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Prenatal inflammation impairs adult neurogenesis and memory related behavior through persistent hippocampal TGFβ$_1$ downregulation

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ABSTRACT

Prenatal exposure to inflammatory stimuli is known to influence adult brain function. In addition, adult hippocampal neurogenesis is impaired by a local pro-inflammatory microenvironment. On this basis, we hypothesized that a pro-inflammatory insult during gestation would have negative effects on adult neurogenesis in the offspring. Pregnant Wistar rats received subcutaneous injections of lipopolysaccharide (LPS; 0.5 mg/kg) or saline every other day from gestational day 14 to 20. The adult offspring prenatally treated with LPS showed a decrease in the proliferating cells and the newborn neurons of the dentate gyrus. Furthermore, prenatal LPS treatment impaired performance in the neurogenesis-dependent novel object recognition test. Maternal care was impaired by prenatal LPS administration but did not contribute to the effects of prenatal LPS on adult neurogenesis. Persistent microglial activation and downregulated expression of transforming growth factor beta-1 (TGFβ$_1$) occurred specifically in the adult hippocampus of animals treated prenatally with LPS. Importantly, chronic hippocampal TGFβ$_1$ overexpression restored neurogenesis as well as recognition memory performance to control levels.

These findings demonstrate that prenatal inflammation triggered by LPS impairs adult neurogenesis and recognition memory. Furthermore, we provide a model of reduced adult neurogenesis with long-lasting defined alterations in the neurogenic niche. Finally, we show that the expression of a single cytokine (TGFβ$_1$) in the hippocampus can restore adult neurogenesis and its related behavior, highlighting the role of TGFβ$_1$ in these processes.

1. Introduction

Long-term effects of prenatal inflammatory stimuli have been extensively described, showing the vulnerability of the central nervous system (CNS) to inflammation at this stage. Epidemiological studies have linked infections during pregnancy to later development of neuropsychiatric disorders in the offspring (Hultman et al., 1999; Mednick et al., 1988). This correlation is supported by experimental data in rodents showing that maternal inflammation or infections during fetal development impair several behaviors and can lead to white-matter damage and even cerebral palsy in the adult offspring (Shi et al., 2003; Yoon et al., 1997). In addition, prenatal administration of lipopolysaccharide (LPS; a bacterial endotoxin) or polyriboinosinic–polyribocytidylic acid (poly I:C; a dsRNA mimicking viral infections), impair behavioral tasks (Meyer et al., 2006; Hao et al., 2010).

Adult neurogenesis, or the birth of new neurons from adult neural stem/progenitor cells (NSCs), occurs in the subgranular zone (SGZ) of the dentate gyrus (DG) at the hippocampus (Gage, 2002). Here, the environment or niche in which NSCs reside, composed of neighboring cells and soluble factors, tightly regulates their proliferation and differentiation (Alvarez-Buylla and Lim, 2004; Gage, 2002).

Among the key factors regulating the NSC niche, microglia, the resident macrophages of the brain, shift to activated states in response to external stimuli; this alters their morphology and their pattern of secreted factors, including cytokines (Simard and Rivest, 2004; Ekdahl et al., 2009; Whitney et al., 2009). Cytokines in turn play a central role as modulators of the NSC niche in different processes, such as immune-mediated responses and neuronal survival (Gage, 2002; Perry et al., 2002). Recently, the pro-inflammatory cytokines interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α, as well as LPS; have been shown to exert anti-neurogenic effects (Iosif et al., 2006; Koo and Duman, 2008; Monje et al., 2003; Vallières et al., 2002). Conversely, transforming growth factor beta-1 (TGFβ$_1$), an anti-inflammatory cytokine, has a pro-neurogenic effect on adult NSCs (Battista et al., 2006).

The neurogenic niche of the DG is programmed during perinatal development. Indeed, it has been shown that the formation of the DG occurs mainly at the early postnatal period, with NSCs proliferating in the hilus and subsequently migrating as neuroblasts

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towards the Granule Cell Layer (GCL) (Namba et al., 2005). Therefore, this process could be altered by insults during the perinatal period. Prenatal administration of LPS (Cai et al., 2000) or poly I:C (Meyer et al., 2006) induces pro-inflammatory cytokine expression and activates microglia in the rat fetal and pup brain. In addition, prenatal and neonatal LPS exposure causes long-term memory impairments (Hao et al., 2010; Bilbo et al., 2005). However, no link has been established yet between prenatal inflammation, adult neurogenesis and adult memory related behaviors.

These data support the idea that prenatal pro-inflammatory insults have deleterious effects in the developing CNS, which are reflected in problems with several functions of the adult brain. Yet the cellular and molecular mechanisms that link prenatal inflammation with long-term CNS impairments have not been elucidated.

Our aim was to determine whether and how prenatal inflammation diminishes adult neurogenesis and neurogenesis-dependent behaviors. Here we show that a pro-inflammatory insult administered during the prenatal period causes a lower neurogenesis concomitantly with an impaired performance in a memory related test, with hippocampal TGFβ_1 levels playing a role in mediating both effects.

This work supports the relevance of early experiences in influencing adult neurogenesis, neurogenesis-dependent behaviors, and the composition of the neurogenic niche. It also presents a molecular link between a prenatal insult, neurogenesis, and a dependent behavior, and highlights the notion of including endogenous immune components of the hippocampus as part of the neurogenic niche.

2. Materials and methods

2.1. Animals

Adult Wistar rats (250–300 g) were bred in the animal house at the Leloir Institute housed under controlled temperature (22 ± 2 °C) and artificial light under a 12-h cycle, with water and food available *ad libitum*. All animal procedures were performed according to the rules and standards of German animal law and the regulations for the use of laboratory animals of the National Institutes of Health, USA. Animal experiments were approved by the Ethical Committee of the Leloir Institute Foundation.

2.2. Prenatal LPS treatment, BrdU staining and stereotaxic injections

Pregnant dams were housed individually. Each received a subcutaneous (sc) injection of LPS (0.5 mg/kg; strain 0111:B4, Sigma–Aldrich, MA, USA) or saline on gestational/embryonic (G/E) days 14, 16, 18, and 20. The litters were culled to 10 pups, weaned at postnatal day (P) 21, and housed with 5 animals per cage until adulthood (P60). One pup from each litter was randomly sampled and assigned to an experimental group to reduce the influence of litter effects on the variables measured, as it has been previously suggested (Zorrilla, 1997). Specifically, we have sampled 1 pup per litter for proliferation and differentiation analyses (5–6 dams per treatment, Fig. 1), for microglia (5–6 dams per treatment, Fig. 3) and astrocytosis (5–6 dams per treatment, Supplementary Fig. 1) quantitation, and for adult behavioral tests (8–9 dams per treatment, Fig. 5D, and 4–8 dams per treatment, Supplementary Figs. 3 and 4). In the rest of the experiments, between 1 and 2 pups were sampled from each litter (3–6 dams per treatment).

For neurogenesis analyses, adult male animals were injected intraperitoneally (ip) with 5-bromo-2′-deoxiuridine (BrdU, 50 mg/kg) daily for 7 days (Fig. 1A). At the 7th, or 30th day, depending on the experiment, the animals were perfused and brains processed for immunohistochemistry (see below). A separate group of adult male rats treated prenatally with LPS or saline received a stereotaxic injection of adenoviral vectors (adenovectors) expressing TGFβ_1 or β-galactosidase at the DG coordinates (see below), followed by 7-daily ip injections of BrdU from days 2 to 9 and perfusion at the 14th day (Fig. 5A).

For stereotaxic injections, the animals were anaesthetized with ketamine chlorhydrate (80 mg/kg) and xylazine (8 mg/kg) and then injected in the DG with 1×10^7 pfu/μL of adenoviral vectors expressing either β-galactosidase or TGFβ_1. When assessing adult neurogenesis, adenoviral vectors were injected on only one side of the DG, and adult neurogenesis levels were determined on that same hemisphere. In the case of Novel Object Recognition test, adenoviral vectors were injected on both sides of the DG. The stereotaxic coordinates of the DG were bregma, −3.3 mm; lateral, 1.7 mm; ventral, −3.5 mm (Paxinos and Watson, 1986).

2.3. Vectors

Adenovectors expressing β-galactosidase and TGFβ_1 were generated, controlled, and used as previously described (Ferrari et al., 2004). Stocks were quantified by plaque assay (final titers: Adβgal = 5.56×10^{11} pfu/μL, AdTGFβ = 1.1×10^{12} pfu/μL), and had less than 1 ng/mL of endotoxin (E-TOXATE Reagents, Sigma). Viral stocks were free of autoreplicative particles. Adβgal was kindly provided by Dr. J. Mallet (Hospital Pitie Salpetriere, Paris, France), and AdTGFβ was kindly provided by J. Gauldie (Department of Pathology, McMaster University, Ontario, Canada).

2.4. Tissue sections

Animals were deeply anesthetized and transcardially perfused with heparinized saline followed by cold 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2. Brains were placed in the same fixative overnight at 4 °C, cryoprotected in 30% sucrose in 0.1 M phosphate buffer, and frozen in isopentane. Serial coronal sections (40 μm) through the extent of the hippocampus (Paxinos and Watson, 1986) were cut on a cryostat. The 40-μm sections were used for free-floating immunostaining.

2.5. Immunostaining

Immunostaining was performed as described elsewhere (Battista et al., 2006). The primary antibodies were rat anti-BrdU (1:200; Abcam, Cambridge, UK), rabbit anti-doublecortin (DCX, 1:400, Abcam), mouse anti-NeuN (1:200, Chemicon) and *Griffonia simplicifolia* lectin (GSAI-B4, Vector). Secondary antibodies used were Cy2-conjugated donkey anti-rat, Cy3-conjugated donkey anti-mouse, Cy3-conjugated donkey anti-rabbit, and Cy2-streptavidin (1:200; Jackson Laboratories, West Grove, PA).

2.6. Quantitation of neurogenesis and microglial activation

The total number of proliferating cells, neurogenesis, and microglial activation were quantified as described elsewhere (Battista et al., 2006). GSAI-B4 was used to assess microglial activation based on cell morphology (Kreutzberg, 1996). Four stages (I–IV) of microglial activation were defined. At stage I, cells have rod-shaped cell bodies with fine, ramified processes and are defined as resting microglial cells. Resting (stage I) microglia were not quantified because the GSAI-B4 staining of the fine microglial processes gives no reliable way of quantifying individual cells in the DG. At stage II, cells have elongated cell bodies with long thick processes; at stage III, cells have small, thick processes and a rounded morphology. At stage IV, cells have a rounded morphology with no processes and vacuolated cytoplasm, and stain for EDI, a

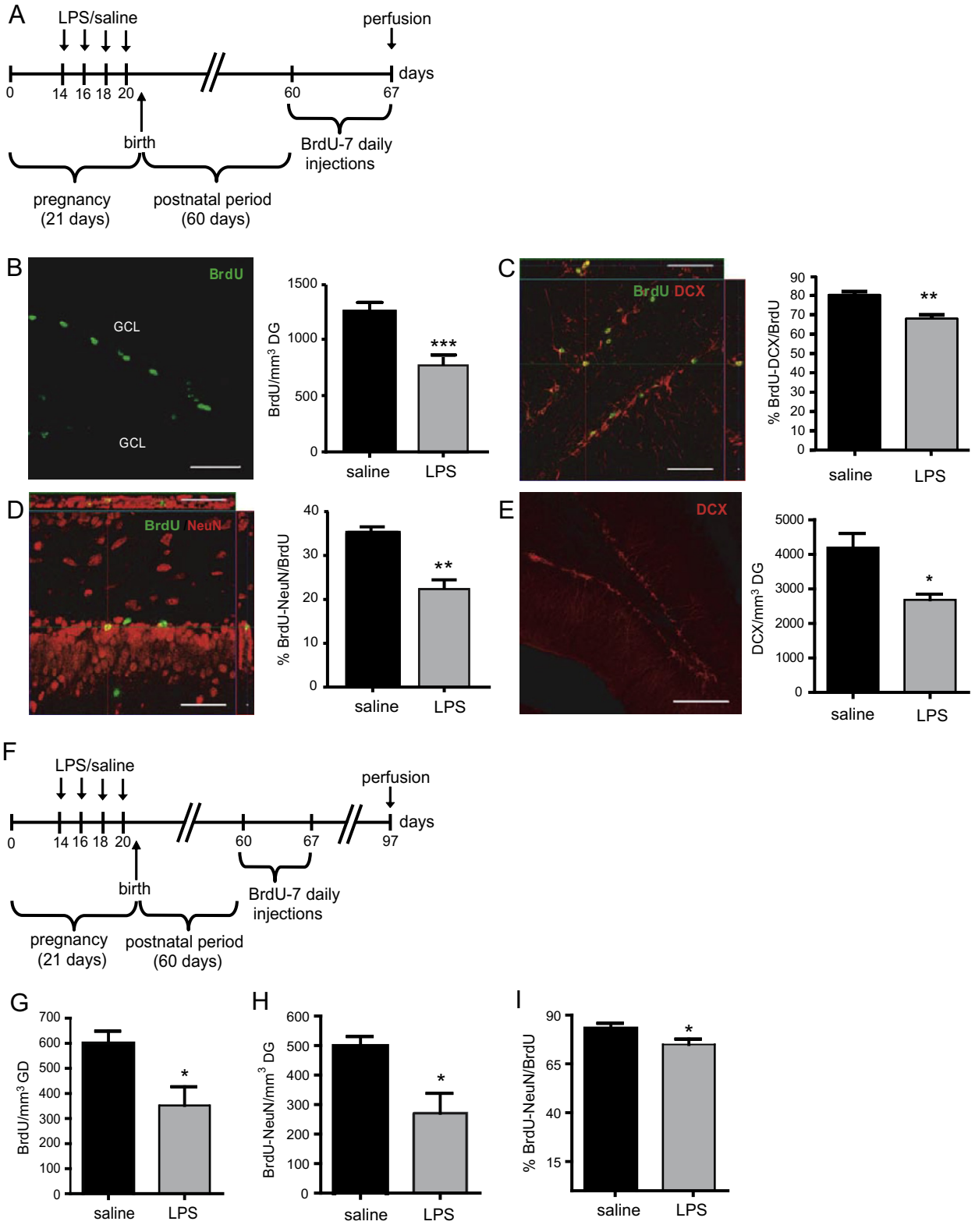

Fig. 1. Prenatal LPS impairs adult neurogenesis. (A) Timeline of the experimental approach. (B–E) Immunohistochemistry and quantitation of newborn neurons. (B) Quantitation of the total number of BrdU-positive cells in the subgranular zone per mm^3 of dentate gyrus (DG). Student's *t*-test, $^{***}p < 0.001$. (C) Percentage of cells labeled with DCX among the BrdU-positive population in the DG. Student's *t*-test, $^{**}p < 0.01$. (D) Percentage of cells labeled with NeuN among the BrdU-positive population in the DG. Student's *t*-test, $^{**}p < 0.01$. (E) Quantitation of the total number of DCX-positive cells in the subgranular zone per mm^3 of dentate gyrus (DG). Student's *t*-test, $^{*}p < 0.05$. (F) Timeline of the experimental approach. Analysis of the BrdU-positive cell population 30 days after BrdU administration. (G) Quantitation of the total number of BrdU-positive cells in the subgranular zone per mm^3 of the DG. Student's *t*-test, $^{*}p < 0.05$. (H) Total number of BrdU/NeuN-positive cell population per mm^3 of DG 30 days after BrdU administration. Student's *t*-test, $^{*}p < 0.05$. (I) Percentage of double-labeled BrdU/NeuN-positive cells relative to the total number of BrdU-positive cells in the DG. Student's *t*-test, $^{*}p < 0.05$. Left columns show representative confocal images. Scale bars: B–D, 50 μm; E, 200 μm. Values are means ± SEM. GCL: Granular cell layer.

marker of phagocytic activity. Stages II, III, and IV are defined as activated microglial cells.

2.7. Maternal behavior

Quantitation of maternal behavior was performed as previously described (Depino et al., 2008). Briefly, maternal observations (60 observations per hour) were performed for 2 h during the light period from P1 to P7. The number of observations of arched-back nursing (ABN), and maternal licking and grooming of the pups (LG) is expressed as their percentage over total observations.

In the cross-fostering study, saline- and LPS-treated dams were allowed to give birth. Within 12 h of birth, the dams were removed from the home cage and similarly sized litters were cross-fostered. The procedure took less than 10 min. The critical groups of interest were animals born to saline-treated mothers and fostered to LPS-treated mothers, and the reciprocal group. The maternal behavior of each dam was then observed for the following 7 days as described above.

2.8. RNA isolation, reverse transcription and real-time PCR

Whole brains from E14 embryos and hippocampal coronal sections from P0 pups were obtained. A group of adult animals was sacrificed, their brains were quickly removed, and the whole hippocampus was dissected out of each brain. The tissues were snap-frozen in liquid nitrogen and stored at −80 °C. RNA isolation, reverse transcription, and real-time PCR were performed as described elsewhere (Battista et al., 2006). Beta-2 microglobulin was used as a house keeping gene as described before (Pitossi et al., 1997; Depino et al., 2003, 2005; Battista et al., 2006).

2.9. Western blotting

A separate group of adult male animals was sacrificed, their brains were quickly removed, and the whole hippocampus was dissected out of each brain. The tissues were snap-frozen in liquid nitrogen and stored at −80 °C until use. Western blotting was performed as described elsewhere (De Lella et al., 2010) with equal amounts of protein samples (30 μg). The primary antibodies used were against TGFβ_1 (1:100; Abcam) and actin (1:2000; Sigma).

2.10. Novel object recognition (NOR)

We analyzed NOR following a previously described protocol (Clark et al., 2000; Jessberger et al., 2009), in an opaque plastic chamber (45 × 45 × 30 cm). A video camera mounted directly above the chamber recorded the testing session for offline analysis. Overhead fluorescent lighting illuminated the testing area (80 lux). A single NOR trial consisted of a *habituation phase* of 5 min in the empty chamber, a *familiarization phase* (encoding) of 5 min, and a prescribed delay interval followed by the *test phase* (retrieval). During the familiarization phase, the rat was allowed to explore and become familiar with two identical objects. Delay intervals of 1 min and 3 h intervened before the test phase. During the test phase, the rat was allowed to explore two objects, one novel object and a third, identical copy of the object from the familiarization phase. Object exploration was scored [nose within 2 cm of object and vibrissae moving (Clark et al., 2000)] for 5 min. The percentage of time that a rat spent exploring the novel object over the total time of exploration was calculated. The delay intervals, which object was novel, and the left/right position of the novel object were counterbalanced within each group. When animals were re-tested two weeks after adenoviral vectors injections, different objects from the ones used in the first NOR test were used. It was previously reported that repeated testing does not interfere with preference for the novel object (Besheer et al., 1999). The experimenter was blind to the treatments during testing and offline data analysis.

2.11. Statistical analysis

Data are presented as means ± standard error of the mean (SEM). Comparisons were performed using Student's *t*-test (two-tailed) and one- and two-way analysis of variance (ANOVA) followed by the post hoc Tukey's test. Differences were considered significant when $p < 0.05$. When required, data were transformed to fulfill normality and homocedacy criteria.

3. Results

3.1. Prenatal LPS exposure results in lower cell proliferation and neurogenesis, in the adult DG

In order to answer the question of whether prenatal LPS affected cell proliferation and neuronal differentiation, we treated rat dams with LPS or saline every other day from G14 to G20. Adult offspring (P60) received daily injections of BrdU for 7 days, and analyses were performed at day 67 (Fig. 1A). Prenatal exposure to LPS reduced the population of cells undergoing proliferation from 0 to 7 days old (BrdU-positive) in the SGZ of the DG of adult rats (Fig. 1B, Student's *t*-test, $t_{10} = 5.49$, $p < 0.001$, $n = 6$ per group).

The newborn neurons among the BrdU-positive population, detected as BrdU/DCX- and BrdU/NeuN-positive cells, were also reduced in the prenatal LPS-treated group (Fig. 1C, Student's *t*-test, $t_8 = 4.76$, $p < 0.01$, $n = 5$ per group; and Fig. 1D, Student's *t*-test, $t_9 = 4.79$, $p < 0.01$, $n = 5$–6 per group). The total number of newborn neurons, stained as DCX-positive cells were diminished as well (Fig. 1E, Student's *t*-test with Welch's correction, $t_6 = 3.44$, $p < 0.05$, $n = 6$ per group). Thus, we can conclude that prenatal LPS reduced net neurogenesis.

Next, we tested any potential effects of prenatal LPS on later cell survival. The same 7-daily BrdU protocol was performed, but the analysis was done 30 days after the last BrdU injection (Fig. 1F). Therefore, we are assessing survival of cells which are from 30 to 37 days old. As with the previous protocol, we found fewer BrdU-positive cells (Fig. 1G, Student's *t*-test, $t_9 = 2.90$, $p < 0.05$, $n = 5$–6 per group) and BrdU/NeuN-positive cells (Fig. 1H, Student's *t*-test, $t_9 = 3.21$, $p < 0.05$, $n = 5$–6 per group) in the prenatal LPS group. The magnitude of the decline in proliferating cells and newborn neurons caused by prenatal LPS was similar at the early and late time points of analysis (Proliferating cells: Fig. 1B vs. G. Newborn neurons: saline, 462.8 ± 79.8; LPS, 232.7 ± 46 vs. Fig. 1H). Thus, when the ratio of BrdU- and BrdU/NeuN-positive cells was compared at the two time points of analysis, no differences were observed between the saline and LPS groups (number of BrdU-positive cells counted 30 days after BrdU incorporation/those counted immediately after BrdU incorporation: saline:601.1/1396 = 0.43; LPS:342/708 = 0.48. Number of BrdU/NeuN-positive cells counted 30 days after BrdU incorporation/those counted immediately: saline:497.8/462.8 = 1.08; LPS:270.5/232.7 = 1.16). Thus, the survival of proliferating cells and of newborn neurons at later time points (between 30 and 37 days) was not affected by prenatal LPS.

The lower percentage of BrdU/NeuN-positive newborn neurons over the total BrdU-positive population in the LPS group persisted 30 days after BrdU administration (Fig. 1I, Student's *t*-test, $t_9 = 2.29$, $p < 0.05$, $n = 5$–6 per group), indicating that the prenatal LPS effect on the neuronal differentiation of adult NSCs is long-lasting.

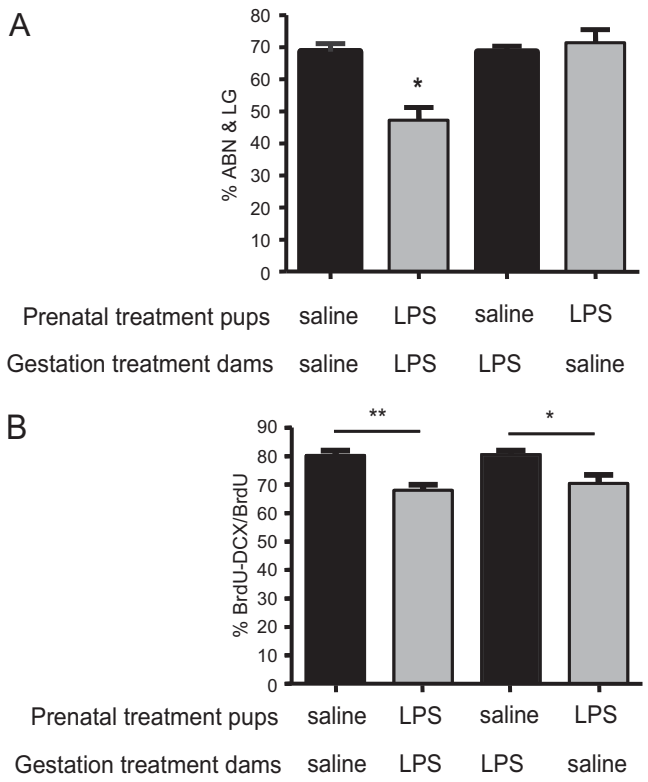

Fig. 2. Prenatal LPS affects adult neurogenesis independently of maternal care of the offspring. (A) Maternal care of the pups measured as percentage of the time in arched-back nursing (ABN) and licking and grooming (LG) of the pups. Tukey's post hoc test, $^{*}p < 0.05$, prenatally treated pups (PrT) LPS + gestationally treated dam (GTD) LPS vs. all other groups. (B) Percentage of double-labeled BrdU/DCX cells relative to total BrdU-positive cells in cross-fostered groups. Tukey's post test, $^{**}p < 0.01$ PrT saline + GTD saline vs. PrT LPS + GTD LPS; $^{*}p < 0.05$ PrT saline + GTD LPS vs. PrT LPS + GTD saline.

3.2. Maternal behavior impairment due to prenatal LPS exposure does not account for reduced adult neurogenesis

Maternal care during early life influences hippocampal development and function (Bredy et al., 2003; Liu et al., 2000). Conversely, maternal separation results in fewer immature neurons in the adult DG (Mirescu et al., 2004), and prenatal restraint stress impairs maternal care (Baker et al., 2008; Champagne and Meaney, 2006). We hypothesized that prenatal LPS would impair maternal care, and that this impairment could contribute to the observed reduction in adult neurogenesis.

Maternal care was scored during the first postnatal week. The dams injected with LPS during pregnancy showed a significant reduction in typical maternal care behaviors. When cross-fostered, both saline- and LPS-treated litters received high levels of maternal care by their foster mothers, regardless of their treatment (Fig. 2A, two-way ANOVA main effects: prenatal treatment pups, $F_{1,6} = 4.9$, $p = 0.07$; gestation treatment dam, $F_{1,6} = 8.26$, $p < 0.05$; interaction $F_{1,6} = 8.19$, $p < 0.05$; $n = 2$–4 per group). Thus, LPS treatment impairs maternal care only in the case of dams that have received LPS during pregnancy towards litters that have received LPS prenatally.

However, the decrease in neurogenesis was observed in litters treated prenatally with LPS, regardless whether they were taken care of by their own mothers or by saline-treated foster mothers (Fig. 2B, two-way ANOVA main effects: prenatal treatment, $F_{1,18} = 25.76$, $p < 0.001$; maternal care, $F_{1,18} = 0.23$, $p = 0.63$; interaction, $F_{1,18} = 0.36$, $p = 0.56$; $n = 5$–6 per group). Thus, LPS administered to pregnant dams impaired adult neurogenesis independently of its effects on maternal care of their offspring.

3.3. Prenatal LPS exposure results in activation of hippocampal microglia

In order to elucidate whether persistent alterations in the adult neurogenic niche could account for the reduced neurogenesis in the prenatal LPS-treated offspring, we examined if prenatal LPS affects the cellular environment of the adult NSCs. Microglial activation can be classified by observing cell morphology (Kreutzberg, 1996; see Methods). Microglial cells of the DG were morphologically shifted to an activated stage (II–III, not IV) in response to prenatal LPS (Fig. 3A–C Student's t-test, $t_7 = 5.62$, $p < 0.001$, $n = 4$–5 per group). Thus, the effects of prenatally administered LPS on microglial activation were also long-lasting, as with neurogenesis. No microglial activation was observed in other brain regions of the brain such as cortex, thalamus, and hypothalamus, indicating that microglial activation in the DG is specific. In contrast, astroglia were not altered in immunoreactivity, or morphology—both indicatives of astrocytocis—in response to prenatal LPS (Supplementary Fig. 1A–C, Student's t-test, $t_{10} = 0.19$, $p = 0.86$, $n = 6$ per group), suggesting that microglial cells are a preferential cellular target of prenatal LPS.

3.4. Prenatal LPS alters the local cytokine expression profile acutely and in adulthood

Next, we examined the cytokine expression levels at different times after LPS injections. At E14 we noticed acute increases of both pro-inflammatory IL-1β (Fig. 4A, one-way ANOVA: $F_{2,7} = 6.49$, $p < 0.05$, $n = 3$–4 per group) and anti-inflammatory TGFβ$_1$ expression (Fig. 4A, one-way ANOVA: $F_{2,9} = 7.48$, $p < 0.05$, $n = 3$–6 per group), but no increase in IL-6 (Fig. 4A, one-way ANOVA: $F_{2,1} = 2.76$, $p = 0.11$, $n = 4$–5 per group). At P0, IL-1β expression was significantly higher in the prenatal LPS group (Fig. 4B, Student's t-test, $t_7 = 3.09$, $p < 0.01$, $n = 7$–12 per group), whereas IL-6 expression was unaltered. TGFβ$_1$ expression was lower in the LPS-treated group at the end of the whole LPS administration protocol (Fig. 4B, Student's t-test, $t_6 = 2.64$, $p < 0.05$, $n = 7$–10 per group). At P60 (adulthood), only TGFβ$_1$ expression in the hippocampus remained significantly lower in the prenatal LPS group (Fig. 4C, Student's t-test, $t_8 = 3.78$, $p < 0.01$, $n = 4$–5 per group). This result was also confirmed at the protein level (Fig. 4D, Student's t-test, $t_6 = 1.99$, $p < 0.05$, $n = 3$–4 per group).

Thus, prenatal LPS administration affected the immune molecular component of the neurogenic niche, initially balancing towards pro-inflammatory factors that have been reported to be anti-neurogenic (Koo and Duman, 2008; Monje et al., 2003), and downregulating TGFβ$_1$ in the adult. This last result indicates a long-lasting effect of prenatal LPS on the local expression on this cytokine.

3.5. TGFβ$_1$ downregulation functionally contributes to the decrease in adult neurogenesis by prenatal LPS

We hypothesized that downregulated TGFβ$_1$ expression levels in the hippocampus could be functionally related to the concomitant impairment in neurogenesis in the prenatal LPS group. Therefore, we speculated that local, chronic TGFβ$_1$ overexpression in the adult DG would at least partially restore adult neurogenesis.

Adenovectors expressing TGFβ$_1$ (AdTGFβ$_1$) or β-galactosidase (AdβGal) were stereotaxically injected into the DG of the adult offspring treated prenatally with LPS or saline. BrdU was administered from days 2 to 9 after inoculation of the viral vectors, and neurogenesis levels were assessed on day 14 (Fig. 5A). Viral vectors

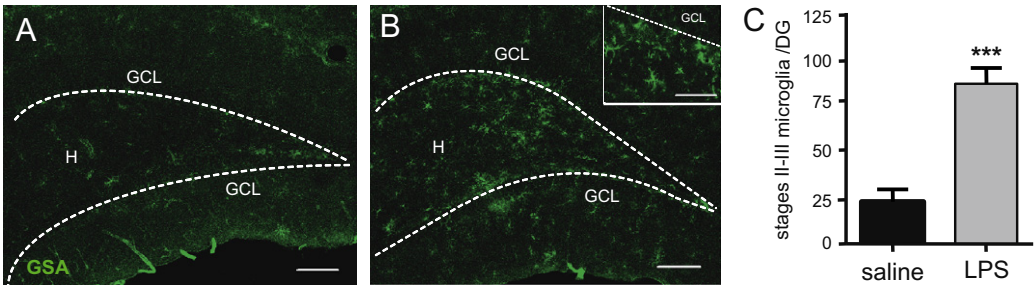

Fig. 3. Long-term hippocampal microglial activation after prenatal LPS, Staining analysis showing *G. simplicifolia* isolectin-B4-positive cells (GSAI-B4 green) in the DG after prenatal saline (A) and LPS (B). (C) Quantification of activated microglia (stages II–III) per DG. ***$p < 0.001$. Scale bars: 200 μm. Inset: 50 μm. GCL: Granule Cell Layer. H: Hilus. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

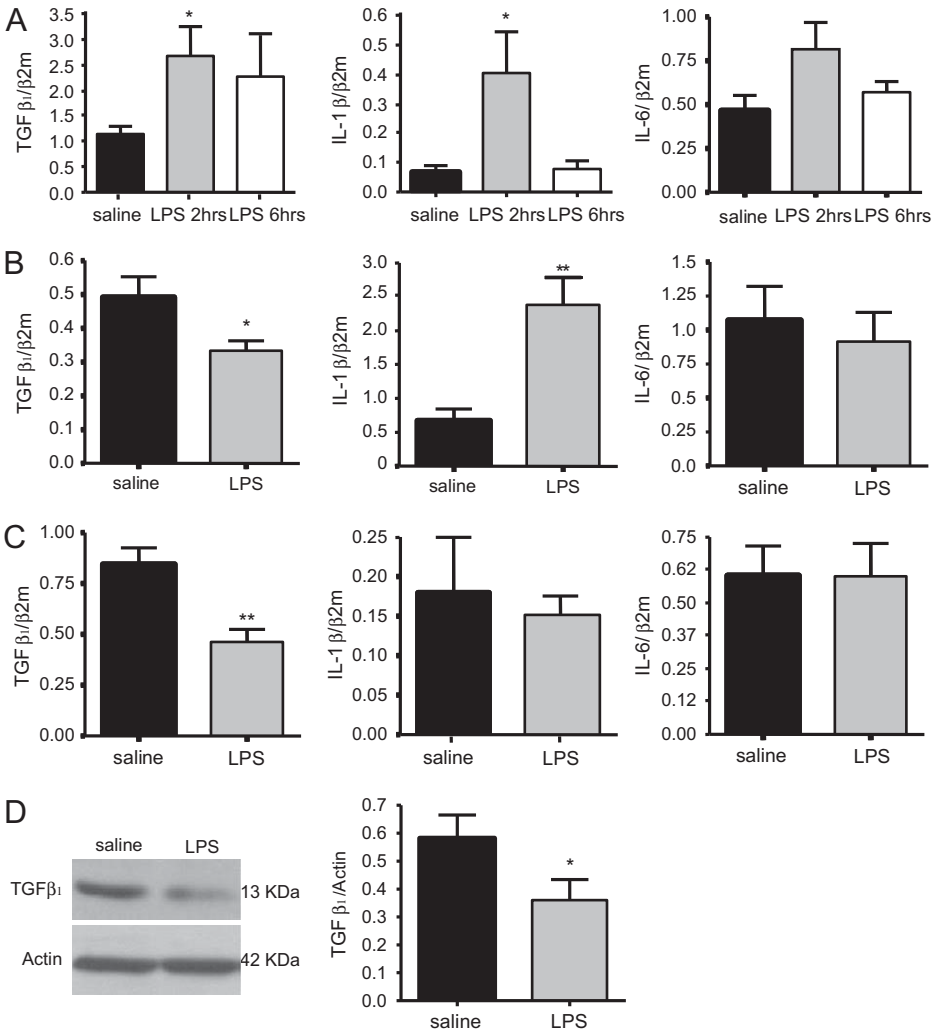

Fig. 4. Prenatal LPS induces acute changes in the neurogenic microenvironment and long-lasting TGFβ$_1$ downregulation. mRNA expression analysis by real-time PCR of the cytokines IL-1β, IL-6, and TGFβ$_1$ in whole brain tissue from fetuses (E14, A), newborn pups (P0, B), and hippocampal tissue from adult rats (P60, C). (D) TGFβ$_1$ protein expression analysis by Western blot of hippocampal tissue from adult rats (P60). Values are means ± SEM. Tukey's post hoc test (E14, *$p < 0.05$) and Student's *t*-test (P0 and P60, *$p < 0.05$; **$p < 0.01$). β2 m: beta-2 microglobulin, used for normalization.

were effectively distributed along the dorsal DG from −2.3 to −5.2 mm from bregma, and the local expression of both transgenes—β-galactosidase and TGFβ$_1$—was confirmed (Supplementary Fig. 2A and B).

Chronic overexpression of TGFβ$_1$ did not significantly increase neurogenesis in the prenatal saline group, but did restore neurogenesis to the control levels in the prenatal LPS group (Fig. 5B, two-way ANOVA main effects: prenatal treatment, $F_{1,20} = 5.19$, $p < 0.05$; adult adenoviral injection, $F_{1,20} = 12.41$, $p < 0.01$, interaction, $F_{1,20} = 4.82$, $p < 0.05$, $n = 5–8$ per group). This suggests that TGF-β$_1$ downregulation is involved in the effects of prenatal LPS on adult neurogenesis.

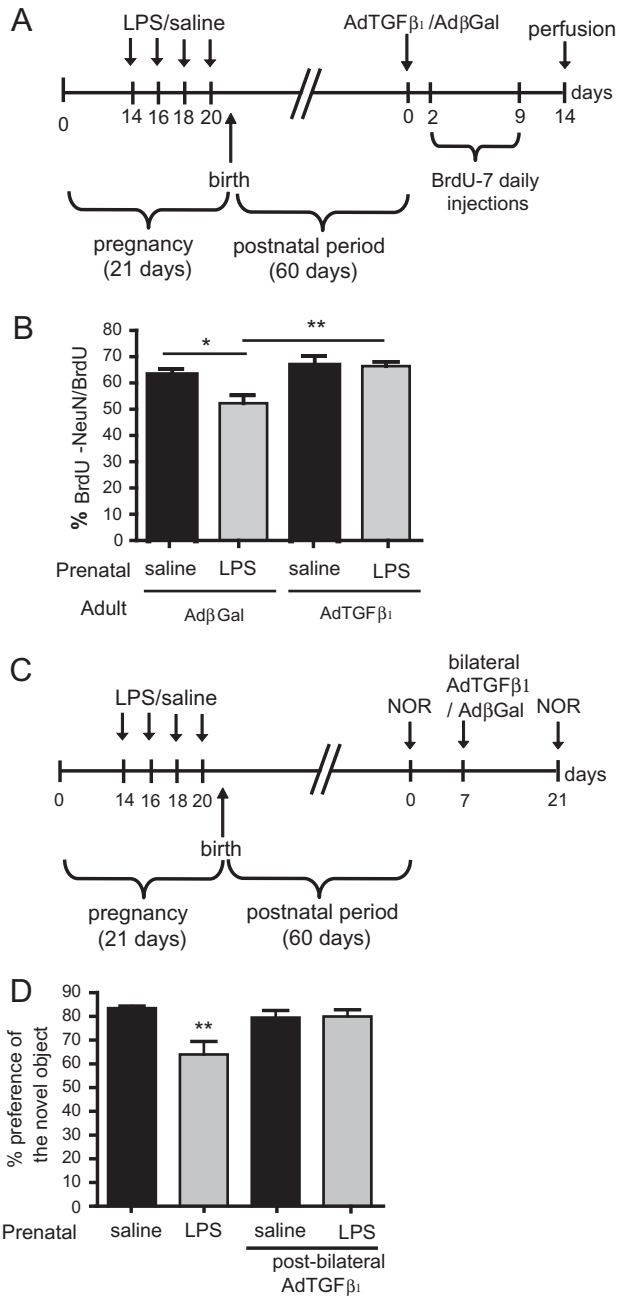

Fig. 5. Local TGFβ$_1$ overexpression reverses neurogenesis levels and Novel Object Recognition test performance. (A) Timeline of the experimental approach for overexpressing TGFβ$_1$ with adenovectors. (B) Percentage of double-labeled BrdU/NeuN-positive cells relative to total BrdU-positive cells. Values are means ± SEM. Tukey's post hoc test, $^{*}p < 0.05$ Saline-AdβGal vs. LPS-AdβGal, $^{**}p < 0.01$ LPS-AdβGal vs. LPS-AdTGFβ$_1$ and Saline-AdTGFβ$_1$ vs. LPS-AdβGal. (C) Timeline of the experimental approach for the NOR test of short-term memory. (D) Percentage of novel object exploration time over total exploration time during the 5-min test phase after a 3-h interval. Values are means ± SEM. Tukeýs post test, $^{**}p < 0.01$ prenatal LPS without AdTGFβ$_1$ vs. all other groups.

3.6. Impairment of novel object recognition by prenatal LPS exposure is reversed by hippocampal TGFβ$_1$ overexpression

Adult neurogenesis is involved in hippocampal-dependent behaviors. It has been linked to depression- and anxiety-related behavioral models in rodents (Santarelli et al., 2003). In addition, the hippocampus plays an important role in memory and learning tasks. Recent work showed that adult neurogenesis is involved in novel object recognition (NOR), a behavioral test for short-term memory (Clark et al., 2000; Jessberger et al., 2009; Clelland et al., 2009). We therefore, hypothesized that the decrease in neurogenesis in our model would have a behavioral correlate in these cognitive functions.

We tested rats for sucrose preference (SP) and Porsolt swimming test (PS), both models of depression; novelty suppressed feeding test (NSF), a model of anxiety, and burrowing (B), a test for sickness behavior. Both experimental groups behaved equally on all of these tests (Supplementary Fig. 3A–D. SP, Student's *t*-test, $t_{10} = 1.40$, $p = 0.19$, $n = 6$ per group; PS, Student's *t*-test, $t_2 = 1.24$, $p = 0.24$, $n = 6$–8 per group; NSF, Student's *t*-test, $t_9 = 0.18$, $p = 0.86$, $n = 4$–7 per group; B, Mann–Whitney test $U_{10} = 17.50$, $p = 0.63$, $n = 5$–7 per group). Therefore, the decrease in neurogenesis by prenatal LPS administration did not result in depression- or anxiety-related behaviors in the adult offspring.

Animals were also tested on the NOR test (Fig. 5C). The groups showed equal increases in novel object exploration when the delay interval was 1 min (Supplementary Fig. 4A, Student's *t*-test, $t_5 = 0.62$, $p = 0.54$, $n = 8$–9 per group). However, only the prenatal saline group differentially explored the novel object when the delay interval was 3 h. In contrast, prenatal LPS exposure impaired recognition memory, as the animals spent similar amounts of time exploring the novel and familiar objects (Fig. 5D).

Because TGFβ$_1$ downregulation was involved in the decrease in neurogenesis caused by prenatal LPS, and the NOR task is dependent on adult neurogenesis (Jessberger et al., 2009), we hypothesized that TGFβ$_1$ downregulation could also be involved in impairing NOR performance. If so, bilateral TGFβ$_1$ overexpression should restore, at least in part, the novel object exploration in the prenatal LPS group. The same animals already tested for NOR were bilaterally injected with AdTGFβ$_1$ into the DG and tested 14 days after the injection (Fig. 5C). Upon TGFβ$_1$ bilateral overexpression, prenatally LPS-treated animals spent the same amount of time exploring the novel object as prenatally saline-treated rats (Fig. 5D). Moreover, the same LPS-treated animals that showed reduced novel object recognition before the AdTGFβ$_1$ injection recognized the novel object after the AdTGFβ$_1$ injection (Fig. 5D, two-way ANOVA main effects: prenatal, $F_{1,26} = 5.61$, $p < 0.05$; AdTGFβ$_1$, $F_{1,26} = 2.22$, $p = 0.15$; interaction, $F_{1,26} = 6.15$, $p < 0.05$, $n = 6$–9 per group). Thus, in addition to modulating adult neurogenesis levels, TGFβ$_1$ modulated NOR behavior. The differences were not due to altered total exploration or locomotion (Supplementary Fig. 4B and C). This result functionally links TGFβ$_1$ levels in the DG with adult neurogenesis and a specific behavioral paradigm (NOR).

4. Discussion

The aims of this study were to investigate whether inflammatory events in the prenatal period alter adult neurogenesis and related behaviors, and which mechanisms underlie these alterations.

We first reported a decrease in the total population of proliferating cells during the first 7 days, as well as fewer total newborn neurons in the DG of prenatal LPS group. Among the proliferating population during this period, the proportion of newborn neurons was reduced as well. Hence, prenatal LPS has specific detrimental effects on neuronal differentiation, in addition to affecting cell proliferation and short-term survival. The lower neuronal commitment persisted after 30 days, suggesting that prenatal LPS affects neurogenesis throughout adulthood. However, the survival of proliferating cells and of newborn neurons between 7 and 30 days was not affected by prenatal LPS.

Nearly half of BrdU-positive cells are lost from the earlier (0–7 days) to the later (30–37 days) times of analysis (Fig. 1B vs. G, saline group). In line with this, it was previously shown that significant (48%) cell death of BrdU-positive cells occurs between 4 and

28 days after BrdU staining (Dayer et al., 2003). This is consistent with our data, and may account for some decline in short survival in addition to cell proliferation at the earlier time of analysis (0–7 days).

It has been reported that 100 μg/kg of prenatally administered LPS failed to decrease adult cell proliferation and differentiation (Cui et al., 2009). Our LPS treatment encompassed a longer period of pregnancy and a higher dose of LPS (500 μg/kg). In addition, our analyses involve a mixture of BrdU populations from 0 to 7 days old instead of a single BrdU-positive population 2 h old. These differences could account for the distinct results observed.

On the other hand, prenatal treatment with PolyI:C at G9, but not at G17, causes lower neurogenesis (Meyer et al., 2006). LPS is a bacterial endotoxin that mimics features of bacterial infections, whereas PolyI:C models components of a viral infection. Therefore, the nature, intensity, and time of exposure of the prenatal stimulus are crucial to determine its effect on the adult brain.

Our observations showing that prenatal LPS affected maternal care were in line with previous studies reporting that maternal behavior is altered upon adverse stimuli to pregnant dams (Baker et al., 2008; Champagne and Meaney, 2006). However, this impairment in maternal care did not influence the levels of adult neurogenesis, which depended only on the prenatal treatment. Thus, maternal care impairment and neurogenesis decrease are two independent effects triggered by prenatal LPS treatment.

The cellular niche was altered in the adult DG of prenatal LPS offspring, with increased activated microglia in this region. Phenotypically, microglia were activated to stages II–III, which are not associated with the typical secretion of pro-inflammatory cytokines detected in other models (Perry et al., 2002); this is in line with the lack of IL-1β and IL-6 overexpression in our animals. We found no induced GFAP expression after prenatal LPS, suggesting only minor participation of astrocytes and highlighting the relevance of microglial activation in this model. To the best of our knowledge, we are providing the first example of long-term, chronic microglial activation in a specific brain region after a prenatal insult.

Acute and long-lasting changes occur at the molecular level in the neurogenic niche of rats treated prenatally with LPS. First, IL-1β and TGFβ$_1$ mRNA expression acutely increased in fetal brain (E14), showing that LPS triggered the expected cytokine expression network (Cai et al., 2000). In adulthood, only pro-neurogenic TGFβ$_1$ expression remained lower in the prenatal LPS group.

Due to the sensitivity of the technique, we were unable to identify the cell population that contributes to basal (prenatal saline group) or diminished (prenatal LPS group) levels of TGFβ$_1$. It is known that the main sources of TGFβ$_1$ in the DG are microglia and astrocytes (Battista et al., 2006; Finch et al., 1993) although in a minority if cases, neuronal production has been reported (Flanders et al., 1998). Therefore, the most likely cellular source of TGFβ$_1$ in the DG may be microglial cells or astrocytes. However, since we could not detect astroglial activation, we favor the idea that activated microglial cells are the source of TGFβ$_1$. Indeed, we have previously shown that activated microglia up to type II/III produces TGFβ$_1$ in the DG in a model of adrenalectomy (Battista et al., 2006).

Prenatal LPS treatment did not affect the performance in depression- and anxiety-related behavioral models. In contrast, prenatal LPS caused deficient NOR performance. Thus, we provide data relating diminished neurogenesis in this model to a specific neurogenesis-related behavior.

Both adult neurogenesis and NOR performance were restored to control levels by hippocampal overexpression of TGFβ$_1$. Therefore, TGFβ$_1$ downregulation at the neurogenic niche could account for the effects of prenatal LPS on adult neurogenesis and its related behavior. This finding reinforces the relevance of brain cytokines as part of the adult neurogenic niche.

Although it has been shown that adult neurogenesis contributes to determining NOR performance (Jessberger et al., 2009), we cannot rule out the possibility that TGFβ$_1$ affects NOR performance via additional, non-excluding mechanisms. For example, TGFβs have been linked to promoting the development and differentiation of synaptic connections, which in turn play a key role in the processes of learning and memory (Fukushima et al., 2007; Packard et al., 2003; Chin et al., 2006; Heupel et al., 2008). Therefore, a possible influence of TGFβ$_1$ on synaptic plasticity can also contribute to the effects observed in the NOR test.

Our results highlight that the prenatal vulnerability of the CNS to pro-inflammatory insults has important consequences in the occurrence of long-term impairments in CNS function. Thus, according to our data, prenatal inflammation should be considered as a risk factor for altered behaviors that depend on adult neurogenesis.

Bacterial vaginosis is a common condition during pregnancy, occurring in 14% of pregnant women, that puts the developing fetus into contact with LPS (Dammann and Leviton, 1997; Thorsen et al., 1998; Purwar et al., 2001; Romero et al., 1989). Importantly, bacterial vaginosis can lead to white-matter damage, cognitive limitations, and cerebral palsy (Yoon et al., 1997; Dammann and Leviton, 1997). We speculate that fetal LPS exposure during pregnancy due to bacterial vaginosis could cause a non-overt effect on the CNS at first, but would impair neurogenesis and related behaviors in adulthood. Studies using non-invasive imaging of adult neurogenesis will certainly be helpful to study the correlation between prenatal bacterial infections and adult neurogenesis levels.

Conflicts of interest

All authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbi.2010.06.005.

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