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Maternal inflammation induces immune activation of fetal microglia and leads to disrupted microglia immune responses, behavior, and learning performance in adulthood



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ABSTRACT

Maternal inflammation during pregnancy can have detrimental effects on embryonic development that persist during adulthood. However, the underlying mechanisms and insights in the responsible cell types are still largely unknown. Here we report the effect of maternal inflammation on fetal microglia, the innate immune cells of the central nervous system (CNS). In mice, a challenge with LPS during late gestation stages (days 15–16–17) induced a pro-inflammatory response in fetal microglia. Adult whole brain microglia of mice that were exposed to LPS during embryonic development displayed a persistent reduction in pro-inflammatory activation in response to a re-challenge with LPS. In contrast, hippocampal microglia of these mice displayed an increased inflammatory response to an LPS re-challenge. In addition, a reduced expression of brain-derived neurotrophic factor (BDNF) was observed in hippocampal microglia of LPS-offspring. Microglia-derived BDNF has been shown to be important for learning and memory processes. In line with these observations, behavioral- and learning tasks with mice that were exposed to maternal inflammation revealed reduced home cage activity, reduced anxiety and reduced learning performance in a T-maze. These data show that exposure to maternal inflammation during late gestation results in long term changes in microglia responsiveness during adulthood, which is different in nature in hippocampus compared to total brain microglia.

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1. Introduction

Microglia are the resident innate immune cells of the brain, and important to maintain or restore tissue homeostasis (Nimmerjahn et al., 2005). Microglia originate from progenitors localized in the embryonic yolk sac early during embryonic development, and colonize the neural epithelium, where they proliferate and mature during embryonic development. Microglia form a self-sustained cell population with, under physiological conditions, very little contribution of bone marrow-derived monocytes (Ginhoux et al., 2013). The microglial population in the mouse brain is replaced however, the exact rate of turnover is unresolved. Where BrdU incorporation experiments indicated that the microglia population is replaced every 100 days (Askew et al., 2017), genetic labelling experiments showed that microglia turnover of with different rates in different brain regions. Hippocampal microglia completely turn over in 15 months and cortical microglia in 41 months

(Tay et al., 2017). Regional differences in microglia are also observed in gene expression profiles and in regional differences in age-related changes in gene expression (Grabert et al., 2016). When exposed to pathogens or tissue damage, microglia adopt a more activated state, release cytokines, chemokines, neurotrophic factors, and change in migratory and phagocytic behavior (Kettenmann et al., 2011). Since microglia, together with astrocytes, are the primary source of cytokine release in the brain, these cells are of main importance in basal functioning as well as pathologies of the brain.

Microglia colonize the developing CNS early during mammalian embryonic development, a period wherein maternal inflammation can greatly affect fetal microglia. In this time, the placenta allows selective exchange of necessary factors including nutrients, endocrine factors and antibodies. At the same time, it protects the embryo against potential harmful factors in the maternal circulation. Although the placenta is an effective barrier, it can be disrupted by certain adverse conditions such as maternal inflammation. This may lead to improper placental functioning, which in turn can impair normal embryonic development (Hsiao and Patterson, 2012). Maternal inflammation is a serious risk factor for several neurological pathologies and in humans, prenatal

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Inflammation has been identified as a risk factor for developing autism (Meldrum et al., 2013; Patterson, 2011), schizophrenia (Patterson, 2009) and cerebral palsy (Yoon et al., 2000). Several animal models have been developed to study this relationship between maternal inflammation and development of these diseases. For example, to mimic a viral infection pregnant animals are treated with polyinosinic:polycytidylic acid (poly-I:C), or to mimic a bacterial infection they are treated with lipopolysaccharide (LPS), a cell wall component of gram negative bacteria. Although these models both induce maternal inflammation and fetal brain inflammation, the physiological consequences of LPS and poly-I:C exposure are different in nature. Where poly-I:C is reported not to affect time of birth and survival of pups, both i.p. and i.v. injection of high dose LPS in pregnant mice had a detrimental effect on both the litter size and survival of the offspring (Arsenault et al., 2013). These observations emphasize that maternal inflammation is not a uniform phenomenon and that potential consequences for the offspring depend on the inflammatory stimulus.

We previously showed that i.p. injection of LPS in adult mice caused a long-term reduction in the pro-inflammatory response to a second LPS challenge in microglia (Schaafsma et al., 2015). Persistent changes induced in these cells by prenatal inflammation might have possible long-term effects in the offspring. Regarding LPS-induced prenatal inflammation, most data were generated using total brain and hippocampus tissue and hence cell type-specific information regarding (cytokine) gene expression and their effects is limited. Furthermore, the effect of LPS-induced maternal inflammation on fetal brain has only been assessed in total fetal brain, where increased levels of pro-inflammatory cytokines such as IL-1 β , TNF- α and IL-6 in the mother as well as in the fetal brain were observed during pregnancy (Elovitz et al., 2011; Liverman et al., 2006). Activation of fetal microglia specifically has been shown in poly-I:C induced inflammation (Pratt et al., 2013). However, it is unknown whether LPS-induced fetal brain inflammation can be attributed to maternal factors, or that fetal microglia themselves are immune activated by LPS, and moreover if this results in an altered response to LPS by these microglia during adulthood. Detrimental effects of prenatal inflammation on behavior, structural changes in the brain, and neurogenesis are reported (Boksa, 2010; Chlodzinska et al., 2011; Graciarena et al., 2010; Hao et al., 2010; Lin et al., 2012). In addition, prenatal inflammation has neuroprotective effects against ischemic events (Wang et al., 2007), which is linked to a more tolerant microglia phenotype with reduced expression of pro-inflammatory cytokines (Halder et al., 2013; Rosenzweig et al., 2004). Impaired learning and memory as a consequence of prenatal inflammation is related to increased pro-inflammatory gene expression, mainly IL-1 β in the hippocampus (Bilbo and Schwarz, 2009; Williamson et al., 2011). The effect of prenatal inflammation seems to depend on the gestation time at the moment of inflammation, the brain regions analyzed and parameter tested. In the study described here, the effect of prenatal inflammation, induced by injection of LPS during late gestation (GD 15–16–17), was investigated in mice with a focus on microglia in fetal and adult brain, and its effects on behavior and learning performance of these mice during adulthood. Regarding learning and memory impairment, it was shown that microglia-derived BDNF is important for hippocampus-dependent learning tasks (Parkhurst et al., 2013). Here, the effect of prenatal inflammation on BDNF expression in total brain and hippocampal microglia was determined.

2. Materials and methods

2.1. Animals

Wild-type pregnant C57Bl/6J01aHsd mice (E12) were purchased from Harlan (Harlan, Horst, the Netherlands). Animals were housed under normal conditions in a 12 h:12 h light dark cycle at the central animal facility or in the animal facility of the Center for Lifesciences of the University of Groningen. Pregnant animals were housed individually as

well as the offspring after weaning and their first treatment. Food and water were available ad libitum throughout the experiments, except during the T maze learning task (see below). Basal home cage activity of offspring mice was measured using a passive infrared monitoring system and analyzed using ACTOVUEW for excel programmed by Dr. C. Mulder (freely available on request).

2.2. Animal treatments

To avoid batch variations in experiments of different LPS lot numbers, one batch of LPS was used in all experiments (*E. coli* 0111:B4, Sigma-Aldrich, Cat# L4391). Prenatal inflammation was induced in pregnant mice by 3 injections with LPS (0.25, 0.10, and 0.05 mg/kg) at GD 15–16–17, control animals were injected with PBS. Young adult offspring (2–4 months) were injected with either PBS or LPS (0.25 mg/kg) and microglia were isolated 3 h post-injection.

2.3. Open field test

To assess whether prenatal inflammation affected the offsprings' explorative activity in a novel environment, the mice were exposed to a so called open field test. Mice were placed in an open field arena with a diameter of 85 cm and height of 30 cm. The arena was subdivided in a center zone and border zone. Mice were introduced in the arena at the outer wall, were allowed to explore the arena for 5 min and were then returned to their home cage. Between sessions the arena was cleaned with 30% ethanol for removal of olfactory cues. The movement of the animals was tracked using ethovision XT videotracking software (Noldus, The Netherlands).

2.4. Elevated plus maze

To assess whether maternal LPS injection affected anxiety levels in the offspring, the mice were subjected to an elevated plus maze test, a commonly used and well-validated anxiety test in rodents. The elevated plus maze consisted of four 90° angle arms with a center region (5 cm \times 5 cm), two open arms (5 cm \times 29 cm) and two closed arms (5 cm \times 29 cm \times 16 cm) and the maze was elevated 80 cm above the floor. The experiments were performed in a separate experimental room during the light phase under bright light conditions (100 lx). Mice were placed in the center region, allowed to explore the plus maze for 5 min, and videotaped for later analysis. In between testing animals, the plus maze was cleaned with 30% ethanol to eliminate olfactory cues. The recordings were analyzed for number of entries into open and closed arms and time spent in open and closed arms. An entry was scored when all 4 paws were in the arm. Entries into open and closed arms and time spent into open and closed arms were expressed as a percentage of total entries and total time spent in the open and closed arms.

2.5. T-maze

To test whether maternal LPS injection affected cognitive performance of the offspring later in life, the offspring mice were subjected to a T maze learning task. The T-maze consisted of 3 tubular, transparent Plexiglas arms (diameter 5 cm, length 27.5 cm): a start arm connected to a start box and two test arms at a 90° angle (left and right test arm). One of the test arms was baited with a food reward consisting of a small crumb of the regular food (0.05–0.1 g). Food crumbs were also placed below perforations at the end of the other test arm to prevent mice from discriminating between baited and non-baited arms by olfactory cues. A 1 cm high rim, 4 cm before the end of the tests arms prevented visual inspection for food presence from a distance. A guillotine door located halfway each arm could be operated manually from the experimenter's position and was used to allow animals only one choice in each training trial. Once the animals chose one arm, the

other arm was closed. To motivate animals for the task, they were food restricted to 90% of their individual body weight, starting 1 week before the beginning of the training. On the starting day, the mice received two habituation trials, where in the first trial one testing arm was baited and open while the other testing arm was closed. After consuming the food, mice were allowed to return to the starting box. A second habituation trial was performed with the other arm open and baited. After habituation, mice were daily subjected to 1 training session consisting of 6 trails. In these training sessions, the same arm was baited and randomly assigned to test animals. When a mouse accessed an arm, the opposing arm was closed, and entry into the baited versus non-baited arms were scored as correct or incorrect, respectively. Training was continued until the animals reached an average of 85–90% correct trails per day. All T-maze sessions were performed in a separate room during the animals' light phase.

2.6. Acute live microglia isolation from adult murine brain

Isolation of live microglia for *in vivo* studies were performed as described previously (Galatro et al., 2017) with some adjustments. After perfusion with PBS, brains were mechanically dissociated with a glass tissue homogenizer in dissection medium consisting of Hanks bovine salt serum, PAA, Cat.nr. H15-010; D-(+)-Glucose solution, Sigma, Cat.nr. G8769; HEPES, PAA, 311-001) and passed over a 70 μ m cell strainer (BD Falcon, Cat.nr. 352350). After centrifugation of the cell suspension (220 RCF, 4 °C) the supernatant was discarded and myelin was removed by centrifugation (20 min 950 RCF, 4 °C, acceleration 4, brake 0) in a PBS/22% percoll (GE Healthcare, Cat.nr. 17-0891-01) gradient. The supernatant was discarded and the cell pellet was resuspended in dissection medium lacking phenol red. The cell pellet was stained with antibodies against CD11b (PE, eBioscience 12-0112), CD45 (FITC, eBioscience, 11-0451) and Ly-6C for 20 min, washed and passed through a 5 ml polystyrene round-bottom tube with a cell strainer cap (12 \times 75 mm style, Cat.nr. 352235). Pure microglia were obtained by FACS sorting sorted using a Mo-flo XDP FACS (Beckman Coulter).

2.7. RNA isolation and quantitative RT-PCR

RNA was isolated using the Qiagen RNeasy Micro Kit according to the manufacturer's instructions. cDNA was generated using Random Hexamers (Fermentas), M-MuLV Reverse Transcriptase and Ribolock RNase inhibitor (Fermentas). Quantitative PCR reactions were carried out using a SYBR supermix (BIORAD) using an ABI 7900HT real time thermal cycler (Applied Biosystems) and primers for genes of interest (see Table S1 for qPCR primer information). Q-PCR primers were designed in PRIMER EXPRESS 2.0. qPCRs were performed in triplicate in a minimum of three independent experiments. HMBS was used as an internal standard to calculate relative gene expression levels with the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001).

2.8. ELISA

TNF- α serum levels were determined by collecting blood samples in minicollect serum/plasma gel tubes (Greiner bio-one, Cat# 450472). After collection, samples were placed at room temperature for 30 min, centrifuged (10 min, 2000 RCF, 4 °C) and serum was collected. ELISAs were performed according to the manufacturer's instructions (Mouse TNF- α ELISA MAXTM Deluxe, Biolegend, Cat# 430906; Mouse IL-1 β ELISA MAXTM Deluxe, Biolegend, Cat# 432606).

2.9. Statistics

All results are presented as means \pm SEM. Data were analyzed using unpaired Student's *t*-test when 2 groups compared or one-way ANOVA followed by a Fisher's Least Significant Difference (LSD) post hoc test to compare several groups. Home cage activity data was analyzed using a

2-way repeated measures ANOVA. Statistical significance was accepted as $p \leq 0.05$ and is indicated as * $p \leq 0.05$, ** $p \leq 0.05$ or *** $p \leq 0.001$.

3. Results

3.1. LPS-induced inflammation in both pregnant mice and fetal microglia

To study the effect of prenatal inflammation on offspring, pregnant mice received three *i.p.* injections with PBS or LPS at gestation days (GD) 15–16–17. In view of reported variability in LPS efficacy (Granholtm et al., 2011; Roumier et al., 2008; Velten et al., 2010; Wang et al., 2010), an LPS dosage titration was performed. Pregnant C57Bl/6J01aHsd mice were injected with 0.05, 0.1 or 0.25 mg/kg LPS on GD 15, 16 and 17. Doses of 0.1 and 0.25 mg/kg LPS induced preterm birth or resorption of the fetuses but viable offspring was obtained with 0.05 mg/kg LPS. This dose significantly elevated TNF- α serum levels in the dams, indicative of an inflammatory response (Fig. 1A; $n = 4$ /group). To assess if embryonic microglia were affected by *i.p.* LPS (0.05 mg/kg) injection of the dam, microglia were FACS isolated from pooled fetal mouse brains, 3 h after the LPS injection on GD17, and RNA was extracted. The expression of pro-inflammatory genes IL-1 β , TNF- α and IL6 was significantly increased in embryonic microglia (Fig. 1B). Exposure to LPS during embryonic development did not affect offspring body weight in the first 4 weeks. Prior to weaning, whole litter weights were determined in week 2 and 3. In this time period, no significant difference was observed in weight gain between litters of PBS and LPS injected dams (Fig. 1C; Ctr $n = 13$, LPS $n = 9$, 2-way ANOVA). After weaning, mice were individually monitored. No overall significant difference was observed between male offspring of PBS and LPS dams. However, an interaction effect of treatment \times time was observed at days 22 and 29 ($F_{10,80} = 4.32$, $p < 0.0001$). Male offspring of LPS dams gained less weight during this period than PBS offspring. No differences in weight gain were observed between female mice. As expected, male offspring gained significantly more weight than female offspring (Fig. 1D; Ctr female $n = 6$, LPS female $n = 6$, Ctr male $n = 7$, LPS male $n = 3$). Furthermore, LPS injection of dams did not significantly affect litter size (Fig. 1E; # of litters: Ctr $n = 6$, LPS $n = 7$). Based on these findings, in subsequent experiments pregnant mice were injected with PBS or 0.05 mg/kg LPS at GD 15, 16 and 17. Their progeny are respectively referred to as LPS or PBS/control offspring and were tested at the young adult age between 2 and 4 months.

3.2. Reduced LPS responsiveness in microglia prenatally exposed to LPS

To determine the effect of prenatal LPS exposure on the response to a subsequent LPS challenge, a titration experiment was performed to determine the LPS dose that induced a robust inflammatory response in microglia in adult mice. Eight-week-old male mice were *i.p.* injected with 0, 0.25, 2.5, and 5 mg/kg LPS ($n = 4$ /group) and microglia were isolated 3 h later using FACS. IL-1 β and TNF- α mRNA expression levels were determined using quantitative RT-PCR and to confirm the induction of systemic inflammation, TNF- α serum levels were determined using ELISA. All LPS doses induced TNF- α levels in serum and 2.5 and 5 mg/kg LPS induced comparable levels of TNF- α (Fig. 2A). In isolated microglia, mRNA expression levels of IL-1 β and TNF- α were significantly increased by all LPS doses used and reached maximal levels at 2.5 mg/kg LPS. Injection of 0.25 mg/kg LPS induced a significant, sub-maximal inflammatory response in microglia (Fig. 2B; all groups $n \geq 4$).

To determine the effect of prenatal LPS exposure on the LPS responsiveness of microglia during adulthood, pregnant mice were *i.p.* injected with LPS (0.05 mg/kg) at GD 15–16–17 and their offspring was injected with PBS or LPS (0.25 mg/kg) at the age of 2 months. Both PBS and LPS offspring showed significantly elevated TNF- α serum levels in response LPS (Fig. 2C; all groups $n \geq 4$). 3 h after injection, microglia were acutely isolated and mRNA expression levels of IL-1 β , TNF- α and IL-6 were determined. In offspring of PBS-injected dams, LPS significantly increased

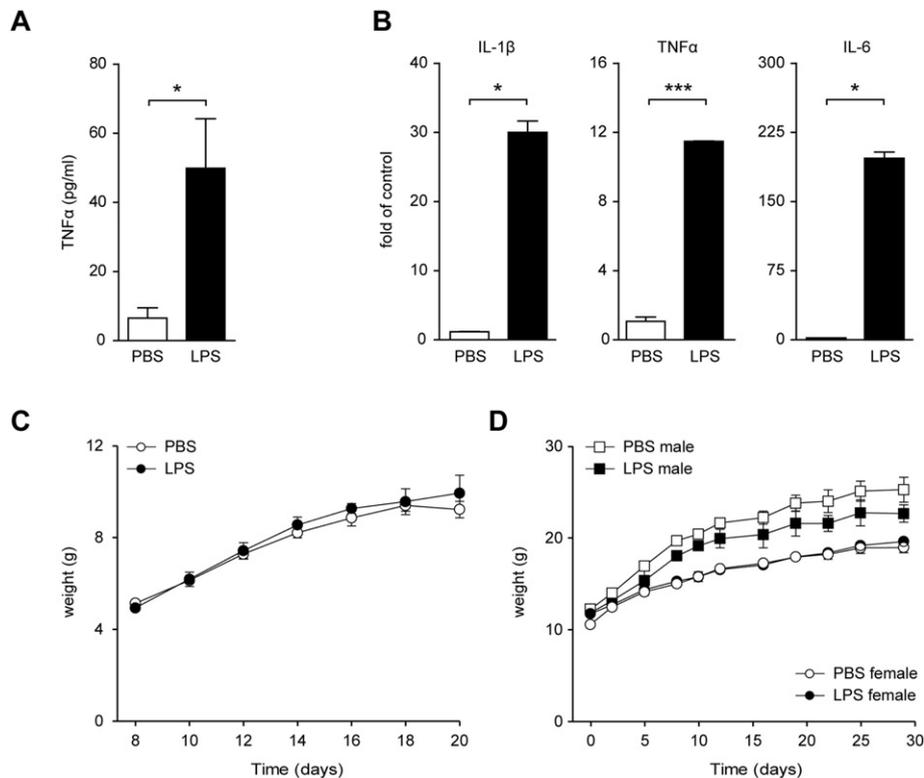


Fig. 1. LPS-induced inflammation in pregnant mice. (A) TNF- α serum levels of pregnant mice injected with PBS or LPS at GD 15, 16 and 17 were determined by ELISA. Pregnant mice injected with LPS (0.05 mg/kg) showed significantly increased TNF- α serum levels after 3 h. Average serum levels (mg/kg) with SEM are depicted; unpaired Student's *t*-test, * $p \leq 0.05$; PBS $n = 4$, LPS $n = 4$. (B) Expression levels of pro-inflammatory cytokines IL-1 β , TNF- α and IL-6 of microglia isolated from embryo's from PBS or LPS injected mothers were determined 3 h after PBS/LPS injection and analyzed by quantitative RT-PCR. Expression levels were normalized to the housekeeping gene hydroxymethylbilane synthase (HMBS). Expression levels are depicted as fold of control with SEM; * $p \leq 0.05$, *** $p \leq 0.001$. (C) Embryonic exposure to LPS did not affect body weight gain during the first 4 weeks of development (2-way ANOVA, $F_{1,6} = 1.31$, $p = 0.27$) and no interaction effect of treatment \times time was observed ($F_{6,120} = 1.22$, $p = 0.30$). (D) No overall significant difference was observed between PBS and LPS offspring groups in weight gain after weaning in male mice ($F_{1,10} = 4.81$, $p = 0.06$). But over time, an interaction effect of treatment \times time was observed ($F_{10,80} = 4.32$, $p < 0.0001$), where post hoc testing showed significance at day 22 and 29 (* $p \leq 0.05$); as expected, male mice gained more weight than female mice. (E) LPS treatment of pregnant dams did not significantly affected litter size (litters: Ctr $n = 6$, LPS $n = 7$).

expression of all three pro-inflammatory cytokines (Fig. 2D). Interestingly, in the offspring of LPS-injected dams, a significantly lower induction of IL-1 β , TNF- α and IL-6 expression in response to LPS was observed (Fig. 2D; all groups $n \geq 5$). These data show that embryonic exposure to LPS resulted in a significantly attenuated response of microglia to a re-challenge with LPS during adulthood.

3.3. Increased LPS responsiveness in hippocampal microglia of prenatally exposed mice

Microglia isolated from total mouse brain showed a reduced LPS responsiveness after prenatal LPS exposure. It is however possible that regional differences in microglia responsiveness exists. A significant body of literature has reported on the effect of pre- and perinatal LPS exposure on the hippocampus, in relation to its role in learning and memory processes. To determine the effect of prenatal LPS exposure on the LPS responsiveness of the hippocampus in adult mice, hippocampal mRNA was isolated from male PBS and LPS offspring 3 h after i.p. injection with LPS or PBS. Gene expression of pro-inflammatory cytokines IL-1 β , TNF- α IL-6 and neurotrophin BDNF were determined in total hippocampal tissue by quantitative RT-PCR. LPS induced significantly higher expression levels of IL-1 β and TNF- α in the offspring of LPS-injected dams compared to PBS-injected dams (Fig. 3A; $n = 4$ /group). In the hippocampus, BDNF has been reported to be important for synaptic plasticity in learning and memory formation (Parkhurst et al., 2013). BDNF mRNA levels were significantly reduced in total hippocampus tissue in response to LPS, both in the offspring of PBS and LPS-injected dams (Fig. 3A; all groups $n = 4$).

The increased inflammatory cytokine and reduced BDNF gene expression levels were determined in total hippocampus tissue. To determine the effect of prenatal LPS exposure on hippocampal microglia specifically, these cells were FACS isolated 3 h after PBS- or LPS-injection from offspring of PBS and LPS injected dams. Where LPS in both PBS and LPS-offspring induced TNF- α levels to similar levels, it resulted in significantly higher levels of IL-1 β in LPS offspring (Fig. 3B; $n = 4$ /group). LPS injection of PBS offspring resulted in a significant reduction of BDNF expression by hippocampal microglia. Interestingly, in hippocampal microglia isolated from LPS offspring, BDNF expression was significantly lower than in PBS offspring, and this was not further reduced by an additional LPS challenge (Fig. 3B; $n = 4$ /group). These data show that prenatal exposure to LPS resulted in drastically reduced BDNF expression in hippocampal microglia; which was not further reduced by an LPS challenge during adulthood.

3.4. Prenatal LPS exposure reduced basal home cage activity

Hippocampal microglia-derived BDNF is important for learning-induced spine formation and performance in various learning tasks (Parkhurst et al., 2013). Since BDNF expression was reduced in hippocampal microglia in LPS offspring, the effect of embryonic exposure to LPS on behavior and learning was determined. As a basal measure, the home cage activity of LPS ($n = 10$) and control ($n = 10$) offspring mice was determined using a passive infrared monitoring system. Mice were continuously monitored over the whole experimental period. Basal home cage locomotor activity is depicted as average activity over a 1 week period without experimentation (Fig. 4A). No significant differences between control- and LPS offspring mice were observed

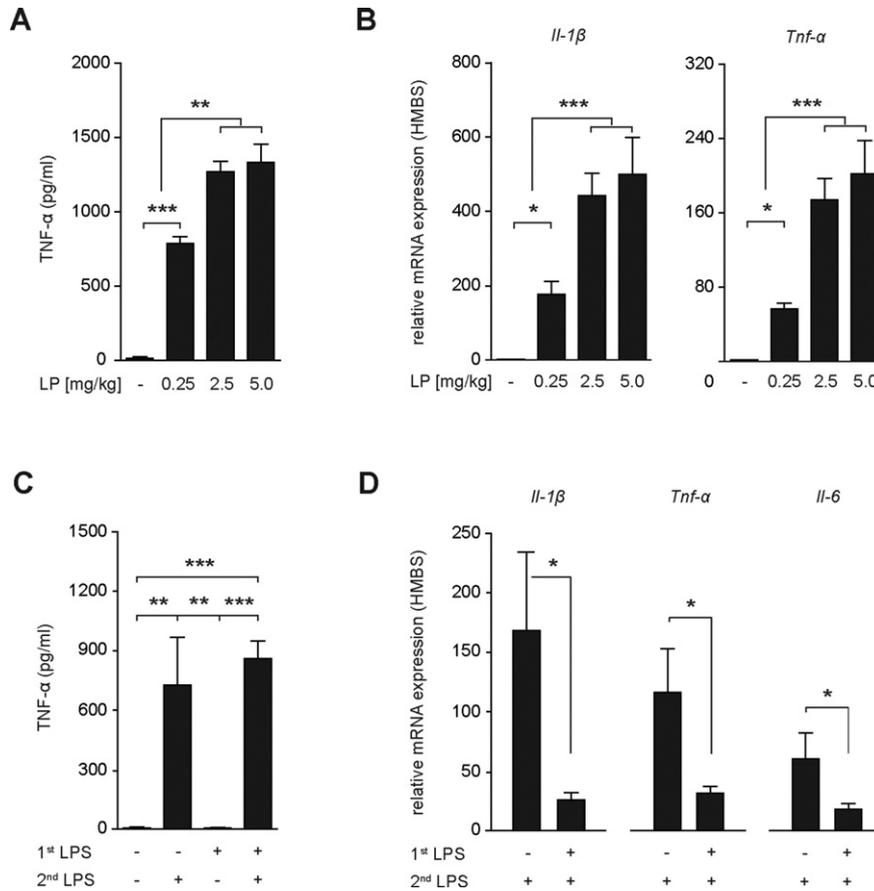


Fig. 2. Embryonic exposure to LPS resulted in reduced responsivity to LPS in adult offspring. (A) TNF- α serum levels of adult mice injected with 0, 0.25, 2.5 and 5 mg/kg LPS were determined by ELISA. Average serum levels (pg/ml) with SEM are depicted; ** $p \leq 0.01$, *** $p \leq 0.001$ ($n = 4$ for every group). (B) Expression levels of pro-inflammatory cytokines IL-1 β and TNF- α in microglia isolated from adult mice injected with 0, 0.25, 2.5 and 5 mg/kg LPS were determined using quantitative RT-PCR. Expression levels were normalized to the housekeeping gene HMBS synthase. Expression levels are depicted as fold of control with SEM; * $p \leq 0.05$, ** $p \leq 0.01$ (all groups $\geq n = 4$). (C) TNF- α serum levels of control and LPS offspring that received a subsequent i.p. PBS or LPS (0.25 mg/kg) injection was determined by ELISA. Average serum levels (pg/ml) with SEM are depicted; ** $p \leq 0.01$, *** $p \leq 0.001$ (all groups $\geq n = 4$). (D) Expression levels of pro-inflammatory cytokines IL-1 β , TNF- α and IL-6 of microglia isolated from male PBS or LPS (0.25 mg/kg) injected offspring were determined using quantitative RT-PCR. Expression levels were normalized to the housekeeping gene HMBS synthase. Expression levels are depicted as fold of control with SEM; * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ (all groups $\geq n = 5$).

during the light, or resting, phase (8 am to 8 pm). During the first 2 h of the dark phase, both groups showed a similar peak in activity. However, during the rest of the dark phase, total activity was significantly lower in LPS offspring mice (Fig. 4B). A 2-way repeated measure ANOVA showed an overall difference between both groups ($F_{1,18} = 4.48$, $p = 0.049$) and an interaction effect between treatment \times time was observed ($F_{23,414} = 1.71$, $p = 0.022$), indicating that over an averaged 24 h period, prenatal LPS exposure significantly reduced basal home cage activity of the offspring.

3.5. LPS and PBS offspring do not differ in exploration behavior

To determine whether prenatal LPS exposure affected general locomotor activity and anxiety to a novel environment, an open field test was performed with offspring from PBS and LPS injected dams. The open field test is commonly used to determine changes in general exploration behavior of novel environments, locomotor activity and is in some cases used as a mild anxiety test in rats and mice (Lipkind et al., 2004; Meerlo et al., 1999; Swiergiel and Dunn, 2007). No differences were observed between PBS- and LPS offspring mice in total distance traveled in the open field test (Fig. 5A). As expected, the distance traveled in the center zone, a measure for mild anxiety for novel environments (Lipkind et al., 2004), was significantly less compared to the distance traveled in the border zone but not significantly different between PBS and LPS offspring mice (Fig. 5A). In addition, the percentage of time spent in the center zone did not differ between PBS and LPS

offspring (Fig. 5B). These data indicate that prenatal exposure to LPS did not affect general locomotor activity and anxiety levels in an open field test.

3.6. LPS offspring showed reduced anxiety in the elevated plus maze

In mice, there is a conflict between exploratory drive and risk avoidance. Where mice prefer dark, protected areas, they also display novelty-seeking behavior. One of the measures for changes in risk taking behavior, or anxiety, is to determine changes in time spent in the illuminated, unprotected and elevated area of an elevated plus maze. To test whether prenatal LPS exposure affected anxiety behavior, offspring of PBS- and LPS-injected dams were subjected to elevated plus maze testing (Hagewoud et al., 2010). The elevated plus maze is a widely used and validated test to measure anxiety behavior in rodents and to some extent exploration and locomotor activity (Lister, 1987; Pellow et al., 1985; Walf and Frye, 2007). In elevated plus maze testing of PBS ($n = 9$) and LPS ($n = 9$) offspring, LPS offspring showed a significantly higher percentage of time spent in the open arms compared to PBS offspring (Fig. 5C, $11.7\% \pm 1.7$ vs 6.7 ± 1.5 , respectively; $p = 0.04$; 2 extreme statistical outliers were removed based on a Grubbs' test for statistical outliers), suggesting a reduced level of anxiety in LPS offspring. The number of entries in the open and closed arms, which can be used as a measure for locomotor and explorative behavior, was not significantly different between groups (Fig. 5D), an observation in agreement with the results obtained in the open field test.

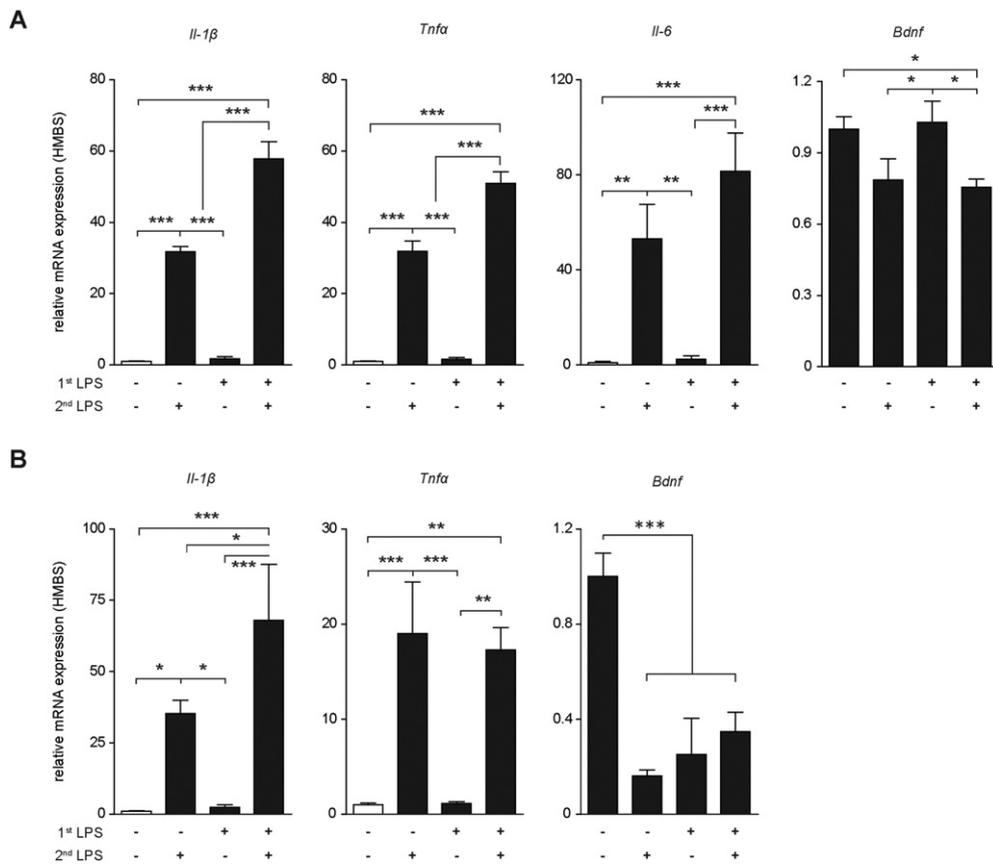


Fig. 3. Prenatal LPS resulted in an exaggerated inflammatory response to LPS and reduced BDNF expression in hippocampal microglia (A) Expression levels of pro-inflammatory cytokines IL-1 β , TNF- α , IL-6 and neurotrophin BDNF in total hippocampus, isolated from PBS- or LPS-injected control and LPS offspring was determined using quantitative RT-PCR. Expression levels were normalized to the housekeeping gene HMBS synthase. Expression levels are depicted as fold of control with SEM; * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ ($n = 4$ for all groups). (B) Expression levels of pro-inflammatory cytokines IL-1 β , TNF- α and neurotrophin BDNF of microglia isolated from hippocampus of PBS- or LPS-injected control and LPS offspring was determined using quantitative RT-PCR. Expression levels were normalized to the housekeeping gene HMBS synthase. Expression levels are depicted as fold of control with SEM; * $p \leq 0.05$, ** $p \leq 0.01$ ($n = 4$ for all groups).

3.7. Impaired learning in prenatally LPS exposed offspring

Previously, we reported that a single i.p. LPS injection in adult mice resulted in a reduced performance in a T-maze learning task

(Schaafsma et al., 2015). We assessed if prenatal LPS exposure also affected learning and memory in a T-maze. Control ($n = 9$; 1 statistical outlier was removed based on a Grubbs' test for statistical outliers) and LPS ($n = 10$) offspring were subjected to 1 training session per

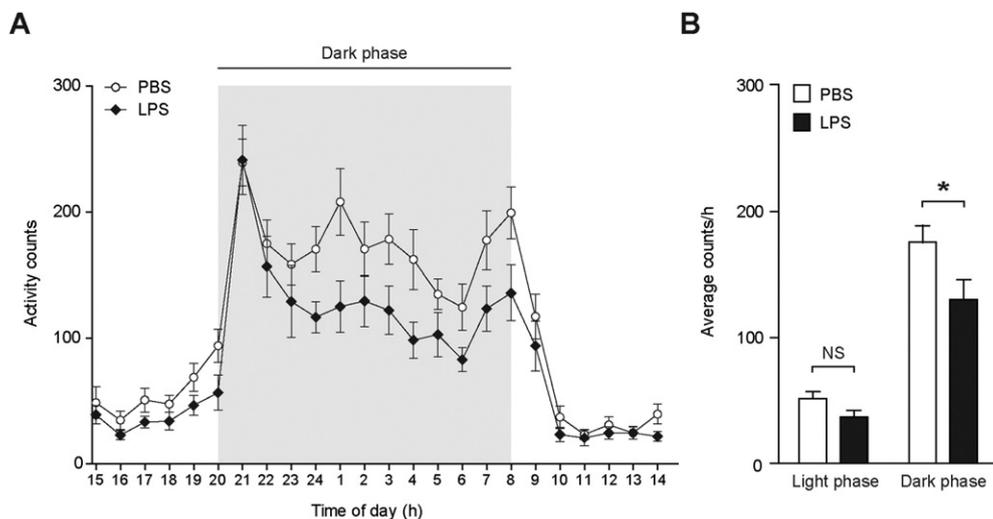


Fig. 4. Prenatal LPS exposure affects general homecage activity. (A) Basal homecage activity of control and LPS offspring was measured using a passive infrared monitoring system. A 24 h average was calculated over a period of 1 weeks during which no experimental procedures were performed. An overall difference in total activity between LPS and PBS offspring was observed using 2-way repeated measures ANOVA ($F_{1,18} = 4.48$, $p = 0.049$) and an interaction effect of treatment \times time was observed ($F_{23,414} = 1.71$, $p = 0.022$) ($n = 10$ for both groups) (B) Quantification of homecage activity shows no significant difference in activity during the light phase ($p = 0.07$). During the dark phase the activity of LPS offspring was significantly lower. Activity is depicted as average counts per h with SEM; * $p \leq 0.05$ ($n = 10$ for both groups).

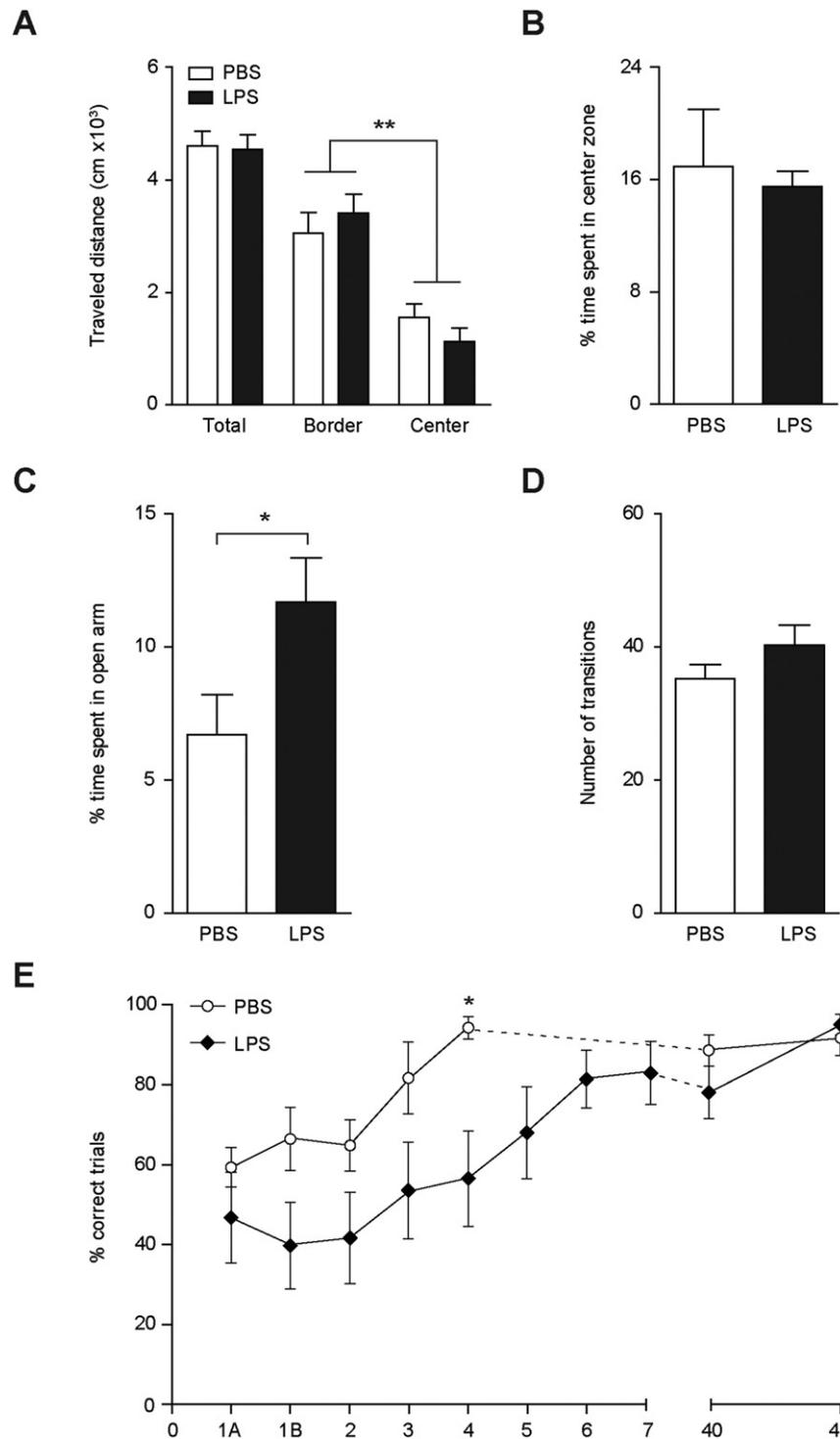


Fig. 5. Prenatal LPS exposure reduces anxiety and performance in a spatial learning task. (A) Control ($n = 10$) and LPS offspring ($n = 10$) were subjected to an open field test in adulthood. Total distance traveled as well as distance traveled in center and border zones during a 5 min trial is depicted with SEM; $**p \leq 0.01$ (B) percentage of time spent in the center zone of the open field was quantified as a measure for anxiety. (C) Elevated plus maze testing was performed on control ($n = 9$) and LPS ($n = 9$) offspring as a specific measure for anxiety. Percentage of time spent in the open arm is depicted with SEM; $*p \leq 0.05$. (D) Locomotor activity in the elevated plus maze during a 5 min trial showed no significant difference between control and LPS offspring. Data are depicted as total number of transitions with SEM. (E) T-maze learning; control ($n = 9$) and LPS ($n = 10$) offspring were subjected to 6 learning trials per day. Day 1 is depicted as day 1A and 1B to illustrate the learning effect observed within trials of day 1 in PBS offspring. Results are depicted as percentage of correct choices with SEM; $*p \leq 0.05$.

day, consisting of 6 trials. For each group, the percentage of correct choices was calculated over each training day until the mice scored $\geq 90\%$ correct choices or after 7 days of training. At the first training day, both control and LPS offspring started at chance levels ($59.3\% \pm 4.9\%$ and $46.7 \pm 11.3\%$, respectively). Control offspring already showed a learning effect during the first day, to visualize this, the 6 learning trials of day one were separated in 1A (first 3 trials) and 1B (second 3

trials). After 4 days of training, control offspring reached a level of $94.4\% \pm 2.8\%$ correct choices, which was significantly different from LPS offspring that reached a level of $56.7\% \pm 12\%$ correct choices at day 4 ($p = 0.011$). LPS offspring required 3 additional training days to reach a level of $83.3\% \pm 7.9\%$ correct choices (Fig. 5E). To determine memory retention, these mice were re-tested in the T-maze 4 weeks after the last training session (day 40–41). Both the PBS and LPS

offspring did not start at chance levels ($88.9\% \pm 3.9\%$ and $78.3 \pm 6.6\%$, respectively) and scored above the 90% criterion the next day ($91.7\% \pm 4.2$ and $95\% \pm 2.6\%$, respectively; Fig. 5E). These data indicate that T-maze learning developed slower in LPS offspring but memory retention for this test was not affected in these animals.

4. Discussion

Detrimental effects of prenatal inflammation on neural development of the developing embryo has been described for a variety of animals including mice, rat, sheep (Boksa, 2010; Wang et al., 2006) and primates (Willette et al., 2011). In humans, it has been identified as a risk factor for autism (Meldrum et al., 2013; Patterson, 2011), schizophrenia (Meyer et al., 2011; Miller et al., 2013; Patterson, 2009), and cerebral palsy (Yoon et al., 2000). Despite extensive description of animal models for prenatal inflammation, information about the effect of prenatal inflammation on specific cell types, such as microglia, is scarce, and models are mostly described in their effects on total fetal brain and behavior of adult offspring. When pregnant mice were injected with poly-I:C at E12.5, IL6 expression of fetal microglia was increased at E16 (Pratt et al., 2013). However, in a study performed by Smolder and co-workers, a single (E11.5) or two (E11.5 and E15.5) poly-I:C injections in mice did not result in fetal microglia activation (Smolders et al., 2015). The role of microglia in maternal inflammation-associated behavioral abnormalities induced by poly-I:C was recently demonstrated and could be completely reverted using the minocycline (Mattei et al., 2017).

Thus far, the effects of LPS-induced prenatal inflammation specifically on microglia in offspring are poorly understood. Graciarena et al. (2013) showed long lasting effects of prenatal inflammation, it reduced neurogenesis in the dentate gyrus but not in other neurogenic regions such as the SVZ. Based on morphology, persistent microglia activation was reported, specifically in the dentate gyrus but not in other brain regions such as cortex (Graciarena et al., 2013). Furthermore, neonatal infection with *E. coli* was shown to induce exaggerated IL-1 β expression by hippocampal microglia upon a subsequent LPS injection in adult mice (Williamson et al., 2011). Microglia are one of the primary sources of cytokines, chemokines and growth factors (Kettenmann et al., 2011), exercising a major influence on brain physiology, from the moment they populate the brain during early embryonal development throughout life. Especially in the case of LPS-induced prenatal inflammation, where cells are immune activated during embryonal development, it is of importance to determine if changes in these microglia persist during adulthood.

4.1. Prenatal LPS exposure negatively affects learning and behavior in offspring

A wide variety of behavioral changes have been reported to occur in prenatal inflammation models ranging from changes in locomotor behavior to alterations in learning and memory function. Overall, most studies consistently show that prenatal inflammation has detrimental effects on learning and memory. In the case of locomotor behavior, varying outcomes have been reported, depending on the maternal LPS injection paradigm, the age of the offspring at the time of behavioral testing, and the method used to assess behavior (Boksa, 2010). To investigate the effect of our prenatal LPS injection paradigm, several behavior and learning performance experiments were performed.

In our study, when general home cage activity of mice was measured by means of a passive infrared monitoring system, a clear difference between LPS and PBS control offspring was evident. LPS offspring were less active particularly during the dark phase, which is the main circadian activity phase in these nocturnal animals. We also assessed locomotor activity of the animals when they were removed from their home cage and transferred to a novel environment. In both the open field test and the elevated plus maze test, the indicators of overall activity

did not differ between control and LPS offspring (total distance covered in the open field and the number of arm crossings in the elevated plus maze test). In other words, the reduced spontaneous activity observed in LPS offspring was no longer present when the mice were placed in a new environment. One explanation might be that LPS offspring were less anxious and less inhibited than control mice when exposed to a novel and potentially threatening environment. Indeed, although the LPS and control mice did not differ in the time spent in the center zone of the open field, which is often used as a mild index of anxiety, there was a clear difference in the time spent on the open arms of the elevated plus maze, a more specific and well-validated indicator of anxiety. The finding that the groups showed no difference in the anxiety measure in the open field test but a clear difference in the more challenging and stressful plus maze test may suggest that this difference in anxiety is more clearly expressed under more stressful conditions.

The T-maze learning paradigm was used to determine if maternal LPS treatment affected learning and memory processes in the offspring mice. LPS offspring showed impaired initial learning. Whereas control animals had clearly learned to locate the food reward in the maze within 4 days (>90% correct arm choices), the group of LPS offspring needed an additional 3 days of training to reach >80% correct arm choices. This attenuated learning in the LPS offspring was likely not due to increased stress levels and anxiety in the T-maze environment because the results from the elevated plus maze test indicated that, if anything, LPS mice are less anxious. The difference in the T-maze performance thus appears to be a true learning deficit. Interestingly, both groups performed equally well after a 4-week interval. These results suggest that LPS offspring have attenuated learning, but their long-term memory consolidation does not seem to be affected.

In summary, these results show that prenatal inflammation can have major developmental effects and ultimately lead to persistent changes in important basal behavior characteristics like spontaneous activity levels, anxiety levels and learning capacity.

4.2. Embryonic microglia are immune activated in response to maternal i.p. LPS injection

Here we show that i.p. LPS injection of pregnant mice at GD 15–16 induced an inflammatory response in both the mother and the embryos. Previous research showed that LPS-induced prenatal inflammation caused an up regulation of pro-inflammatory cytokines in fetal brain in rat (Cai et al., 2000) and mice (Elovitz et al., 2011; Liverman et al., 2006). The imbalance of cytokines in the fetal brain, especially in the case of excess pro-inflammatory cytokines, has been proposed to be a fundamental cause of the detrimental effects of prenatal inflammation (Boksa, 2010; Meyer et al., 2009). Whether or not these pro-inflammatory cytokines originate exclusively from the mother or also from the fetus (brain) is unknown. Here, for the first time, we show that maternal inflammation induced by i.p. injection of LPS during late pregnancy caused an increased expression of pro-inflammatory cytokines IL-1 β , TNF- α and IL-6 in fetal microglia. This indicates that fetal brain microglia themselves are a source of pro-inflammatory cytokines in response to maternal inflammation, and might contribute to possible detrimental effects of prenatal inflammation.

4.3. Total brain microglia and hippocampal microglia are differentially affected by prenatal LPS exposure

Pre- and neonatal LPS exposure is mainly used to investigate its detrimental effects on learning and memory and neurodevelopmental aspects to model pathologies such as schizophrenia, autism and Parkinson's disease. In these models, the hippocampus is the main point of focus since it plays a paramount role in learning and memory processes. Hippocampal microglia of perinatal LPS exposed offspring are in this respect described as highly activated cells that release a higher than normal amount of pro-inflammatory cytokines such as IL-

IL-1 β (Bilbo and Schwarz, 2009; Graciarena et al., 2013; Williamson et al., 2011). A key issue of the research presented here was to gain more insight in the specific microglia response after LPS induced prenatal inflammation. We previously showed that microglia in vivo displayed a reduced inflammatory response to a second LPS challenge, up to 32 weeks after the first challenge (Schaafsma et al., 2015). With the observation that fetal microglia were immune activated, based on these previous results, we hypothesized that microglia in adult LPS-offspring would be immune-suppressed. Indeed, our results show that total brain microglia from adult LPS-offspring showed a reduced pro-inflammatory response to a subsequent LPS injection. Gene expression profiling of microglia isolated from different brain regions from mice of different ages revealed microglia heterogeneity both in relation to brain region and the effect of aging on gene expression (Grabert et al., 2016). In addition, microglia turnover rates differ between brain regions (Tay et al., 2017). The observation suggest that the effect of a prenatal LPS challenge on microglia might vary between brain regions. Indeed, most literature describes a more activated phenotype of microglia in the hippocampus in models of perinatal immune challenge. In agreement with this, in response to LPS, we observed that induction of pro-inflammatory cytokines IL-1 β , TNF- α and IL-6 was more pronounced in total hippocampus tissue of LPS offspring compared to control mice. When pure hippocampal microglia samples from LPS offspring mice were analyzed, exaggerated IL-1 β transcription levels were observed in response to LPS challenge. Surprisingly, LPS-induced TNF- α expression levels in microglia did not differ between control- and LPS-offspring (Fig. 3B) suggesting that the TNF- α mRNA detected in total hippocampus tissue was, in part, not microglia-derived (Fig. 3A). Astrocytes express TNF- α in response to LPS (Chung and Benveniste, 1990), and can be primed in their inflammatory response to subsequent stimuli by preceding inflammatory challenges (Henn et al., 2011). Recently it was reported that activated, neuroinflammatory microglia can induce reactive astrocytes with neurotoxic properties (Liddelow et al., 2017). Embryonic exposure to LPS might have led to an altered inflammatory responsiveness of hippocampal astrocytes resulting in the observed exaggerated TNF- α levels after a second LPS challenge.

In summary, our study is the first to report that the effect of LPS-induced prenatal inflammation on microglia is different in the hippocampus and the rest of the brain. Where whole brain microglia isolated from prenatally LPS stimulated mice showed a tolerant, immune-suppressed phenotype, hippocampal microglia showed a sensitized phenotype with regard to pro-inflammatory cytokine IL-1 β expression. These observations possibly explain how prenatal inflammation on the one hand provides neuroprotection against injury and ischemic events (total brain microglia) and on the other hands leads to learning and memory impairment (hippocampal microglia).

4.4. Prenatal LPS causes a stable microglia-specific reduction in BDNF expression

In agreement with previous studies, our LPS treatment paradigm caused significant behavioral changes. Where prenatal LPS inflammation induced reduced home cage activity, reduced anxiety and impaired learning in the T-maze paradigm but no changes in long-term memory formation. Together with these observations, we show that prenatal LPS exposure causes fetal microglia activation and thereby long-term and stable changes in microglial expression of cytokines important for learning and memory. In agreement with previous observations (Bilbo and Schwarz, 2009; Williamson et al., 2011), hippocampal microglia of mice prenatally exposed to LPS showed an exaggerated IL-1 β expression when receiving a subsequent LPS injection. However, it should be noted that this difference was only observed when mice were injected with LPS and no expression differences were observed in basal expression of IL-1 β between groups before the second LPS injection. Also in a study by Williamson et al. (2011), learning impairment was only observed when animals were subsequently injected with LPS, thereby

causing exaggerated IL-1 β expression in the hippocampus (Williamson et al., 2011). Our behavioral and learning tasks were performed before mice received a second PBS/LPS injection. These data indicate that the observed exaggerated IL-1 β expression by microglia in the hippocampus after a second LPS challenge was not responsible for the observed impairment in T-maze learning in LPS-offspring. Interestingly, here we report that prenatal LPS-induced inflammation caused a persistent reduction in BDNF expression, specifically in microglia in the hippocampus. In total hippocampal tissue, a reduction in BDNF expression levels were only observed after LPS injection in both prenatally LPS- and control-offspring. Where control offspring and prenatally LPS exposed offspring, which received a PBS injection during adulthood, showed comparable levels of BDNF. This restoration of BDNF expression levels in total hippocampus tissue of LPS offspring mice can probably be attributed to the production of BDNF by neurons and astrocytes, which in general express the highest levels of BDNF in the brain (Zhang et al., 2014). BDNF is a key player in neuronal survival, maintenance of neuronal circuits, is implicated in learning and memory and is highly involved in synaptic plasticity (Zagrebelsky and Korte, 2014). Although the critical role of BDNF in learning and memory is evident, the exact mechanisms of action of BDNF, mainly through signaling through the TrkB receptor, are still under extensive investigation. The fact that BDNF is able to regulate excitatory and inhibitory synapses (Bolton et al., 2000; Cunha, 2010) makes delineating these mechanisms even more complicated. In hippocampal synaptic plasticity, BDNF has been shown to promote LTP induction, which is considered to reflect learning and memory. Heterozygous BDNF KO mice display reduced LTP induction and impaired memory but also mice with BDNF overexpression showed impaired LTP induction and substantial learning and memory deficits in behavioral paradigms (Cunha, 2010). This shows that regulation of BDNF is a complex and tightly regulated process in learning and memory. In regard to microglia-specific BDNF, Parkhurst et al. (2013) showed that microglia depletion in mice resulted in impaired learning and decreased motor learning-dependent synapse formation. Furthermore, removal of specifically microglial BDNF had similar effects (Parkhurst et al., 2013). Because we only observed impairment in T-maze learning but not in memory retention, these data suggest that microglial BDNF is involved in learning, but possibly not in the formation of long-term memory.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.nbd.2017.07.017>.

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