice. Microglia are long-living cells that can start to proliferate under pathological conditions as an activated response (Tay et al., 2017), and this reduction in cell proliferation therefore adds to the altered microglial profile in ES APP/PS1 mice. However, we previously showed that the total number of microglia in the hippocampus of APP/PS1 mice at 10 months was not affected by ES (Hoeijmakers et al., 2017). Indeed, the number of proliferating microglia was fairly low and these few cells might therefore not per se lead to a significant change in the microglial population numbers between Ctrl and ES mice. Interestingly, also the number of non-microglial dividing cells (BrdU+/Iba1-) was elevated in the 10-month-old ES APP/PS1 mice and this population of cells might reflect for instance proliferating astrocytes. Indeed, next to microglia, astrocytes also have a proliferative response to the Aβ exposure (Kamphuis et al., 2012), but we did not further investigate this hypothesis.

We further addressed the number of accumulated cells at plaques and indicated that there was no significant difference in the number of clustered Iba1+ cells at Aβ deposits between Ctrl and ES APP/PS1 mice. Considering that our previous study showed a decrease in clustered Iba1 immunoreactivity in these same ES APP/PS1 mice (Hoeijmakers et al., 2017), this reduction does thus not likely reflect reduced cell numbers, but rather a difference in morphology. Plaque-associated microglia obtain a dystrophic (neuro-degenerative) phenotype, which is associated with reduced process complexity and altered inflammatory signaling (Gyoneva et al., 2016; Krasemann et al., 2017; Yin et al., 2017). Whether plaque-associated microglia indeed show a differential (dystrophic) phenotype and how this relates to the overall aberrant neuroinflammation in ES APP/PS1 mice remains to be studied.
4.4 Conclusion
This ongoing study stresses the important influences of ES on later neuroinflammatory processes and microglia in the adult and aged hippocampus. Acute challenges in culture conditions show that ES might sensitize the neuroinflammatory and microglial responses. In addition, a more chronic exposure to Aβ elicits a less severe neuroinflammatory phenotype in APP/PS1 mice at 10 months of age.

Intervention studies should clarify if the dysregulation of microglia after ES contributes to cognitive deficits in WT mice and to the altered Aβ progression in AD mouse models, or vice versa. Additionally, it will be of interest to study which early-life and later-life interventions could change these neuroinflammatory components in ES, and if such intervention will also be beneficial in counteracting the ES-induced increased vulnerability to develop AD.

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Early-life stress sensitization of the neuroinflammatory response to acute and chronic challenges

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