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Differential vulnerability of adult neurogenesis by adult and prenatal inflammation: role of TGF-β1

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Abstract

Peripheral inflammation, both during the prenatal period and in adulthood, impairs adult neurogenesis. We hypothesized that, similar to other programming effects of prenatal treatments, only prenatal inflammation causes long-term consequences in adult neurogenesis and its neurogenic niche. To test this, pregnant Wistar rats were subcutaneously injected with lipopolysaccharide (LPS; 0.5mg/kg) or saline solution every other day from gestational/embryonic day (GD) 14 to 20. In addition adult animals were injected with a single intraperitoneal saline or LPS injection (1mg/kg) and the effects on neurogenesis were assessed 7 days later. Alternatively, to evaluate long-term consequences of adult LPS injections, LPS (1mg/kg) was administered peripherally to adult rats 4 times every other day, and the effects on neurogenesis were assessed 60 days later.

Prenatal and adult LPS treatments reduced adult neurogenesis and provoked specific microglial (but not astroglial) activation in the dentate gyrus (DG). However, only prenatal inflammation-mediated effects were long-lasting (at least 60 days). Moreover, these effects were specific to the DG since the Subventricular Zone (SVZ) and the Rostral Migratory Stream (RMS) were not affected. In addition, these stimuli caused differential effects on the molecular components of the neurogenic niche; only prenatal LPS treatment reduced the local levels of TGF-β1 mRNA in the DG. Finally, TGF-β1 exerted its pro-neurogenic effects via the Smad2/3 pathway in a neural stem cell culture.

Taken together, these data add evidence to the duration, regional specificity and dramatic consequences of prenatal immune programming on CNS physiology, compared with the limited response observed in the adult brain.

Keywords: prenatal programming, inflammation, adult neurogenesis, microglial activation, neurogenic niche, TGF-β1.

Research highlights

Prenatal, but not adult inflammation, elicits long-term reduction on adult neurogenesis in the hippocampus (but not the SVZ or the RMS) via TGF-β1

INTRODUCTION

Neurogenesis from adult neural stem/progenitor cells (NSC) occurs in the subgranular zone (SGZ) of the dentate gyrus (DG) and in the subventricular zone of the lateral ventricle (SVZ). Several studies have shown that pro- and anti-inflammatory stimuli occurring in the CNS or in the periphery modulate adult neurogenesis (Ekdahl et al, 2003, Graciarena et al, 2010, Mathieu et al, 2010b, Monje et al, 2003, Ormerod et al, 2012, reviewed in Mathieu et al, 2010a).

NSCs reside within an environment or niche which modulates NSC proliferation and differentiation. The cellular and molecular factors that modulate these effects need to be further characterized. Microglial cells are components of this niche and are capable of altering adult neurogenesis via net changes in the pattern of secreted factors, including immune cytokines (Ekdahl et al, 2009, Mathieu et al, 2010a, Mathieu et al, 2010b, Perry et al, 2002, Simard & Rivest, 2004, Whitney et al, 2009, Ziv et al, 2006). Cytokines are part of the molecular components of the neurogenic niche in normal conditions and have shown to play a central role in processes affecting NSCs biology (Gage, 2000, Mathieu et al, 2010a, Perry et al, 2002). Among them, pro-inflammatory Interleukin (IL)-6 has shown to exert anti-neurogenic effects in the adult DG (Monje et al, 2003, Vallieres et al, 2002). Conversely, the anti-inflammatory cytokine Transforming Growth Factor beta-1 (TGF-β1) has proven to be pro-neurogenic *in vitro* and *in vivo* (Battista et al, 2006, Graciarena et al, 2010, Mathieu et al, 2010a, Mathieu et al, 2010b). Nevertheless, the role of local cytokine expression on adult neurogenesis, the regional and temporal specificity of these effects, as well as the intracellular pathways involved in this modulation awaits further characterization.

Despite the particular regions that retain neurogenic potential, the adult CNS is mainly characterized by its limited plasticity. On the contrary, the developing CNS is characterized by a series of temporal windows where key proliferation, differentiation and migration events occur, which will in turn determine many of the CNS functions in the adult. These critical developmental steps are prone to be altered as a consequence of external influences (de Graaf-Peters & Hadders-Algra, 2006) which can be reflected on diverse spectra of CNS dysfunctions in the adult. The concept of programming has been built considering the long term effects that these early external influences have on the physiology and behavior of adult individuals. Programming includes alterations in adult neuronal CNS functions or immune responses caused by early (pre- or early postnatal) administration of inflammatory stimuli. In this regard, experimental studies have shown that inflammation during the prenatal period can lead to diverse alterations in CNS functions in the adult, including behavioral and cognitive impairments (Bilbo & Schwarz, 2009), alterations of the Hypothalamic-Pituitary-Adrenal axis activity (Reul et al, 1994), white matter damage and even cerebral palsy (Shi et al, 2003). In particular, we and others have shown that adult neurogenesis in the DG is impaired by prenatal inflammation triggered by lipopolysaccharide (LPS), a bacterial endotoxin (Graciarena et al, 2010); or polyriboinosinic-polyribocytidylic acid (PolyI:C), a synthetic analog of double-stranded RNA that mimicks components of a viral infection (Meyer et al, 2006). In particular, prenatal LPS administration may model bacterial vaginosis, a condition during pregnancy that put the developing fetus into contact with LPS (Dammann & Leviton, 1997, Purwar et al, 2001, Romero et al, 1989, Thorsen et al, 1998). Approximately 14% of pregnant women get bacterial vaginosis, which leads to a pro-

inflammatory environment and has been shown to lead to white matter damage, cognitive limitations and even cerebral palsy (Dammann & Leviton, 1997, Yoon et al, 1997). In addition, epidemiological studies have linked infections during pregnancy to later development of neuropsychiatric disorders in the offspring (Hultman et al, 1999, Mednick et al, 1988). These observations increase the relevance of possible prenatal LPS effects on the human population.

Since inflammation can impair adult neurogenesis both at the prenatal period and in the adulthood, here we aimed to compare the duration and magnitude of this effect when LPS is administered prenatally or in adult animals. Also we studied whether both adult neurogenic regions (DG and SVZ) are affected by LPS treatment. Finally, we explored the cellular and molecular alterations in the neurogenic niche that could explain the different effects of adult vs pre-natal LPS.

METHODS

Animals

Adult Wistar rats (250-300 g) were bred in the animal house at the Leloir Institute. Animals were housed under controlled temperature (22 ± 2°C) and artificial light under a 12-h cycle, with water and food available *ad libitum*. All dams were naive breeders. All animal procedures were performed according to the rules and standards 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.

LPS treatment and BrdU staining

Prenatal LPS treatment

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 days (GD) 14, 16, 18, and 20. The litters were culled to 10 pups, weaned at postnatal day (PD) 21, and housed with no more than 5 animals per cage until adulthood (PD60). One to two pups from each litter were 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). The time of appearance of typical developmental parameters such as eye opening, ear detachment and testis descent was unaffected by prenatal LPS administration, as it was the weight of the pups between birth and weaning**.** In depth characterization of the prenatal LPS model in terms of maternal behavior and other parameters can be found in (Graciarena et al, 2010).

Adult LPS treatment and BrdU injections

Adult male rats prenatally treated with saline received a single intraperitoneal (ip) injection of either LPS (1 mg/kg; Saline/LPS7 group) or saline (Saline/Saline7 group). Adult male rats prenatally treated with LPS received a single ip injection of saline to control for the effect of injection (LPS/Saline7 group). The adult injection was followed immediately by 7 daily ip injections of 50 mg/kg 5-bromo-20-deoxiuridine (BrdU, Sigma–Aldrich) and perfusion at the 7th day (Figure 1a) for immunohistochemistry studies as described below. For RNA isolation animals were decapitated on the 7th day.

To study the DG, a total number of 16 animals (Saline/Saline7, n=6; Saline/LPS7, n=4; LPS/Saline7, n=6) were processed for immunostaining procedures and 21 animals (Saline/Saline7, n=7; Saline/LPS7, n=6; LPS/Saline7, n=8) for mRNA quantitation protocols. For the SVZ and RMS, a total number of 9 animals (n=3/group) were processed for immunostaining procedures.

Alternatively, adult male rats with no prenatal treatment received 4 ip injections of LPS (1 mg/kg, LPS60 group) or saline (Saline60 group) every other day. After 60 days, rats received 7 daily ip injections of BrdU (50 mg/kg), were perfused or decapitated at the 7th day (Figure 2a). A total number of 16 animals (8 for immunohistochemistry and 8 for RNA isolation, 4 in each group) were processed for this experimental protocol.

Tissue sections

Animals were deeply anesthetized and transcardially perfused with heparinized saline followed by cold 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH=7.2). Brains were placed in the same fixative overnight at 4°C, cryoprotected in 30% sucrose in PB 0.1 M, frozen in isopentane. Serial coronal sections throughout the hippocampus and serial sagittal sections throughout the SVZ and RMS were cut in a cryostat. The 40 µm sections were used for free floating immunostaining.

Immunostaining

Co-localization of BrdU with other markers was performed as previously described (Graciarena et al, 2010). Briefly, every sixth coronal section was incubated for 2 hrs in 50 % formamide at 65 °C, washed once in 2x saline-sodium citrate (SSC; 0.3 M NaCl, 0.03 M sodium citrate), incubated for 30 min at 37°C in 2 M HCl, rinsed in 0.1 M borate

buffer pH 8.5, and thoroughly washed in Tris-buffered saline (TBS; Tris-HCl 50 mM pH 7.4, NaCl 150 mM). Sections were then blocked in 0.1 % Triton X-100, 1% donkey serum in TBS (blocking solution) for 45 min at room temperature. Primary antibodies were diluted in blocking solution and sections were incubated for 48 hrs at 4°C. After 2 washes with 0.1 M PB, sections were incubated in secondary antibodies diluted in 0.1 M PB for 2 hrs at room temperature. Sections were then washed with 0.1 M PB and mounted in Mowiol (Calbiochem, La Jolla, CA, USA).

Primary antibodies used were rat anti-BrdU (1:200; Abcam, Cambridge, UK); mouse anti-polysialic acid neuronal cell adhesion molecule (PSA-NCAM, 1:200, kindly provided by Dr. Seki, Department of Anatomy, Juntendo University School of Medicine, Tokyo, Japan); mouse anti-NeuN (1:200, Chemicon); rabbit anti-doublecortin (Dcx, 1:400, Abcam); Griffonia simplicifolia lectin (GSAI-B4, 1:200, Vector) and rabbit anti-glial fibrillary acidic protein (GFAP, 1:500; Chemicon, Temecula, CA).

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).

For staining with Dcx, GSAI-B4 or GFAP alone, every sixth coronal section were incubated in the primary antibody diluted in blocking solution for 24 hrs at 4°C. After 2 washes with 0.1 M PB, sections were incubated with secondary antibodies diluted in 0.1 M PB for 2 hrs at RT. Sections were then washed with 0.1 M PB and mounted in Mowiol (Calbiochem).

Quantitation of neurogenesis and microglial activation

Neuronal differentiation. One-in-six series of coronal or sagittal sections (40 µm-thick) from the entire Hippocampus or SVZ were observed at 40x magnification in a Zeiss LSM 510 laser scanning confocal microscope equipped with an argon and He/Ne laser that emitted at 488 nm and 543 nm, respectively. Four fields were randomly selected for each rat and scanned along the entire z-axis. Between 50 and 100 total BrdU positive cells have been counted in each rat for the hippocampus and between 50 and 400 total BrdU positive cells for the SVZ.

Neurons born in the adult brain were identified as cells labeled with BrdU and a neuronal marker, such as PSA-NCAM or Dcx (young neurons), or NeuN (mature neurons). Neurogenesis was expressed as the percentage of double-labeled cells over the total BrdU-positive cell population. Sections were counted using a double-blind procedure.

Total newborn neurons. For the DG, one-in-six series of coronal sections (40 µm-thick) spanning the entire hippocampus (-2.3 mm to –5.2 mm from bregma) were stained for Dcx by immunofluorescence. Dcx-labeled cells in the SGZ were quantified at 40X magnification under a fluorescence microscope. The total number of positive cells through the entire GCL was estimated and then divided by the volume of the DG. For the calculation of the volume, one-in-six sections were photographed using a Nikon Eclipse E600 microscope and a CX900 camera (MicrobrightField Inc., USA) with a 10x magnification, and Image J software (Media Cybernetics, Silver Spring, MD) was used to delineate the GCL area. The volume was estimated by multiplying each section area by the section's width (0.04 mm) and the total number of sections obtained for the whole DG.

For the SVZ and RMS, one-in-six series of sagittal sections (40 µm-thick) were stained for BrdU and Dcx immunofluorescence. The percentage of double-labeled cells over the total BrdU-positive cell population was quantified at 40X magnification. The total number of BrdU-positive cells through the entire SVZ and RMS was divided by the volume of the SVZ or the RMS. The volume was calculated using the LSM Image Browser. The analyzed area was defined manually and the numeric area value given by the program was multiplied by the thickness of the z-stack analysis.

Microglial activation. GSAI-B4 was used to assess microglial activation based on positive staining and cell morphology (Kreutzberg, 1996). Four stages (I to IV) of microglial activation were defined as already described (Mathieu et al, 2010a). At stage I, cells have rod-shaped cell bodies with fine, ramified processes and are defined as resting microglial cells. At stage II, cells have elongated cell bodies with long thick processes; at stage III cells had 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 marker of phagocytic activity. Stages II, III and IV are defined as activated microglial cells. Stages II and III were counted throughout all the DG in one-in-six coronal sections of the hippocampus. Total activated cells per mm3 of DG were calculated by extrapolating cell number to the volume of the DG.

RNA isolation, reverse transcription and real time PCR (RT-PCR)

Adult animals were decapitated, their brains were quickly removed and the whole hippocampus dissected. Tissue was 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). Briefly, the tissue was homogenized in TRI Reagent (Sigma) and then extracted with chloroform. The aqueous phase was precipitated with isopropanol, washed with ethanol and suspended in 20 µl of RNAse-free milliQ water. Reverse transcription was performed by incubating 10 µg of the RNA with oligo-dT (Invitrogen, San Diego, CA, USA) and SuperScript First-strand Synthesis System (Invitrogen), according to manufacturer's protocol. For quantitative RT-PCR reaction, 24 µl of SYBR Green Master Mix was added to 1 µl of cDNA. The specificity of PCR primers was tested, and a single DNA band of the expected molecular size was observed in a 1.5 % agarose gel electrophoresis stained with ethidium bromide. In addition, a single peak in the iCycler plot was observed after a melting curve was performed. Each sample was tested in triplicate. To compare mRNA expression among treatments, the amount of mRNA was expressed as the ratio between TGF-β1 or IL-6 and β2-microglobulin levels.

NSC culture and immunocytochemistry

Adult hippocampal NSC culture was prepared as described elsewhere (Battista et al, 2006). Cells were maintained in DMEM/F12 medium supplemented with FGF-2 (Peprotech, DF, Mexico) and N2 1X (Invitrogen), in dishes previously coated with poly-L-ornithine and laminin (Sigma).

For differentiation experiments: 3.5 10^4 cells⁄ml were plated on poly-L-ornithine⁄laminin-coated cover slides. One day after plating, cells were transduced with equivalent doses of adenovectors recombinant for Smad7 or β-galactosidase ($5x10^8$ infective particles/ml) in DMEM/F12 alone. After 2 hs the medium was replaced by DMEM/F12

with N2, and a low FGF concentration (0.5 ng/ml). The next day, cells were incubated with the same medium with or without TGF-β1 (Peprotech) at a concentration 1 ng/ml. The medium with or without TGF-β1 was replaced every 48 hrs and on the fifth day after adenovector transduction, cells were fixed in 4% paraformaldehyde for 10 min at 4°C.

For β-III-tubulin (Tuj I) immunofluorescence, fixed cells were blocked for 30 min in 1% donkey serum in PBS with 0.1% Triton X-100, and then incubated in mouse anti-Tuj I, 1:1000 (Promega Corporation, Madison, USA) for 2 h at RT. Cells were then washed three times with PBS and incubated in Cy3-conjugated donkey anti-mouse, 1:1000 (Jackson) for 2 h at RT. Cells were then washed three times with PBS and cell nuclei were stained with Hoescht 1:1000 (Sigma) in PBS for 5 min, followed by three washes with PBS. Cells were finally mounted in Mowiol (Calbiochem).

The coverslides were observed in a fluorescence microscope, and four random areas were photographed within each coverslide. Each treatment was performed in three coverslides. Within each area, total nuclei (positive for Hoescht) and cells of a neuronal phenotype (positive for β-III-tubulin) were counted. Then, for each coverslide, the percentage of positively stained cells over the total number of cells was calculated. Cell counting were performed using the Image Pro Plus software, in a double blind procedure.

Western blot

Protein extracts were obtained from NSC lysates at 24 hs after adenovector transduction, and 30 min after addition of TGF-β1 to the medium at concentrations of 0.1 or 1ng/ml. Briefly, cells were disaggregated, centrifuged and suspended in sample

buffer (62.5 mM pH 6.8 Tris-HCl, 2% SDS, 10% glycerol, 0.01% 50mM DTT in MilliQ water) with addition of protease inhibitors (GE Healthcare, USA) on ice. The samples were then briefly sonicated (5 sec). Bradford protein assays were performed to determine total protein concentration in each sample. 30 µg of protein samples were separated in a 12% SDS-polyacrylamide gel and transferred onto Nitrocellulose membranes using a Trans-blot apparatus (BioRad, Hercules, CA). Membranes were blocked with 5% w/v skim milk in PBS with 0.05% v/v Tween 20 for 1 h, and incubated in appropriate dilutions of primary antibodies (rabbit anti-rat Smad7 1:500 and rabbit anti-rat p-Smad2/3 1:500, both purchased at Cell signaling; mouse anti-rat actin 1:1000, purchased at Sigma) overnight at 4°C. After extensive washing, membranes were incubated with peroxidase-conjugated secondary antibodies (HRP Goat anti-rabbit 1:2000, HRP Goat anti-mouse 1:2000, Jackson) for 2 hrs, and washed before detection with ECL chemoluminescence (GE). Optical Density (OD) of p-Smad2/3 and actin bands was measured using Image J software (Rasband WS. Image J. Bethesda, Maryland, USA: US National Institutes of Health). Values were normalized according to total protein loading and expressed as percentage of the density corresponding to the control group. OD quantitations show an average of three independent experiments.

Adenoviral vector preparation

Adenovectors expressing β-galactosidase and Smad7 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, AdSmad7 = 1.05×10^{12} pfu/µL), and had less than 1 ng/mL of endotoxin (E-TOXATE Reagents, Sigma). Viral stocks were free of

autoreplicative particles. AdSmad7 was kindly provided by M. Fujii (Department of Biochemistry, The Cancer Institute, Tokyo, Japan).

Statistical Analysis

Data is presented as the mean ± standard error of the mean (SEM). Comparisons were performed using Student's t-test (two-tailed), One- and Two-way analysis of variance (ANOVA) followed by Tukey´s post-hoc test. Differences were considered significant when $p<0.05$. Data were previously checked to fulfill normality and homocedacy criteria.

RESULTS

Prenatal and adult LPS treatments reduce adult neurogenesis in the DG to the same extent, but only prenatal LPS effects are long-lasting.

We first sought to compare the effects of the prenatal and adult LPS treatment on adult neurogenesis. Pregnant rats were treated with 0.5 mg/kg LPS or saline during GD 14, 16, 18 and 20 (Figure 1A). The adult offspring of dams treated with saline received one i.p. injection of saline solution (Saline/Saline7 group) or LPS (1mg/kg, Saline/LPS7 group) at postnatal day (PD) 60, whereas the adult offspring prenatally treated with LPS received saline at PD 60 (LPS/Saline7 group). This experimental design allowed us to control for changes in adult neurogenesis elicited by handling and injection of the animals. The adult treatment was immediately followed by 7 daily i.p. BrdU injections from PD60 to PD66, and the animals were sacrificed and perfused at PD67 for posterior immunostaining analyses (Figure 1A). Both LPS/Saline7 and Saline/LPS7 groups showed a reduced neuronal differentiation rate, identified as cells stained for both BrdU and PSA-NCAM (Figure 1B, One-way ANOVA; $F(2,8) = 17.42$, $p < 0.01$; $n = 3$ per group) or BrdU and NeuN (Figure 1C, One-way ANOVA; $F(2,8) = 8.12$, $p < 0.05$; $n = 3$ per group) over the total BrdU positive population. Furthermore, both treatments decreased the total newborn neuron population, identified by Dcx expression (Figure 1D, One-way ANOVA; $F(2,14) = 13.27$, $p = 0.01$; $n = 3$-6 per group). Thus, we conclude that LPS either administered prenatally or during adulthood decreases adult neurogenesis to the same extent.

To rule out any effect of the adult saline injection, animals prenatally treated with LPS (LPS-only) or saline (saline-only) were analyzed without further manipulation in the adulthood. The ratio of adult newborn neurons (% BrdU-PSA-NCAM/total BrdU cells) observed in the prenatal LPS and saline groups without further manipulation was similar to the LPS/Saline7 or Saline/Saline7 groups, respectively (prenatal saline-only: 71.60 ± 2.22; n=5, prenatal LPS-only: 50.47 ± 4.04; n=6; Student's t test $t(9) = 4.31$, $p < 0.01$). These results observed in the prenatal-only treatments discard any effect of the injection of saline solution in the adult and are in agreement with previous results using the same experimental model but other cell markers (Graciarena et al, 2010).

Based on these results, we wondered whether the detrimental effects of adult LPS exposure on adult neurogenesis in the DG had long-lasting effects, as observed for prenatal LPS treatment. To address this issue, adult animals (PD60) were injected with a comparable LPS regime than prenatally treated animals: adult animals received 4 injections, every other day, of either saline (Saline60 group) or LPS (ip, 1 mg/kg; LPS60 group). At PD120, 60 days after the last LPS injection (the same time period for the prenatally treated rats to reach adulthood), the animals received 7 daily injections of BrdU and later sacrificed and their brains analyzed (Figure 2A). The neuronal differentiation rate, represented as the percentage of double stained BrdU/NeuN cells over the BrdU positive population in the DG, was analyzed. The neuronal differentiation rate was equivalent 60 days after adult LPS or saline administration (Figure 2B, Student´s t-test $t(6) = 1.3$ $p = 0.24$, $n = 4$ per group). Moreover, total adult newborn neuron population, detected as cells positive for Dcx throughout the SGZ of the DG, was unchanged in LPS60 group relative to Saline60 group (Figure 2C, Student´s t-test t

(6) = 1.71, p= 0.14, n = 4 per group). These results indicate that adult LPS exposure no longer affects adult neurogenesis after 60 days of administration.

Hence, these data show that long lasting effects of LPS treatment on adult neurogenesis occurs when it is administered prenatally, not in adulthood, suggesting that this gestational period is a critical window for programming of adult neurogenesis in the DG.

Prenatal LPS treatment decreases adult neurogenesis specifically in the DG but not in the SVZ or the migrating neuroblasts in the RMS.

We next examined the regional specificity of the prenatal LPS effect and analyzed the SVZ, the other well-characterized adult neurogenic zone and the RMS, the region where neuroblasts migrate to the olfactory bulb. We quantified the percentage of cells positive for BrdU and Dcx over the total BrdU-positive population in adult rats treated as previously described. Neither prenatal LPS (LPS/Saline7) nor adult LPS (Saline/LPS7) treatment affected adult neurogenesis in the SVZ (Figure 3A:One-way ANOVA; F (2,8) = 0.11, p = 0.89; n = 3 per group) or the population of cells undergoing proliferation (BrdU-positive/mm^3) (Figure 3D: One-way ANOVA; F (2,8) = 0.35, p = 0.71; n = 3 per group). In order to study if the LPS treatment could affect the migrating neuroblasts along the RMS, the same quantitation was performed. No significant differences in the number of migrating neuroblasts (% of BrdU-Dcx/total BrdU cells) or cells undergoing proliferation (BrdU-positive/mm^3) were observed among the groups studied (Figure 3B: One-way ANOVA; F (2,8) = 2.26, p = 0.18; n= 3 per group; Figure 3E: Total BrdU/mm3, One-way ANOVA; F (2,8) = 1.81, p = 0.24; n= 3 per group and Figures 3C, F and G).

Taken together these results show that prenatal LPS treatment decreases adult neurogenesis preferentially in the DG but not in the SVZ and does not influence the number of migrating neuroblasts in the RMS.

Microglial but not astrocytic activation correlates with reduced adult neurogenesis regionally and temporally

Next, we sought to determine the differences in the adult neurogenic niche that would explain the duration of the effects of prenatal vs. adult LPS. Microglia and astrocytes are main mediators of the LPS effects and important players in regulating NSC functions. Hence, we first wondered whether any of these cell types showed morphological alterations in response to LPS administration, along with the observed effects on adult neurogenesis.

Microglia activation can be classified according to their morphology (Kreutzberg, 1996); see Methods). We aimed to compare the extent and duration of microglial activation upon prenatal or adult LPS exposure in the DG and the SVZ. We use the lectin GSAI-B4 to detect microglial cells in all stages of activation in the tissue sections. Microglial cells of the DG were morphologically shifted to an activated state to the same extent in LPS/Saline7 and Saline/LPS7 groups compared to the Saline/Saline7 group (Figure 4A-D, One-way ANOVA; $F(2,12) = 21.97$, $p < 0.001$; $n = 4$-5 per group). No significant differences were observed among the LPS treated groups, also all activated cells were at the activation stages II or III, but not IV (Figure 4 A to C). On the contrary, no significant microglial activation was observed in animals injected with LPS or saline in adulthood and analyzed 60 days later (Figure 4 E and F, n=4 per group). We also tested

the SVZ for microglial activation after prenatal LPS injection. No differences were observed in morphological changes among the different groups. (Supplementary Figure 1 A and C, n=3/group). Finally, microglial activation was not seen in other brain regions such as cortex, thalamus, or hypothalamus, indicating that microglial activation in the DG is specific.

We then searched for any evidence of astrocytosis in the DG in prenatally or adult LPS-treated animals 7 days after treatment. We evaluated astrocyte morphology as well as GFAP-labeling intensity in the DG or SVZ of animals treated with LPS prenatally or during adulthood. No differences were observed among the Saline/Saline7, Saline/LPS7 or LPS/Saline7 experimental groups analyzed in the DG (Supplementary Figure 2A to C) or the SVZ (Supplementary Figure 1B and D). Thus, neither prenatal nor adult LPS exert any effect on astrocytes in the DG or the SVZ of adult animals at the time points tested.

These results indicate that, microglial activation was observed only in the experimental groups were reduced neurogenesis was detected (prenatal and adult LPS after 7 days) and specifically in the brain region with reduced neurogenesis (DG) and not in another neurogenic region (SVZ). Importantly, long-lasting effects of LPS on microglial activation were also in accordance with the effects on adult neurogenesis, i.e. only the animals treated prenatally with LPS showed activated microglial cells in the DG 60 days after treatment. Astroglia did not present morphological alterations in response to prenatal or adult LPS treatment either in the DG or the SVZ, showing that microglial cells are the preferential cellular targets of LPS in the DG in our experimental paradigm.

Prenatal and adult LPS administration causes differential alterations in TGF-β1 mRNA levels in the adult

In order to analyze the molecular alterations in the neurogenic niche that could underlie the reduction observed in adult neurogenesis, we analyzed the expression of transforming growth factor β1 (TGF-β1) and Interleukin-6 (IL-6) in the adult hippocampus. TGF- β1 is an anti-inflammatory cytokine which has been shown to be a key mediator of the reduced neurogenesis caused by prenatal LPS administration (Graciarena et al, 2010; Mathieu et al, 2010a) and IL-6 is a pro-inflammatory cytokine involved in adult LPS-mediated reduction in adult neurogenesis (Monje et al, 2003; Vallieres et al, 2002).

Therefore, we aimed to compare TGF-β1 mRNA levels in the hippocampi of adult animals that received LPS either prenatally or 7 days earlier in adulthood by real time RT-PCR. As shown in Figure 5A, when compared with the Saline/Saline7 group, the LPS/Saline7 group showed lower TGF-β1 mRNA levels in the hippocampus, while the Saline/LPS7 group did not show altered TGF-β1 mRNA levels after 7 days (Figure 5A, One-way ANOVA; $F(2,20) = 3.74$, $p = 0.04$; n = 6-8 per group). Moreover, TGF-β1 mRNA levles did not change 60 days after adult saline or LPS treatment (Saline60: 7.18 ± 1.07; LPS60: 8.02 ± 0.64, Student´s t test $t(6) = 0.67$, $p = 0.53$; n=4/group).

Neither prenatal nor adult LPS treatment caused any alterations in IL-6 mRNA levels in the hippocampus (Figure 5B, One-way ANOVA; $F(2,15) = 0.16$, $p = 0.85$; n = 4-7 per group). These results indicate that the immune molecular component of the neurogenic niche is differentially affected by LPS administration at the prenatal or adult period:

prenatally administered LPS did cause a lower TGF-β1 mRNA levels in the hippocampus of the adult offspring, while LPS administered in adulthood did not, independently from the time of analysis. Interestingly, the diminution of TGF-β1 mRNA levels correlates with the only treatment where the LPS effects on neurogenesis are long-lasting (prenatal LPS).

The pro-neurogenic effect of TGF-β1 is exerted through its canonical Smad pathway.

TGF-β1 signals through different pathways, Smad2/3 being the most frequent and the canonic one (Derynck & Zhang, 2003, Massague & Gomis, 2006). We next examined the possibility that TGF-β1 could be exerting its pro-neurogenic properties via the Smad signal transduction pathway. To this aim, we first attempted to study p-Smad 2/3 expression in whole hippocampi but the Western Blot was not sensitive enough to detect p-Smad 2/3 in any sample tested, most likely due to the dilution of the signal with other cell types in the sample. Therefore, NSC cultures were used to test the involvement of the Smad pathway on the pro-neurogenic effects of TGF- β1. NSC were prepared from the adult DG and transduced with a replication-deficient adenovector expressing Smad7 (AdSmad7), an endogenous inhibitor of the Smad2/3 pathway (Zhu & Burgess, 2001), or β-galactosidase (Adβgal), as control. TGF-β1 (0.1 or 1 ng/ml) was added to NSCs media 24hs after the adenovector transduction.

First, we tested whether the Smad2/3 pathway was functional on these cells, and whether the pre-incubation with AdSmad7 prevented Smad2/3 pathway activation upon TGF-β1 incubation. We performed Western blot analyses for Smad7, phosphorylated

Smad2/3 (p-Smad2/3), and actin as a load control (Figure 6A), at 30 min after TGF-β1 addition to the media. Smad7 expression was evident from 24 hs (Figure 6A, upper panel) to 5 days after NSC transduction with AdSmad7 (data not shown), confirming the expression of the Smad7 protein by the adenoviral vector. The quantitation of the p-Smad2/3 and actin bands by optic densitometry showed that 1ng/ml TGF-β1 provoked an increase in p-Smad2/3, while pre-incubation of NSCs with AdSmad7 partially prevented this increase (Figure 6A and B, Two way ANOVA. Adenoviral treatment: $F(1,12) = 28.47$, $p < 0.001$; TGF-β1 treatment: $F(2,12) = 53.51$, $p<0.001$; Interaction: $F(2,12) = 4.06$, $p = 0.045$; n = 3 per group). Thus, we can conclude that the Smad2/3 pathway is activated by TGF-β1 in NSCs, and that pre-incubation of NSCs with AdSmad7 prevents Smad2/3 pathway activation upon this treatment.

Next, we tested the functional effects of the activity of the Smad2/3 pathway in NSC neuronal differentiation. To this aim, NSCs were incubated with 1 ng/ml TGF-β1 for 4 days, and then fixed for immunocytochemistry against β-III-tubulin, a marker of neuronal lineage (Figure 6C to 6F). The percentage of β-III-tubulin positive cells/total number of cells was quantified (Figure 6G). As expected, TGF-β1 increased the percentage of β-III-tubulin-positive cells in the preparation with no adenovector transduction as well as in cells previously transduced with Adβgal. On the contrary, pre-incubation of NSCs with AdSmad7 prevented the increase in the neuronal population upon TGF-β1 incubation (Figure 6G, Two way ANOVA. Adenoviral treatment: $F(1,10) = 7.71$, $p < 0.05$; TGF-β1 treatment: $F(2,10) = 10.81$, $p < 0.01$; Interaction: $F(2,10) = 10.96$, $p < 0.01$; n = 2-3 per group). Based on these results, we can conclude that TGF-β1 exerts its pro-neurogenic effects on NSCs through the Smad2/3 signaling pathway.

DISCUSSION

In this study we aimed to investigate the regional and temporal specificity of the effects of peripheral inflammation on adult neurogenesis.

First, we found that both prenatal and adult LPS administration impair adult neurogenesis in the DG to the same extent. However, while prenatal LPS decreases adult neurogenesis in the adult offspring, and hence its effects are long lasting; adult LPS effects are acute and reversible, and no longer evidenced 60 days after treatment. The same dynamics of LPS effects on adult neurogenesis are also evidenced on microglial activation in the DG: prenatal LPS causes long term microglial activation in the DG of the adult offspring; while adult LPS causes acute microglial activation, evidenced after 7 days but no longer after 60 days. We show evidence of differential molecular mechanisms underlying LPS effects at prenatal or adult stages: while prenatal LPS involves TGF-β1 downregulation in the DG, LPS administered in adulthood does not cause any changes in TGF-β1 levels concomitant to the decrease in adult neurogenesis. The effects of prenatal LPS were specific for the DG: no effects on adult neurogenesis, cell proliferation or microglial activation were observed in the SVZ, the other well-characterized neurogenic region in the adult rodent brain (see Table 1) or the RMS. Finally, we further explored the intracellular pathway involved in TGF-β1 pro-neurogenic effects and found that Smad2/3, the canonical TGF-β1 signaling pathway, is involved in its pro-neurogenic effects *in vitro*.

Hence, the prenatal period represents a temporal window of vulnerability of the DG to LPS administration with long-term consequences in adult neurogenesis levels and

microglia activation, involving downregulation of TGF-β1 expression, which probably exerts its effects on NSCs via activating the Smad2/3 pathway.

Other groups have previously reported that LPS administered in adulthood impairs neurogenesis acutely (Fujioka & Akema, 2010; Monje et al, 2003). However, no previous work has assessed the duration of those effects. We decided to evaluate adult neurogenesis 60 days after adult LPS treatment, since it is the same period after which animals treated prenatally with LPS were analyzed. We show that 60 days after LPS injection, no effects on neuronal differentiation or the total number of newborn neurons in the DG were observed. In addition, we have corroborated previous results on the effects of prenatal LPS exposure on adult neurogenesis in the DG (Graciarena et al, 2010) using a different marker of neuronal lineage (PSA-NCAM). These results altogether indicate that whereas prenatal LPS effects on neurogenesis are persistent and long lasting, adult LPS effects are acute and transient (see Table 1). Interestingly, a previous report has shown that adult LPS had no effect on adult neuronal differentiation (Bastos et al, 2008). This discrepancy could be due to different experimental designs. In the work by Bastos and colleagues, the animals received only one BrdU injection 5 hrs after LPS injection, and were analyzed 7 days later. Therefore, the neuronal differentiation rate studied represents the immediate time point after LPS injection, rather than the period of 7 days between LPS treatment and analysis that we report here. These different results might be explained by hypothesizing that adult LPS may cause its effects on neuronal differentiation of NSCs with a certain delay (which may allow for microglial activation to occur), and hence, would not be reflected by the neuronal differentiation of NSC population stained with BrdU immediately (5 hrs) after

LPS stimulus. Curiously, in our work, the magnitude of adult neurogenesis reduction in the prenatal and adult LPS–treated animals was similar (see Figure 1). It is tempting to speculate that there is a specific NSC subpopulation that is affected by LPS irrespective of its time of inoculation and additional factors render the effect of LPS long-lasting or acute. One such factor could be the reduced levels of TGF-β1 expression (as discussed below).

We did not observe any differences in the GCL area at PND60 upon prenatal LPS treatment (data not shown), as it could be expected as a consequence of a chronic reduction in adult neurogenesis. Our data is in agreement with those of Lemaire and collaborators, where cumulative differences in total granule cell number owing to a lesser extent of adult neurogenesis could not be observed before 3 months of age in prenatally stressed rats (Lemaire et al., 2000). Thus, taking into account the relatively small proportion of adult-newborn granule neurons as compared to the 1-2 million neurons already present in the GCL, we favor the hypothesis that only a longer period of decreased adult neurogenesis (at least 3 months) than the one analyzed in this study will modify the total granule cell number.

The same dynamics and regional specificity of LPS effects on adult neurogenesis were also evidenced on microglial activation: prenatal LPS causes long term microglial activation in the DG of the adult offspring, while adult LPS causes acute microglial activation, evidenced after 7 days but no longer after 60 days. In addition, the astrocyte population does not seem to play a major role in mediating the LPS effects since no significant differences in astrocyte activation were found among the prenatally or adult treated animals at any time point studied, either in the DG or the SVZ (see Table 1).

Based on these results we can conclude that i) microglial but not astrocytic activation occurs concomitantly with adult neurogenesis decrease; and ii) long term microglial activation only occurs in the DG when LPS is administered prenatally. Hence, these observations favor the possibility that adult TGF-β levels were down-regulated in hippocampal microglial cells rather than in astrocytes.

Several hypotheses could be envisaged to explain the specificity of the effects of pre-natal LPS on adult neurogenesis and microglial activation in the DG but not in the SVZ. First, the hippocampal region has been previously shown to respond differentially to prenatal inflammation compared to other brain regions (Fatemi et al, 2008, Golan et al, 2009). For example, Golan and co-workers have shown that LPS injected at GD17 affects the hippocampus but not the cerebral cortex or cerebellum, which correlates with impaired forms of learning and memory but no alteration in motor function in the adult offspring. In addition, the adult offspring of influenza virus-infected pregnant mice at GD18 showed specific different gene expression patterns in the cortex, hippocampus or cerebellum, suggesting a specific regional response to inflammation. In addition, the timing and characteristics of neuronal development in both regions are not similar (Kriegstein & Alvarez-Buylla, 2009). The generation of hippocampal neurons in the fetal brain starts around the time of the first LPS injection in our model (GD14) (Angevine, 1965). Interestingly, microglial cells start to enter the brain parenchyma around a similar time period (Dalmau et al, 1997). On the other hand, the embryonic SVZ play a major role in the generation of neurons in the whole brain from the beginning of neuronegenesis (Kriegstein & Alvarez-Buylla, 2009). Therefore, there could be different windows of susceptibility to inflammation for the developing DG and SVZ. The

experimental exploration of this issue, albeit of importance, is beyond the scope of this work.

At the molecular level, we observed that TGF-β1 expression in the adult hippocampus is downregulated when LPS is administered prenatally (LPS/Saline7) but not when injected in adulthood (Saline/LPS7). On the other hand, IL-6 expression, which has been proposed as a microglial produced cytokine upon adult LPS exposure with an anti-neurogenic role (Monje et al, 2003), was not affected by prenatal or adult LPS affected in our experimental paradigm (see Table 1). Thus, we conclude that the effects of LPS on adult neurogenesis involve differential molecular mechanisms whether it is administered prenatally or in the adulthood. It is interesting to note that the downregulation of TGF-β1 expression is the only variable tested that correlates with the long-term effects of prenatal LPS on adult neurogenesis and microglial activation and is not changed in the adult LPS paradigm irrespective of the time points studied. These observations add proof to previous observations demonstrating a functional role of TGF-β1 downregulation in mediating long-term effects of LPS (Graciarena et al, 2010). In conclusion, the mechanisms by which pre-natal and adult LPS diminish adult neurogenesis are different and our data suggest that TGF-β expression levels play a major role in the final outcome of these treatments. Previously, we have observed TGF-β, IL-1β and IL-6 induction in the brain of pups whose dams have been treated with the same dose of LPS as in this study (Graciarena et al, 2010), indicating a typical response to LPS. However, we cannot rule out that the dams play an important role in conveying different LPS-derived signals to the pups than the one that is received directly by LPS administration in the adult brain. In turn, these additional signals could

be responsible for the chronic downregulation of TGF-β in the hippocampus of prenatally-treated animals compared with the unaltered TGF-β levels after LPS treatment in the adults.

Finally, we aimed to characterize the pro-neurogenic effects of TGF-β1. We have previously shown that TGF-β1 increases neuronal differentiation of adult NSC in vitro (Battista et al, 2006). Here, we studied whether the canonic signaling pathway triggered by TGF-β1 was involved in this effect. We showed that Smad2/3, TGF-β1 main signaling pathway, mediates its pro-neurogenic effects on a NSCs culture. Smad2/3 has been shown to be a transcriptional modulator of several target genes whose functions include cell cycle modulation and cell differentiation (Chang et al, 2002, Moustakas et al, 2001, Shi & Massague, 2003). The actual molecular targets of Smad2/3 deserved further investigation to pinpoint the molecules responsible for the pro-neurogenic effect of TGF-β1 in vitro. In addition, whether the LPS effects on NSCs are cell autonomous or not could be explored by analyzing NSC derived from LPS-treated animals.

Overall, our results contribute to highlighting the differential vulnerability of the prenatal CNS, as opposed to the adult CNS, to suffer long-term functional alterations due to pro-inflammatory insults, and thus supporting the relevance of the programming concept occurring during the prenatal period but not in adulthood. In addition, it provides regional and temporal specificity for the anti-neurogenic effect of inflammation triggered by LPS.

Importantly, the effects of prenatal LPS may reflect a non-overt concern on adult neurogenesis. As stated in the Introduction, approximately 14% of pregnant women get bacterial vaginosis, a condition during pregnancy that put the developing fetus into

contact with LPS (Dammann & Leviton, 1997, Purwar et al, 2001, Romero et al, 1989, Thorsen et al, 1998, Yoon et al, 1997). It is then tempting to speculate that fetal exposure to inflammation could affect the developing CNS, specifically reflecting in a lower adult neurogenesis in the DG. In this context, future studies that consider the differential vulnerability of the developing DG upon inflammatory stimuli, will be helpful to reach a better understanding of this relation and for early prevention of diverse adult hippocampal dysfunctions.

Table 1

	Adult neurogenesis	Microglial activation	Astrocyte activation	TGF-b1 mRNA levels	IL-6 mRNA levels
Prenatal LPS (DG)	Reduced	Increased (types II and III)	Unaltered	Reduced	Unaltered
Prenatal LPS (SVZ)	Unaltered	Unaltered	Unaltered	ND	ND
Prenatal LPS (RMS)	Unaltered (migrating neuroblasts)	ND	ND	ND	ND
Adult LPS (7 days analysis) (DG)	Reduced	Increased (types II and III)	Unaltered	Unaltered	Unaltered
Adult LPS (7 days	Unaltered	Unaltered	ND	ND	ND

analysis) (SVZ)					
Adult LPS (60 days analysis)	Unaltered	Unaltered	Unaltered	ND	ND

Table 1: Summary of the results obtained using the different inflammatory paradigms and times and regions of analysis. ND= not determined

FIGURE LEGENDS

Figure 1. Prenatal or adult LPS exposure after 7 days impairs adult neurogenesis to the same extent. A. Timeline of the experimental approach. **B, C, E, F, H and I**: Immunohistochemistry and quantitation of newborn neurons. D. Percentage of double-labeled BrdU-PSA-NCAM cells over total BrdU positive cells in the SGZ of the DG. G. Percentage of double-labeled BrdU-NeuN cells over total BrdU positive cells in the SGZ of the DG. J. Total number of Dcx-positive cells in the SGZ per mm3 of DG. Left column shows representative confocal/fluorescent images. GCL: Granular cell layer, H: Hilus. Scale bar: 50 μm. Values are the mean ± SEM. Tukey's post-hoc test *p<0.05, **p<0.01 vs. Saline/Saline7 group.

Figure 2. Adult LPS does not impair adult neurogenesis after 60 days. A. Timeline of the experimental approach. **B.** Percentage of double-labeled BrdU-NeuN over total BrdU positive cells in the DG. **C**. Total number of Dcx-positive cells in the SGZ per mm3 DG.. Values are the mean ± SEM.

Figure 3. SVZ neurogenesis and neuroblast migration in the RMS are not affected by the prenatal LPS treatment. Immunohistochemistry and quantitation of newborn neurons. **A-B**. Percentage of cells labeled with Dcx among the BrdU-positive population in the SVZ (A) and RMS (B). Values are the mean ± SEM Tukey's post-hoc test, no statistical significance. **C.** Representative confocal image of cells quantified in B. **D-E**. Quantitation of the total number of BrdU-positive cells in the SVZ (D) and RMS (E) per mm3. Values are the mean ± SEM. Tukey's post-hoc test, no statistical significance. **F**. Representative diagram of a sagittal section of the rat brain showing the SVZ, RMS and

OB. The migration of the neuroblasts from the SVZ towards the OB is represented with a red line. The analyzed area is the one between the two vertical lines. **G.** Composite image of the analyzed area in the SVZ and RMS. Scale bars: **C**, 50 µm; **G**, 100 µm SVZ, Subventricular zone; RMS, rostral migratory stream; LV, lateral ventricule; OB, olfactory bulb.

Figure 4. Prenatal LPS causes long term microglial activation, while adult LPS provokes transient microglial activation. A-C. Histochemistry analysis using GSAI-B4 showing microglial activation in the DG in: Saline/Saline7 (A), LPS/Saline7 (B), and Saline/LPS7 (C) groups. Inset in A shows a larger image of a typical microglial cell activated to Stage I. Insets in B and C show a larger image of typical microglial cells activated to Stages II-III. **D**. Quantitation of activated microglial cells (Stages II-III) per mm3 DG. **E-F**. Histochemistry analysis using GSAI-B4 showing microglial activation in the DG in the Saline60 group (E), and the LPS60 group (F). Scale bar: 50 µm. Values are the mean ± SEM. Tukey´s post-hoc test, **p<0.01, ***p<0.001 vs. Saline/Saline7 group.

Figure 5. Prenatal and LPS exposures cause differential alterations in local inflammatory cytokine expression. Analysis by real time RT-PCR of the mRNA levels in the hippocampal tissue of adult rats from Saline/Saline7, LPS/Saline7, and LPS/LPS7 groups. **A.** TGF-β1. **B.** IL-6. Values are the mean ± SEM. Tukey's post-hoc test, *p<0.05 vs. Saline/Saline7 group. β_2m: β_2-microglobulin.

Figure 6. TGF-β1 exerts its pro-neurogenic effects on NSCs via its canonic pathway, Smad 2/3. A-B. Protein expression analysis of NSCs transduced with AdSmad7 or Adβgal recombinant adenovectors, 30 min after TGF-β1 or mock

treatment. **A.** Representative Western blot of Smad7, phosphorylated (p-) Smad 2/3 and actin. **B.** OD quantitation of the p-Smad2/3 relative to actin bands, corresponding to 3 independent experiments. Values are the mean ± SEM. Tukey's post-hoc test, **p<0.01; ***p<0.001. **C-G.** Immunocytochemistry and cell quantitation of NSCs transduced with AdSmad7 or Adβgal recombinant adenovectors, 5 days after TGF-β1 or mock treatment. **c.** Control medium (No Ad) **D.** Control medium (No Ad) + TGF-β1 **E.** AdSmad7 + TGF-β1 **F.** Adβgal + TGF-β1 **G.** Quantitation of the percentage of β-III-tubulin, averaged from 3 independent experiments, Violet: cell nuclei stained with Hoescht; pink: β-III-tubulin-positive cells. Values are the mean ± SEM. Tukey's post-hoc test, *p<0.05; ***p<0.001. Scale bar: 50 μm.

References

Angevine JB, Jr. 1965. Time of neuron origin in the hippocampal region. An autoradiographic study in the mouse. *Experimental neurology. Supplement*: Suppl 2:1-70

Bastos GN, Moriya T, Inui F, Katura T, Nakahata N. 2008. Involvement of cyclooxygenase-2 in lipopolysaccharide-induced impairment of the newborn cell survival in the adult mouse dentate gyrus. *Neuroscience* 155: 454-62

Battista D, Ferrari C, Gage F, Pitossi F. 2006. Neurogenic niche modulation by activated microglia: transforming growth factor beta increases neurogenesis in the adult dentate gyrus. *Eur J Neurosci* 23: 83-93

Bilbo SD, Schwarz JM. 2009. Early-life programming of later-life brain and behavior: a critical role for the immune system. *Frontiers in behavioral neuroscience* 3: 14

Chang H, Brown CW, Matzuk MM. 2002. Genetic analysis of the mammalian transforming growth factor-beta superfamily. *Endocr Rev* 23: 787-823

Dalmau I, Finsen B, Tonder N, Zimmer J, Gonzalez B, Castellano B. 1997. Development of microglia in the prenatal rat hippocampus. *J Comp Neurol* 377: 70-84

Dammann O, Leviton A. 1997. Does prepregnancy bacterial vaginosis increase a mother's risk of having a preterm infant with cerebral palsy? *Developmental medicine and child neurology* 39: 836-40

de Graaf-Peters VB, Hadders-Algra M. 2006. Ontogeny of the human central nervous system: what is happening when? *Early human development* 82: 257-66

Derynck R, Zhang YE. 2003. Smad-dependent and Smad-independent pathways in TGF-beta

family signalling. *Nature* 425: 577-84

Ekdahl CT, Claasen JH, Bonde S, Kokaia Z, Lindvall O. 2003. Inflammation is detrimental for neurogenesis in adult brain. *Proc Natl Acad Sci U S A* 100: 13632-7

Ekdahl CT, Kokaia Z, Lindvall O. 2009. Brain inflammation and adult neurogenesis: the dual role of microglia. *Neuroscience* 158: 1021-9

Fatemi SH, Reutiman TJ, Folsom TD, Huang H, Oishi K, et al. 2008. Maternal infection leads to abnormal gene regulation and brain atrophy in mouse offspring: implications for genesis of neurodevelopmental disorders. *Schizophr Res* 99: 56-70

Ferrari CC, Depino AM, Prada F, Muraro N, Campbell S, et al. 2004. Reversible demyelination, blood-brain barrier breakdown, and pronounced neutrophil recruitment induced by chronic IL-1 expression in the brain. *Am J Pathol* 165: 1827-37

Fujioka H, Akema T. 2010. Lipopolysaccharide acutely inhibits proliferation of neural precursor cells in the dentate gyrus in adult rats. *Brain Res* 1352: 35-42

Gage FH. 2000. Mammalian neural stem cells. *Science* 287: 1433-8

Golan MH, Mane R, Molczadzki G, Zuckerman M, Kaplan-Louson V, et al. 2009. Impaired migration signaling in the hippocampus following prenatal hypoxia. *Neuropharmacology* 57: 511-22

Graciarena M, Depino AM, Pitossi FJ. 2010. Prenatal inflammation impairs adult neurogenesis and memory related behavior through persistent hippocampal TGFbeta1 downregulation. *Brain Behav Immun* 24: 1301-9

Hultman CM, Sparen P, Takei N, Murray RM, Cnattingius S. 1999. Prenatal and perinatal risk factors for schizophrenia, affective psychosis, and reactive psychosis of early onset: case-control study. *Bmj* 318: 421-6

Kreutzberg GW. 1996. Microglia: a sensor for pathological events in the CNS. *Trends Neurosci* 19: 312-8

Kriegstein A, Alvarez-Buylla A. 2009. The glial nature of embryonic and adult neural stem cells. *Annu Rev Neurosci* 32: 149-84

Lemaire V, Koehl M, Le Moal M, Abrous DN 2000. Prenatal stress produces learning deficits associated with an inhibition of neurogenesis in the hippocampus. *PNAS* 97: 11032-37

Massague J, Gomis RR. 2006. The logic of TGFbeta signaling. *FEBS Lett* 580: 2811-20

Mathieu P, Battista D, Depino A, Roca V, Graciarena M, Pitossi F. 2010a. The more you have, the less you get: the functional role of inflammation on neuronal differentiation of endogenous and transplanted neural stem cells in the adult brain. *J Neurochem* 112: 1368-85

Mathieu P, Piantanida AP, Pitossi F. 2010b. Chronic expression of transforming growth factor-beta enhances adult neurogenesis. *Neuroimmunomodulation* 17: 200-1

Mednick SA, Machon RA, Huttunen MO, Bonett D. 1988. Adult schizophrenia following prenatal exposure to an influenza epidemic. *Archives of general psychiatry* 45: 189-92

Meyer U, Nyffeler M, Engler A, Urwyler A, Schedlowski M, et al. 2006. The time of prenatal immune challenge determines the specificity of inflammation-mediated brain and behavioral pathology. *J Neurosci* 26: 4752-62

Monje ML, Toda H, Palmer TD. 2003. Inflammatory blockade restores adult hippocampal neurogenesis. *Science* 302: 1760-5

Moustakas A, Souchelnytskyi S, Heldin CH. 2001. Smad regulation in TGF-beta signal transduction. *J Cell Sci* 114: 4359-69

Ormerod BK, Hanft SJ, Asokan A, Haditsch U, Lee SW, Palmer TD. 2013. PPARγ activation prevents impairments in spatial memory and neurogenesis following transient illness. *Brain Behav Immun*. 29:28-38

Perry V, Cunningham C, Boche D. 2002. Atypical inflammation in the central nervous system in prion disease. *Current opinion in neurology* 15: 349-54

Purwar M, Ughade S, Bhagat B, Agarwal V, Kulkarni H. 2001. Bacterial vaginosis in early pregnancy and adverse pregnancy outcome. *The journal of obstetrics and gynaecology research* 27: 175-81

Reul JM, Stec I, Wiegers GJ, Labeur MS, Linthorst AC, et al. 1994. Prenatal immune challenge alters the hypothalamic-pituitary-adrenocortical axis in adult rats. *J Clin Invest* 93: 2600-7

Romero R, Sirtori M, Oyarzun E, Avila C, Mazor M, et al. 1989. Infection and labor. V. Prevalence, microbiology, and clinical significance of intraamniotic infection in women with preterm labor and intact membranes. *American journal of obstetrics and gynecology* 161: 817-24

Shi L, Fatemi SH, Sidwell RW, Patterson PH. 2003. Maternal influenza infection causes marked behavioral and pharmacological changes in the offspring. *J Neurosci* 23: 297-302

Shi Y, Massague J. 2003. Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell* 113: 685-700

Simard AR, Rivest S. 2004. Role of inflammation in the neurobiology of stem cells. *Neuroreport* 15: 2305-10

Thorsen P, Jensen IP, Jeune B, Ebbesen N, Arpi M, et al. 1998. Few microorganisms associated with bacterial vaginosis may constitute the pathologic core: a population-based microbiologic study among 3596 pregnant women. *American journal of obstetrics and gynecology* 178: 580-7

Vallieres L, Campbell IL, Gage FH, Sawchenko PE. 2002. Reduced hippocampal neurogenesis in adult transgenic mice with chronic astrocytic production of interleukin-6. *J Neurosci* 22: 486-92

Whitney NP, Eidem TM, Peng H, Huang Y, Zheng JC. 2009. Inflammation mediates varying effects in neurogenesis: relevance to the pathogenesis of brain injury and neurodegenerative disorders. *J Neurochem* 108: 1343-59

Yoon BH, Kim CJ, Romero R, Jun JK, Park KH, et al. 1997. Experimentally induced intrauterine infection causes fetal brain white matter lesions in rabbits. *American journal of obstetrics and gynecology* 177: 797-802

Zhu HJ, Burgess AW. 2001. Regulation of transforming growth factor-beta signaling. *Mol Cell Biol Res Commun* 4: 321-30

Ziv Y, Ron N, Butovsky O, Landa G, Sudai E, et al. 2006. Immune cells contribute to the maintenance of neurogenesis and spatial learning abilities in adulthood. *Nat Neurosci.* 9(2):268-75

Zorrilla EP. 1997. Multiparous species present problems (and possibilities) to developmentalists. *Developmental psychobiology* 30: 141-50

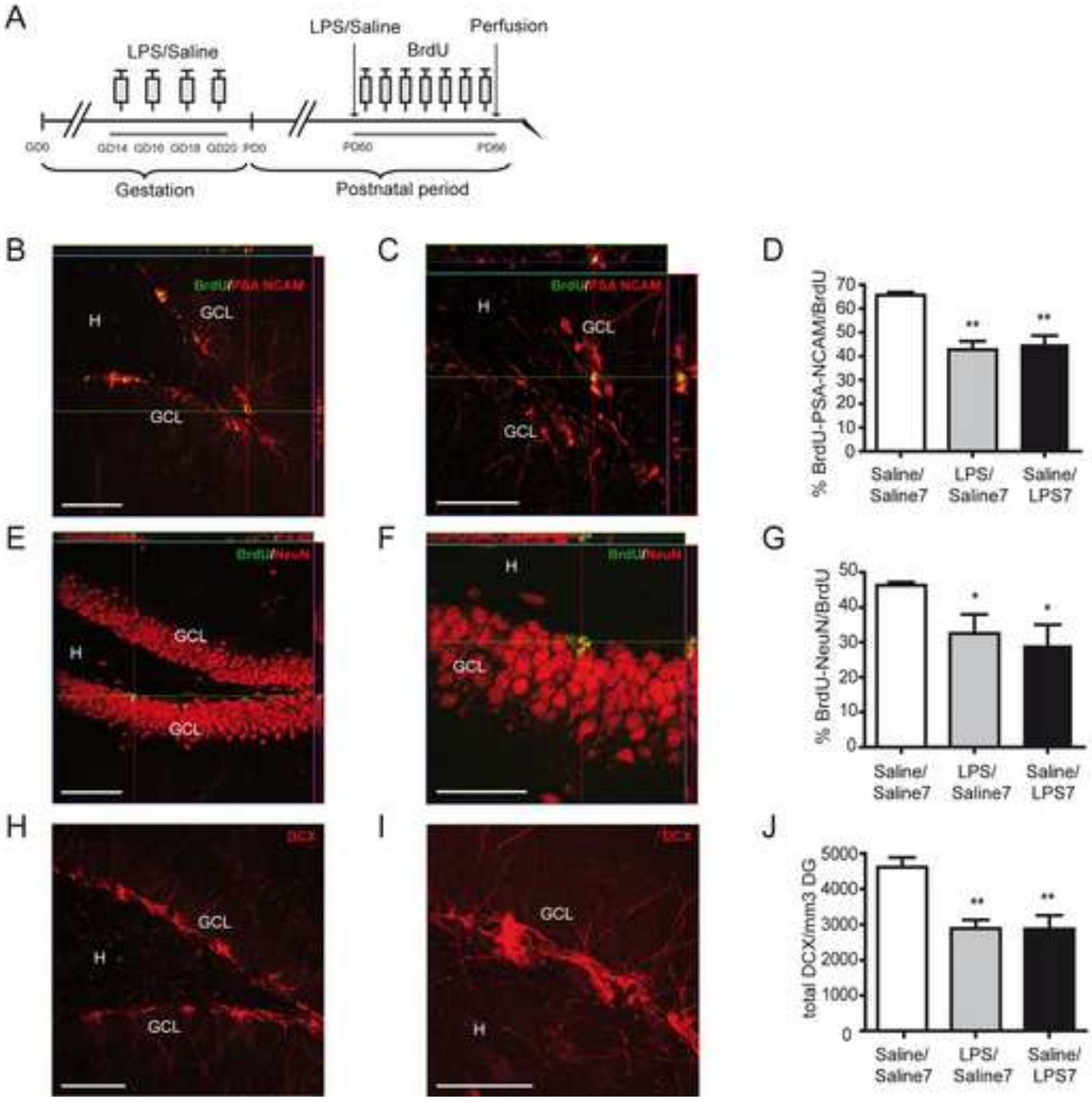
Table 1

	Adult neurogenesis	Microglial activation	Astrocyte activation	TGF-b1 mRNA levels	IL-6 mRNA levels
Prenatal LPS (DG)	Reduced	Increased (types II and III)	Unaltered	Reduced	Unaltered
Prenatal LPS (SVZ)	Unaltered	Unaltered	Unaltered	ND	ND
Prenatal LPS (RMS)	Unaltered (migrating neuroblasts)	ND	ND	ND	ND
Adult LPS (7 days analysis) (DG)	Reduced	Increased (types II and III)	Unaltered	Unaltered	Unaltered
Adult LPS (7 days analysis)	Unaltered	Unaltered	ND	ND	ND

(SVZ)					
Adult LPS (60 days analysis)	Unaltered	Unaltered	Unaltered	ND	ND

Table 1: Summary of the results obtained using the different inflammatory paradigms and times and regions of analysis. ND= not determined

Figure 1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LPS/Saline
LPS/Saline
BrdU
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GD0
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PD0
PD50
PD66
Gestation
Postnatal period
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C
D
BrdU/PSA NCAM
H
GCL
GCL
% BrdU-PSA-NCAM/BrdU
70
60
50
40
30
20
10
0
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Saline/ Saline7
LPS/ Saline7
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E
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BrdU/NeuN
% BrdU-NeuN/BrdU
50
40
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20
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I
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DCX
total DCX/mm3 DG
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4000
3000
2000
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Figure 2

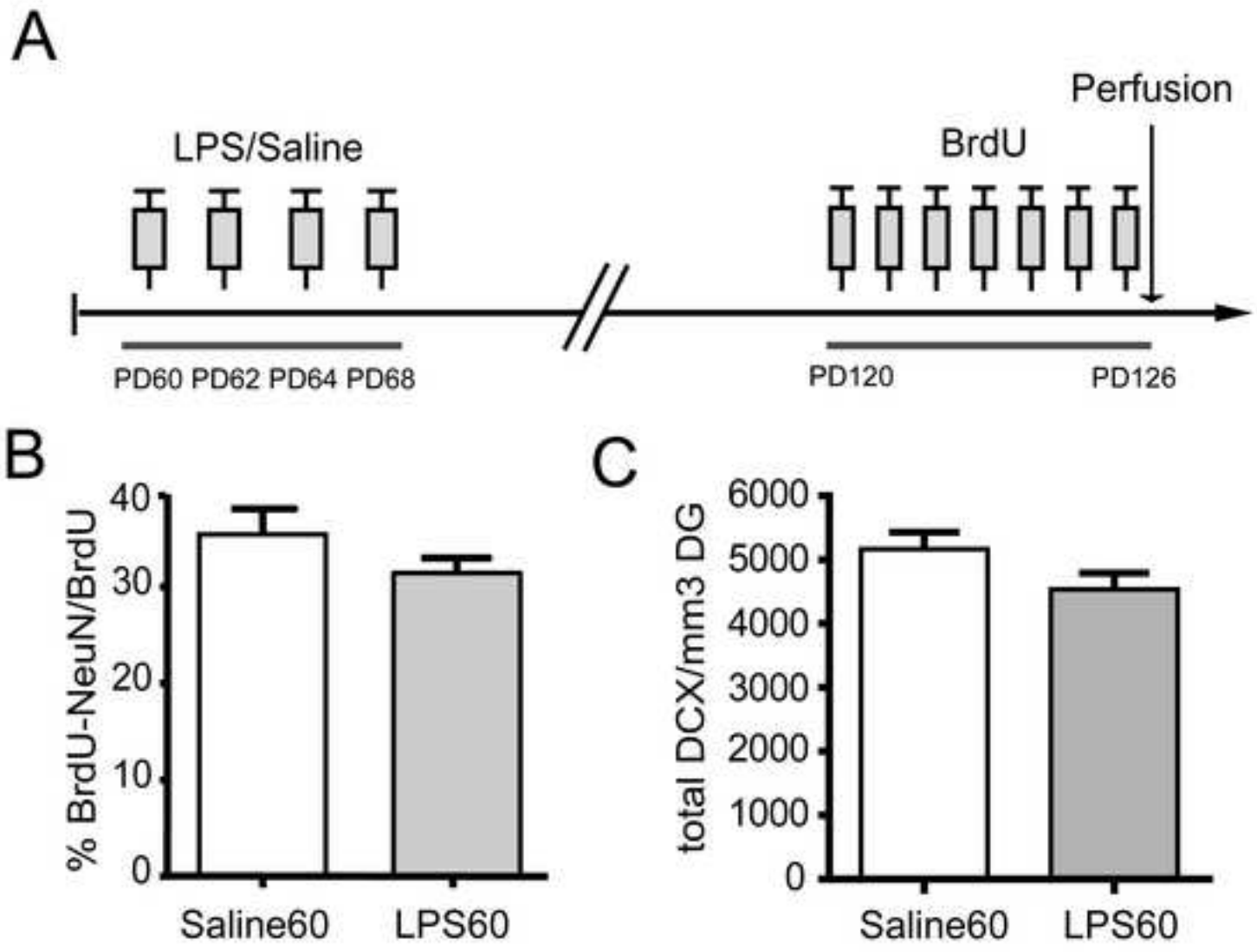
A
LPS/Saline
BrdU
Perfusion
PD60 PD62 PD64 PD68
PD120
PD126
B
% BrdU-NeuN/BrdU
40
30
20
10
0
Saline60
LPS60
C
total DCX/mm3 DG
6000
5000
4000
3000
2000
1000
0
Saline60
LPS60

Figure 3

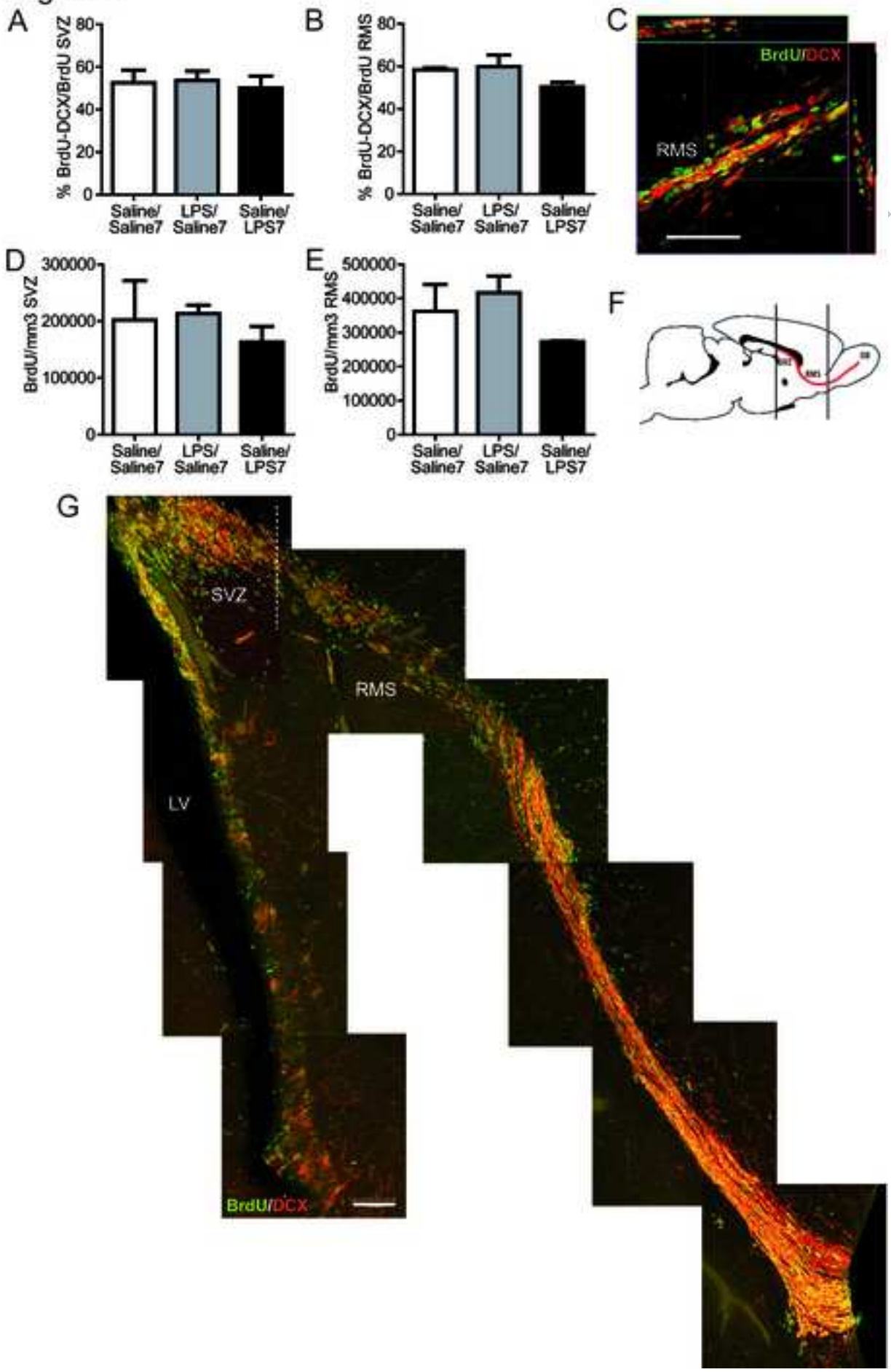
A
% BrdU-DCX/BrdU SVZ
80
60
40
20
0
Saline/ Saline7
LPS/ Saline7
Saline/ LPS7
B
% BrdU-DCX/BrdU RMS
80
60
40
20
0
Saline/ Saline7
LPS/ Saline7
Saline/ LPS7
C
BrdU/DCX
RMS
D
BrdU/mm3 SVZ
300000
200000
100000
0
Saline/ Saline7
LPS/ Saline7
Saline/ LPS7
E
BrdU/mm3 RMS
500000
400000
300000
200000
100000
0
Saline/ Saline7
LPS/ Saline7
Saline/ LPS7
F
G
SVZ
RMS
LV
BrdU/DCX

Figure 4

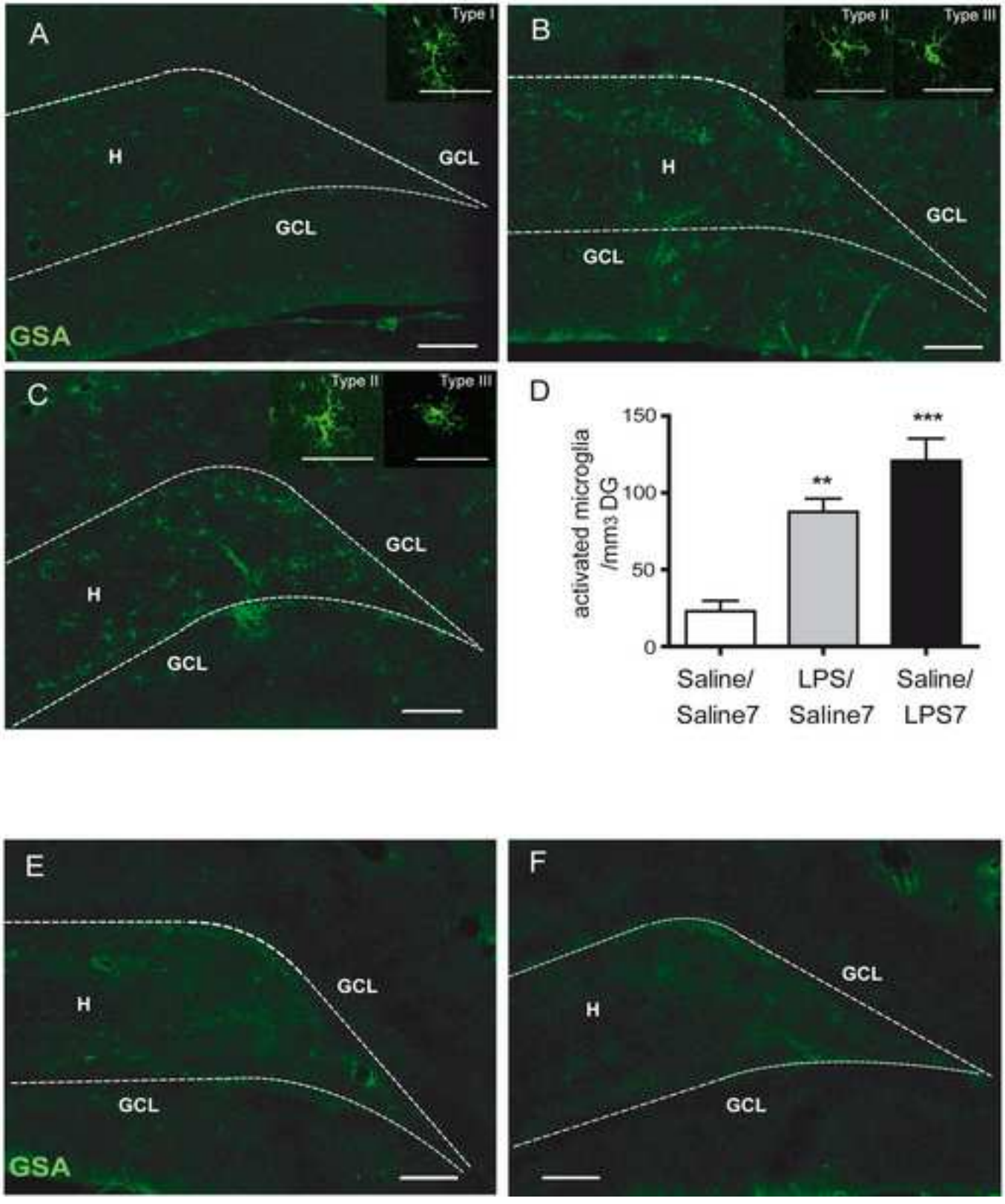
A
Type I
H
GCL
GCL
GSA
B
Type II
Type III
H
GCL
GCL
C
Type II
Type III
GCL
H
GCL
D
activated microglia /mm3 DG
150
100
50
0
**
***
Saline/ Saline7
LPS/ Saline7
Saline/ LPS7
E
H
GCL
GCL
GSA
F
GCL
H
GCL

Figure 5

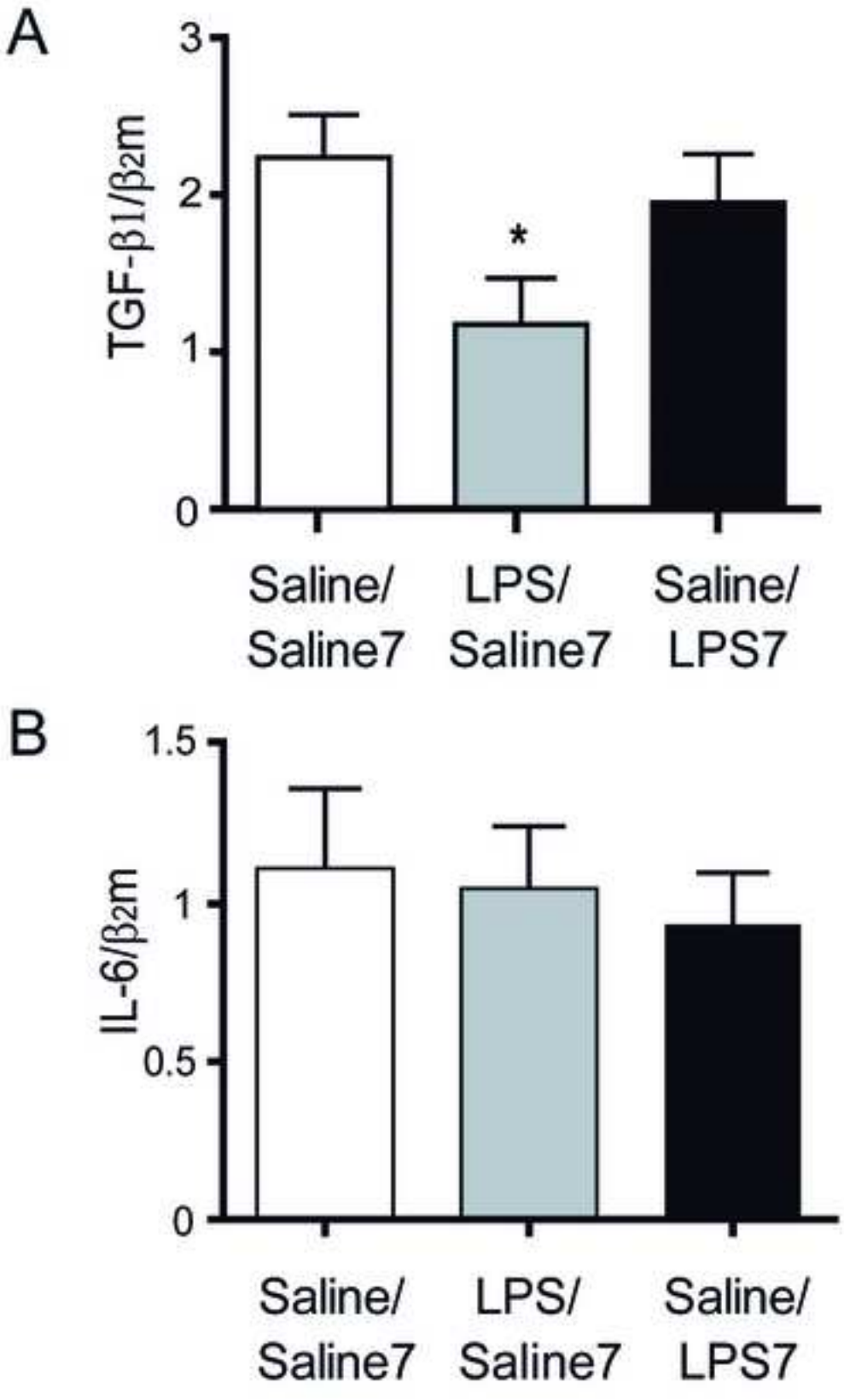
A
TGF-β1/β2m
3
2
1
0
*
Saline/
Saline7
LPS/
Saline7
Saline/
LPS7
B
IL-6/β2m
1.5
1
0.5
0
Saline/
Saline7
LPS/
Saline7
Saline/
LPS7

Figure 6