Neonatal Peripheral Immune Challenge Activates Microglia and Inhibits Neurogenesis in the Developing Murine Hippocampus

Peter L.P. Smith^a Henrik Hagberg^{a, c} Andrew S. Naylor^{a, b} Carina Mallard^a

^aInstitute of Neuroscience and Physiology, Sahlgrenska Academy, and ^bCentre of Brain Research and Rehabilitation, Institute of Neuroscience and Physiology, University of Gothenburg, Gothenburg, Sweden; ^cCentre for the Developing Brain, King's College, London, UK

Key Words

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Abstract

The early postnatal period represents an important window in rodent hippocampal development with peak hilar neurogenesis and widespread microgliogenesis occurring in the first week of life. Inflammation occurring during this period may negatively influence development, potentially facilitating or increasing susceptibility to later-life pathology. We administered the Gram-negative bacterial coat protein lipopolysaccharide (LPS) systemically at postnatal day 5 (1 mg/kg i.p.) and assessed potential effects on microgliogenesis, inflammation and neurogenesis in the developing hippocampus. LPS administration led to an acute but transient increase in absolute number and density of ionized calcium-binding adaptor molecule 1-immunoreactive microglia, a change attributable to increased proliferation of central nervous system-resident microglia/microglial precursor cells but not infiltration of peripheral monocyte-derived macrophages. qRT-PCR analysis of hippocampal gene expression

showed these LPS-mediated changes to be associated with persistent dysregulation of genes associated with both M1 and M2 microglial phenotypes, indicating prolonged alteration in hippocampal inflammatory status. Further, analysis of progenitor cell regulation in the hippocampal subgranular zone revealed a transient inhibition of the neuronal differentiation pathway up to 2 weeks after LPS administration, a change occurring specifically through effects on type 3 neural progenitor cells and independently of altered cell proliferation or survival of newly born cells. Together, our results show that systemic inflammation occurring during the early neonatal period is sufficient to alter inflammatory status and dysregulate the ongoing process of neurogenesis in the developing hippocampal germinal niche.

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Introduction

The neuronal population of the adult central nervous system (CNS) is almost exclusively developmentally generated; as such, subtle alterations to the perinatal immune environment may alter the normal course of development, potentially increasing susceptibility to secondary

CNS insult and predisposing to adult neuropsychiatric disorders such as schizophrenia and autism [1–4]. During early neonatal life, neurogenic potential is progressively restricted to the subventricular zone of the lateral ventricles and the subgranular zone (SGZ) of the hippocampal dentate gyrus, where its persistence throughout adulthood [5–7] is hypothesized to support the important role of the hippocampus in learning and memory.

Under physiological conditions, microglia have numerous functions including synaptic maintenance [8] and phagocytosis of apoptotic newborn cells in the adult hippocampal stem cell niche [9]. In response to pathological alterations to the CNS microenvironment, microglia rapidly assume an ‘upregulated’ or ‘activated’ phenotype; undergoing changes in morphology and surface antigen expression, and synthesizing numerous pro- and anti-inflammatory cytokines [10–13]. At the broadest level this upregulated phenotype can be classified as either proinflammatory (M1) or anti-inflammatory (M2) [14], a categorization dependent on the nature of each cell’s overall inflammatory profile, which is influenced by the nature and duration of the inflammatory stimulus [15, 16]. Microglia display both neuroprotective and neuroregenerative potential [15, 17] and additionally harbour microbicidal activity, the latter being facilitated by the production and release of reactive oxygen species [18].

Experimentally, systemic inflammation as induced by intraperitoneal administration of the Gram-negative bacterium-derived lipopolysaccharide (LPS) [19–24] or subcutaneous administration of *Escherichia coli* [25–27] has been shown to upregulate proinflammatory cytokine production and increase microglial activation in the rodent CNS. Furthermore, neonatal induction of central inflammation by subcutaneous *E. coli* administration reduces adult performance in hippocampus-dependent memory tasks, but only if a second peripheral LPS immune challenge is given in close temporal proximity to the test-learning phase [25], an effect attributed to reduced hippocampal neurogenesis [27]. Similarly, neonatal administration of *E. coli* reduces both the survival and neurogenic potential of hippocampal progenitors born within 24 h of *E. coli* injection [27]. While such research has clearly demonstrated acute consequences of neonatal inflammation on hippocampal progenitors, the subacute and longer-term consequences remain to be investigated.

In this study we stimulated central inflammation in mice through peripheral (intraperitoneal) administration of LPS at postnatal day (P)5, an age when murine developmental neurogenesis is highly active in the hilar germi-

nal zone [28], and yolk sac-derived microglial precursors are highly proliferative [29]. We hypothesized that peripheral inflammation occurring during this period would instigate a central inflammatory response, altering microgliogenesis and having a negative impact on the neurogenic potential of SGZ-resident neural progenitor cells (NPCs). Specifically, we investigated the acute, subacute and chronic effects of neonatal LPS-induced systemic inflammation on microgliogenesis, inflammation and various aspects of neurogenesis in the developing murine hippocampus.

Methods

Animals

C57Bl/6j mice were sourced from Charles River Laboratories (Germany), and *Lys-EGFP-ki* mice were obtained from Dr. Tomas Graf, Autonomous University of Barcelona (Spain). Animals were bred at the University of Gothenburg’s Laboratory for Experimental Biomedicine and housed with ad libitum access to food and water on a 12-hour light-dark cycle (illuminated 19:00–07:00) at constant temperature (24°C) and relative humidity (50–60%). All experimental procedures were approved by the University of Gothenburg’s animal ethics committee (No. 344/09, 280/12).

Experimental Manipulations

Male mice received intraperitoneal injections of LPS at a dose of 1 mg/kg (Serotype O55:B5; Sigma-Aldrich) or equivalent volumes of saline at P5. We selected this dose on the basis of previous studies demonstrating effects of this dose and administration route on rodent neurogenesis [21, 23, 24].

BrdU Administration and Tissue Collection

Mice were randomly allocated to 1 of 4 groups and tissue was collected at the following time points: P7 (acute); P21 and P28 (subacute); and P74 (chronic). Animals composing the acute group received single injections of BrdU (5-bromo-2'-deoxyuridine; 50 mg/kg i.p.) at P6 and were sacrificed 24 h later (P7); the subacute group received single injections of BrdU (50 mg/kg i.p.) at P19 and were sacrificed 48 h later (P21). Neuronal fate specification was investigated in animals injected with BrdU (50 mg/kg i.p.) at P21 and sacrificed 7 days later (P28). Long-term survival was assessed in a further group receiving BrdU injections (50 mg/kg i.p.) once daily from P22 to P25 and sacrificed 49 days later (P74).

Immunohistochemistry

Mice were deeply anaesthetized and transcardially perfused with ice-cold 0.9% saline followed by 4% paraformaldehyde. Brains were rapidly removed, postfixed in 4% paraformaldehyde for 24 h at 4°C, and cryoprotected in 30% sucrose for a minimum of 3 days. Cryoprotected brains were snap frozen on liquid nitrogen and sectioned serially at 40 or 25 µm (for P7 or older animals, respectively) on a Leica CM3050 S cryostat (Leica, Sweden). Cut sections were transferred to cryoprotectant solution (25% ethylene glycol, 25% glycerine, in 0.1 M phosphate buffer) and stored at –20°C. Stereological estimations of absolute cell number and cell density

were made on tissue stained for BrdU, phosphohistone-H3 (PhH3), ionized calcium-binding adaptor molecule 1 (Iba1) and doublecortin (DCX). Antigen retrieval steps were included when staining for Iba1, BrdU and DCX; sodium citrate antigen retrieval (10 mM sodium citrate; pH 6; 97°C; 10 min) was performed prior to Iba1 and DCX staining, and hydrochloric acid (HCL) pretreatment (2 M HCL; 37°C; 30 min) followed by a single wash in borate buffer (0.1 M; pH 7.5; room temperature; 10 min) was performed in advance of BrdU staining. With exception pretreatment, all steps proceeded as follows: endogenous peroxidase activity was blocked by a 30-min incubation with TBS containing 0.6% hydrogen peroxide, and blocking of non-specific binding sites and tissue permeabilization were achieved by 30-min incubation in TBS containing 3% donkey serum and 0.1% Triton X-100 (hereafter referred to as blocking buffer). Sections were then incubated at 4°C overnight with either rat monoclonal anti-BrdU (1:1,000; Accurate Chemical, Westbury, N.Y., USA), goat polyclonal anti-Iba1 (1:500; Abcam, Cambridge, UK), rabbit polyclonal anti-PhH3 (1:1,000; Millipore, Billerica, Mass., USA) or goat anti-DCX (1:200; Santa Cruz, Heidelberg, Germany) diluted in blocking buffer. The sections were subsequently incubated at room temperature for 2 h with appropriate biotinylated (BT) secondary antibodies diluted in blocking buffer: donkey anti-rat BT (1:1,000; Jackson, Newmarket, UK), donkey anti-goat BT (1:1,000; Jackson) and donkey anti-rabbit BT (1:1,000; Jackson), followed by application of the Vectastain standard ABC kit (Vector, Burlingame, Calif., USA). Visualization was performed with DAB (3,3'-diaminobenzidine; 0.25 mg/ml; Saveen & Werner, Sweden), 10 µl per 1 ml TBS containing 0.3 µl hydrogen peroxide (30%) and 5 µl of 8% NiCl₂ (Sigma-Aldrich, Sweden).

Phenotyping of BrdU-labelled cells was performed via double immunofluorescence staining combining either rat monoclonal anti-BrdU with antibodies to Iba1 (microglial proliferation), DCX (neuronal commitment) and NeuN (neuronal survival), or by quadruple staining combining rat monoclonal anti-BrdU with antibodies to nestin, glial fibrillary acidic protein (GFAP) and DCX (precursor development). Sodium citrate and HCL pretreatment was performed prior to immunofluorescence staining; importantly, it was necessary to conduct the sodium citrate step in advance of HCL pretreatment to preserve BrdU antigenicity. Blocking buffer was then applied for 30 min, and the sections were incubated at 4°C overnight with relevant combinations of the following primary antibodies diluted in blocking buffer: rat monoclonal anti-BrdU, goat anti-Iba1, goat anti-DCX (all as described above), mouse monoclonal anti-NeuN (1:250; Millipore), mouse anti-nestin (1:100; Abcam) and chicken anti-GFAP (1:1,000; Covance, Princeton, N.J., USA). Secondary antibodies were diluted to 1:1,000 in blocking buffer and applied for 2 h at room temperature: donkey anti-rat Cy3 (Jackson), donkey anti-mouse Alexa 488 (Molecular Probes/Invitrogen, Carlsbad, Calif., USA), donkey anti-goat Alexa 488 (Molecular Probes/Invitrogen), donkey anti-mouse CF647 (VWR, Lutterworth, UK), donkey anti-goat CF555 (VWR), donkey anti-chicken CF488 (Sigma-Aldrich, Sweden) and donkey anti-rat DyLight 405 (Jackson).

Staining for goat anti-Iba1 and goat anti-CD31 (1:200; R&D Systems, Abingdon, UK) on tissue derived from *Lys-EGFP-ki* mice was performed in the absence of Triton X-100 to limit quenching of EGFP fluorescence.

Microscopy and Quantification Strategies

Stereological estimations of absolute cell number and cell density were performed on serially spaced sections representing the entire hippocampal formation, using a Leica DM600 B microscope (Leica, Heidelberg, Germany) equipped with Stereo Investigator (MBF Biosciences, Williston, Vt., USA). BrdU-, DCX- and PhH3-positive cells were counted exhaustively, absolute cell numbers were derived via multiplication of total counted cells by number of series per brain (the latter value varying depending on the time point and section thickness). At P7, the absolute number of BrdU^{pos} cells was quantified in the hippocampal granule cell layer (GCL) and SGZ as at this stage of development, proliferative potential is not limited to the SGZ. At P21 and P74, BrdU- and PhH3-immunoreactive cells, respectively, were quantified in the SGZ as proliferation was limited to this region. In the P21–P28 and P21–P74 differentiation studies, cells were quantified in the SGZ and GCL as newly born BrdU-labelled cells migrate into the GCL during maturation. Absolute number and density of Iba1^{pos} cells were assessed using the optical fractionator. Representative images of DAB-stained sections were captured on a Zeiss Axio Imager Z2 equipped with Zeiss AxioVision image capture software (Zeiss, Oberkochen, Germany). The percentage of newly born microglia at P7 was assessed by investigation of BrdU immunoreactivity in a minimum of 100 Iba1^{pos} cells randomly sampled from reconstructed tile-scanned z-stacks of entire hippocampi captured on a Zeiss Axio Observer upright microscope equipped with an ApoTome module and Zen Blue software (Zeiss); resultant z-stacks were imported to Stereo Investigator for analysis. Neuronal precursor phenotyping studies involved analysis of immunohistochemical marker coexpression in a minimum of 100 BrdU^{pos} cells; percentage coexpression values were then combined with stereologically derived estimates of the absolute BrdU^{pos} cell number to provide an estimate of the absolute number of double-labelled cells. BrdU/DCX expression was investigated using a Leica TCS SP2 confocal microscope (Leica, Heidelberg), and effects on particular hippocampal NPC populations were assessed in BrdU/nestin/GFAP/DCX-costained tissue (progenitor populations characterized as follows: type 1, BrdU/nestin/GFAP; type 2a, BrdU/nestin; type 2b, BrdU/nestin/DCX; type 3, BrdU/DCX [30]); here, coexpression was investigated using a Zeiss LSM 700 inverted confocal microscope. Z-projections and orthogonal reconstructions were produced in ImageJ (NIH, Bethesda, Md., USA; <http://rsbweb.nih.gov/ij/>).

Tissue Collection for RNA Isolation

Mice were deeply anaesthetized and transcardially perfused with ice-cold 0.9% saline, and brains rapidly removed and microdissected. Whole hippocampi were either snap frozen on liquid nitrogen and subsequently stored at –80°C or transferred to ice-cold Hanks' buffered salt solution (Life Technologies, Stockholm, Sweden) prior to magnetic antibody-based cell sorting (MACS).

Isolation of Hippocampal Microglia

CD11b^{pos} microglia were isolated from hippocampi microdissected from P21 mice. Briefly, isolated hippocampi were dissociated into single cell suspensions using a gentleMACS Dissociator and papain-based Neural Tissue Dissociation kit (Miltenyi Biotec Norden AB, Lund, Sweden), myelin debris was removed from the single-cell suspensions with Myelin Removal

Table 1. Primers used for qRT-PCR

Gene	Official symbol	NCBI ID	Primer	Product number
Gapdh	Gapdh	NM_008084	Mm_Gapdh_3_SG	QT01658692
Hprt	Hprt	NM_013556	Mm_Hprt_1_SG	QT00166768
Cd86	Cd86	NM_019388	Mm_Cd86_1_SG	QT01055250
Il1b	Il1b	NM_008361	Mm_Il1b_2_SG	QT01048355
iNos	LOC673161	XM_001004823	Mm_LOC673161_1_SG	QT01547980
Il6	Il6	NM_031168	Mm_Il6_1_SG	QT00098875
YM1	Chi3L3	NM_009892	Mm_Chi3l3_2_SG	QT02241722
CD206	Mrc1	NM_008625	Mm_Mrc1_1_SG	QT00103012
Arg1	Arg1	NM_007482	Mm_Arg1_1_SG	QT00134288
Il10	Il10	NM_010548	Mm_Il10_1_SG	QT00106169

Beads II (Miltenyi Biotec) and microglia were isolated with anti-CD11b microbeads (Miltenyi Biotec). Separations were performed with an autoMACS Pro Separator (Miltenyi Biotec). All procedures were conducted in accordance with the manufacturer's guidelines. Isolated microglia were subject to direct RNA processing.

RNA Extractions and qRT-PCR

Total RNA was isolated from the left hippocampus using a Qiagen RNeasy Lipid Tissue Mini Kit (Qiagen, Sollentuna, Sweden) or from MACS-isolated microglia using a Qiagen RNeasy Micro Kit (Qiagen) in accordance with the manufacturer's instructions. RNA concentrations were assessed using a NanoDrop 1000 Spectrophotometer (NanoDrop, Wilmington, Del., USA). Reverse transcription was performed in duplicate for each sample with a QuantiTect Reverse Transcription Kit (Qiagen); in the case of MACS-isolated microglia, duplicates were later pooled due to low RNA yield. qRT-PCR was performed using a QuantiFast SYBR Green PCR Kit (Qiagen) following a 2-step cycling protocol (5 min of initial denaturation followed by 10 s of denaturation at 95°C and 30 s of annealing/extension at 60°C for 25 cycles) on a Roche LightCycler 480 (Roche, Bromma, Sweden). Amplification specificity was confirmed by melting curve analysis. All primers were purchased from Qiagen (table 1). Quantification was conducted in accordance with the standard curve method, and expression values were normalized to the geometric mean of the reference genes glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and hypoxanthine phosphoribosyltransferase 1 (*Hprt1*).

Statistics

Stereological data are presented as group means $\pm$ SEM and qRT-PCR data are presented as mean percent changes (relative to control) $\pm$ SEM. Comparisons were performed by Student's *t* test and differences were considered significant at * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$. Analyses were performed using SPSS (PASW Statistics, release 17.02).

Results

Perinatal LPS Administration Acutely Increases Microglial Density in the Developing Hippocampus through Increased Proliferation, Not Infiltration

We first sought to assess the effect of peripheral immune stimulation on the microglial population of the developing hippocampus. LPS was administered intraperitoneally at a dose of 1 mg/kg to P5 pups and tissue harvested at P7, P21 and P74. Stereological estimation revealed a transient 342.1% increase in absolute number ($p < 0.05$; fig. 1a) and 214.4% increase in density ($p < 0.05$; fig. 1b) of Iba1^{pos} microglia 48 h after LPS administration; this change was coupled with a shift towards a more active but not phagocytic morphology, with cells displaying characteristically thickened processes (fig. 1c, d, 2d, e). With developmental progression and concomitant increase in microglial cell number, differences in absolute cell number and density were rapidly lost (fig. 1a, b; P21, P74). Interestingly, LPS administration appeared to prematurely boost microglial cell density to a level similar to that observed in more mature animals (fig. 1b).

We next examined whether the additional Iba1^{pos} cells observed 48 h after LPS administration were derived from increased proliferation of primitive microglial progenitors/microglia or infiltration of peripheral monocytes. To assess proliferation, BrdU was administered (50 mg/kg i.p.) at P6 (24 h after LPS administration) and tissue was harvested 24 h later (P7); infiltration was assessed in *Lys-EGFP-ki* mice, which express EGFP only in mature peripheral granulomyelomonocytic cells and not in microglia [31, 32], 48 h after LPS administration (P7). Our findings show a 53.1% increase ($p < 0.05$; fig. 2a–e) in the percentage of newly proliferated Iba1^{pos}/BrdU^{pos} cells in

Fig. 1. Systemic administration of LPS (1 mg/kg i.p.) at P5 acutely increases the absolute number and density of microglial cells in the hippocampal formation. Stereological assessment reveals a significantly increased absolute number (**a**) and density (**b**) of Iba1-immunoreactive cells at P7 (saline: $n = 8$; LPS: $n = 6$) but not at P21 or P74 following LPS administration ($n = 8$ per group). **a, b** Values are presented as means $\pm$ SEM. Student's t test between groups at each time point. * $p \leq 0.05$. **c, d** Representative images showing hippocampal Iba1 immunoreactivity 48 h after saline (**c**) or LPS (**d**) administration. Scale bar = 200 μm .

the hippocampus 48 h after LPS administration. Whilst a small number of *Lys*-EGFP^{pos} cells could be observed in the hippocampus following saline or LPS administration (fig. 2f, g), these cells did not coexpress Iba1 (*Lys*-EGFP^{pos}/Iba1^{neg}; fig. 2h, i) and were localized within the lumen of CD31^{pos} cerebral blood vessels (fig. 2j) most likely representing residual blood-borne granulocytes not cleared during perfusion [31, 33]. We hence conclude that peripheral monocytes do not contribute to the CNS microglia population following systemic LPS stimulation in this model. Together these data strongly indicate that peripheral administration of LPS during the early neonatal period increases the proliferation of primitive microglial progenitor cells, transiently increasing absolute population size and leading to premature attainment of adult microglial density in the developing hippocampus.

Peripheral LPS Alters Hippocampal Expression of Genes Associated with Microglial Phenotype

With the observation of increased acute-phase microglial proliferation transiently increasing the hippocampal microglial population size, we hypothesized that a proportion of the stable microglial population in LPS-administered animals may have been born during the acute inflammatory period and, hence, might display altered inflammatory phenotypes. We employed qRT-PCR

to assess mRNA expression of genes associated with M1 (*Cd68*, *Il1b*, *iNOS* and *Il6*) and M2 (*Ym1*, *Cd206*, *Arg1* and *Il10*) microglial phenotypes in the developing hippocampus. Our results indicate a mixed M1/M2 response following LPS administration with increased expression of both the M1 gene *Cd86* (+133.9%; $p < 0.001$) and the M2 gene *Ym1* (+85.0%; $p < 0.05$) at P7 (fig. 3a), and regulation of the M1 genes *iNOS* (+53.6%; $p < 0.01$) and *Il6* (−70.0%; $p < 0.05$) at P21 (fig. 3b).

As astrocytes may also produce and elaborate the cytokines *Il6* and *iNOS* [34, 35], we further analysed gene expression levels in MACS-isolated hippocampal microglia from P21 animals (fig. 3c). Here we observed a strong but statistically insignificant upregulation of *Il6* (158.8%; $p = 0.16$) and *iNOS* (121.8%; $p = 0.22$) occurring in response to LPS administration.

Hippocampal Progenitor Proliferation Is Unaltered by Perinatal LPS Treatment

As LPS administration acutely increased microglial density and altered expression of several inflammation-associated genes in the developing hippocampus, we further examined the potential effects of LPS on hippocampal cell proliferation. At acute (P8) and subacute (P21) time points, BrdU was administered 24 h (P8) and 48 h (P21) prior to sacrifice, and proliferation was determined

Fig. 2. Increased microglial cell density at P7 is caused by microglial proliferation and not infiltration of peripheral monocytes/macrophages. Animals received LPS (1 mg/kg i.p.) at P5 followed by 50 mg/kg BrdU at P6. Tissue was harvested at P7. **a–e** P5 LPS administration significantly increased the percentage of Iba1^{pos}/BrdU^{pos}, newly born microglia in the P7 hippocampus (saline: $n = 6$; LPS: $n = 5$; **c**). Representative photomicrographs of the hippocampus in saline- (**a**) and LPS-treated (**b**) animals. **c** Values are presented as means $\pm$ SEM. Student's t test between groups at each time point. * $p \leq 0.05$. **d, e** Enlargements of insets in **a** and **b**, respectively. **e** Arrows: BrdU/Iba1 coimmunoreactivity. **f–i** Few EGFP-positive peripheral myeloid-derived cells can be detected in the hippocampus of *Lys-EGFP-ki* mice following either saline (**f, h**) or LPS (**g, i**) administration. **h, i** Enlargements of insets in **f** and **g**, respectively, showing that detectable EGFP is never present in Iba1^{pos} microglia. **j** Immunostaining for CD31 confirms the intraluminal localization of EGFP^{pos} cells. Scale bars = 200 μ m (**a, f**), 20 μ m (**d, h, i**) and 5 μ m (**j**).

Fig. 3. Peripheral LPS administration alters hippocampal expression of genes associated with pro- (M1) and anti-inflammatory (M2) microglial phenotypes. Animals received LPS (1 mg/kg i.p.) at P5 and were sacrificed at P7 and P21; gene expression, as assessed by qRT-PCR, is presented as percent change (relative to saline-injected control animals). Values are presented as means $\pm$ SEM. Student's *t* test between groups at each time point. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. **a, b** Regulation of gene expression in whole microdissected hippocampi at 48 h ($n = 6$ per group) and 14 days ($n = 12$ per group), respectively, after LPS administration. **c** Regulation of gene expression in MACS-isolated (CD11b^{pos}) hippocampal microglia at P21 ($n = 10$ per group).

by stereological quantification of the absolute number of BrdU^{pos} cells (fig. 4a, b). The endogenous mitotic marker PhH3 was used to identify proliferating cells at P74 (fig. 4c). Neonatal peripheral immune stimulation had no effect on cell proliferation in the hippocampus at any of the time points examined (fig. 4a–c).

Perinatal LPS Administration Inhibits Neuronal Differentiation Two Weeks after Administration

We next addressed LPS effects on the stem and progenitor cell populations of the developing SGZ. Here we chose to perform analyses 2 weeks after peripheral administration of LPS, aiming to build upon previous work by Bland et al. [27] which showed reduced neurogenesis among hippocampal progenitors born within 24 h of subcutaneous *E. coli* stimulation in P4 rats.

Focussing on neurogenesis, we first undertook a short-term differentiation study with BrdU being administered at P21 and cell fate assessed via DCX coimmunoreactivity (type 2b and 3 NPCs) 7 days later. Absolute numbers

of BrdU^{pos} (all labelled cells; fig. 5a) and BrdU^{pos}/DCX^{pos} cells (type 2b and type 3 progenitors; fig. 5b–d) in the hippocampal SGZ were stereologically assessed at P28. LPS administration led to a significant ($p < 0.05$) 24% reduction in the number of newly born DCX^{pos} type 2b and type 3 progenitor cells in the P28 hippocampus (fig. 5b). This effect occurred in the absence of altered survival of BrdU^{pos} cells (fig. 5a; $p > 0.05$).

As the proliferating stem and progenitor cells of the SGZ can be divided into at least 4 subpopulations based on their expression of the stem cell/astrocyte markers nestin and GFAP, and the transiently expressed early neuronal marker DCX [30], we further investigated which specific subset of SGZ progenitors was susceptible to LPS-mediated changes (fig. 6). Here, mice received LPS at P5; proliferating cells were labelled with BrdU at P19 and tissue was analysed at P21. We observed a 35% ($p < 0.05$) reduction in type 3 (BrdU^{pos}/DCX^{pos}) late-stage precursor cells in response to LPS stimulation (saline: $1,764.2 \pm 432.6$; LPS: $1,133.1 \pm 570.5$) with no detectable effect on

Fig. 4. LPS administration (1 mg/kg i.p.) at P5 has no effect on proliferation of cells present in the hippocampus at acute (**a**, **d**), subacute (**b**, **e**) or chronic time points (**c**, **f**). BrdU (50 mg/kg) was administered 24 h (P7; saline: $n = 6$; LPS: $n = 5$) and 48 h (P21; saline: $n = 7$; LPS: $n = 8$) prior to sacrifice, and immunoreactivity was assessed as an indicator of cell proliferation (**a**, **b**, respectively). At P74 (saline: $n = 10$; LPS: $n = 11$) proliferation was quantified via immunoreactivity of the mitotic marker PhH3 (**c**). Please note: im-

munoreactive cells were quantified in the GCL and SGZ at P8 as proliferation is widespread at this age; at P21 and P74, immunoreactive cells were only quantified in the SGZ. The photomicrographs display immunoreactivity for BrdU at P7 (**d**) and P21 (**e**), and for PhH3 at P74 (**f**). Values represent means $\pm$ SEM. Student's t test between groups for cell number at each time point. Scale bar = 50 μ m.

Fig. 5. LPS administration (1 mg/kg i.p.) at P5 significantly impairs neuronal fate specification among SGZ progenitors in the subacute postinflammatory environment. A single BrdU injection (50 mg/kg i.p.) was given at P21 and neuronal commitment examined 7 days later by assessment of BrdU/DCX coimmunoreactivity. **a** LPS administration had no effect on short-term survival of proliferating cells labelled at P21 (saline: $n = 8$; LPS: $n = 8$). **b** LPS significantly reduced neuronal fate specification among cells born at P21 (saline: $n = 7$; LPS: $n = 8$). **a**, **b** Values are presented as means $\pm$ SEM. Student's t test between treatments. * $p \leq 0.05$. **c** Z-projection displaying BrdU/DCX coimmunoreactivity. **d** Orthogonal reconstructions showing coexpression of BrdU and DCX. Scale bars = 10 μ m (**c**) and 5 μ m (**d**).

Fig. 6. LPS administration (1 mg/kg i.p.) at P5 significantly reduced the number of type 3 neuronal precursors in the early postnatal hippocampal SGZ. A single injection of BrdU (50 mg/kg) was made at P19 and tissue harvested at P21. Immunoreactivity for nestin, GFAP and DCX was used to assess fate specification and maturation of newly proliferated (BrdU^{pos}) precursor cells. **a** Response of hippocampal precursor populations to LPS ($n = 8$ per group; precursor classification as follows: type 1 – $\text{BrdU}/\text{nestin}/\text{GFAP}$, type 2a – $\text{BrdU}/\text{nestin}$, type 2b – $\text{BrdU}/\text{nestin}/\text{DCX}$, type 3 – BrdU/DCX). Values are presented as means $\pm$ SEM. Student's t test between treatments. * $p \leq 0.05$. **b** Representative maximum intensity projection of $\text{BrdU}/\text{nestin}/\text{DCX}/\text{GFAP}$ immunoreactivity in the P19 hippocampus. **c–g** Enlargement of **inset** in **b**, displayed as individual channels. Arrows: locations of BrdU^{pos} nuclei; bottom-left arrow: $\text{BrdU}^{\text{pos}}/\text{nestin}^{\text{pos}}/\text{GFAP}^{\text{pos}}$ type 1 NPC extending a characteristic radial glia-like process through the molecular layer; remaining arrows: $\text{BrdU}^{\text{pos}}/\text{nestin}^{\text{pos}}/\text{DCX}^{\text{pos}}$ type 2b NPCs. Scale bars = 50 μm (**b**) and 10 μm (**c**).

Fig. 7. LPS (1 mg/kg i.p. at P5) does not affect long-term cell ($\text{BrdU}^{\text{pos}}/\text{NeuN}^{\text{neg}}$) or neuronal ($\text{BrdU}^{\text{pos}}/\text{NeuN}^{\text{pos}}$) survival. **a**, **b** Cell (**a**) and neuronal (**b**) survival ($n = 10$ per group) of cells labelled between P22 and P25 (50 mg/kg i.p. once per day) and analysed at P74. Values are presented as means $\pm$ SEM. Student's t test between treatments. **c** Z-projection displaying BrdU/NeuN coimmunoreactivity. **d** Orthogonal reconstructions showing coexpression of BrdU and NeuN in the GCL/SGZ. Scale bars = 10 μm (**c**) and 5 μm (**d**).

Fig. 8. Perinatal LPS (1 mg/kg i.p. at P5) does not alter the number of DCX^{pos} neuroblasts present in the P74 hippocampus. **a** Absolute number of DCX^{pos} cells in the P74 hippocampus (n = 10 per group). Values are presented as means $\pm$ SEM. Student's t test between treatments. **b, c** Representative photomicrographs of DCX-immunoreactive cells lining the hippocampal SGZ at P74 in saline- (**b**) and LPS-treated (**c**) animals. Scale bar = 50 μ m.

the absolute number of type 1 (BrdU^{pos}/nestin^{pos}/GFAP^{pos}; saline: 50.2 $\pm$ 45.8; LPS: 84.9 $\pm$ 76.36), type 2a (BrdU^{pos}/nestin^{pos}; saline: 75.3 $\pm$ 99.0; LPS: 42.6 $\pm$ 53.7) or type 2b (BrdU^{pos}/nestin^{pos}/DCX^{pos}; saline: 1,903.3 $\pm$ 768.1; LPS: 2,142.4 $\pm$ 1,163.4) cells (fig. 6a).

Together these data indicate an LPS-mediated decrease in absolute number of newly born SGZ progenitors committed to neuronal fate, a change manifest in the reduced size of the type 3 progenitor cell population.

Reduced Neuronal Differentiation between P21 and P28 Coupled to Enhanced Long-Term Neuronal Survival

Having shown that neonatal peripheral immune stimulation inhibits neuronal fate commitment amongst NPCs entering the S phase of the cell cycle at P21 and P19 (fig. 5b, 6a, respectively), we investigated the long-term survival and fate of cells labelled with BrdU daily between P22 and P25; the absolute number of BrdU^{pos} (survival; fig. 7a) and BrdU^{pos}/NeuN^{pos} cells (neuronal maturation/survival; fig. 7b–d) in the hippocampal GCL and SGZ were stereologically assessed at P74. Our analyses showed no effect of LPS administration on total cell survival (fig. 7a) or neuronal survival (fig. 7b). These results suggest that the reduced neurogenic potential following LPS administration (fig. 5b) is compensated for by enhanced neuronal survival.

Peripheral LPS Effects on Neurogenesis Are Transient

We further asked whether the peripheral LPS-mediated effect on the numbers of type 2b and type 3 progenitors was persistent. Reasoning that the lower presence of DCX^{pos} type 2b and type 3 cells in the mature hippocampus would make them amenable to exhaustive counting, we quantified the absolute number of DCX^{pos} cells in the adult (P74) hippocampal SGZ (fig. 8a–c). No significant difference could be observed between the

LPS and saline groups, highlighting the transient nature of peripheral LPS-induced inhibition of neuronal differentiation.

Discussion

In this study we investigated the acute, subacute and chronic effects of experimentally induced neonatal peripheral inflammation on the developing hippocampus. Our results show that peripheral LPS stimulation induces an acute expansion of the hippocampal microglia population, which occurs through increased proliferation in the absence of peripheral monocyte infiltration. Further, qRT-PCR analysis of hippocampal gene expression indicates dysregulation of several genes associated with both M1 and M2 microglial phenotypes, a response which persists for at least 14 days. Repetition of these analyses on MACS-isolated microglia from the hippocampus gave results partially supportive of our hypothesis of long-term changes to microglial inflammatory status. We next screened for perturbations of the ongoing neurogenic process in the hippocampal neurogenic niche. Our analyses indicate that neonatal LPS administration transiently reduces neurogenesis 2 weeks after LPS administration in a proliferation-independent manner, specifically affecting the type 3 NPC population. Paradigms tracing the fate of cells born during this period through to P74 indicate that these differentiation deficits are insufficient to reduce the number of mature neurons derived from progenitors entering the cell cycle during this period, leading us to posit that the observed inhibition of neuronal fate commitment is compensated for by enhanced neuronal survival in this model.

Common opinion holds that yolk sac-derived myeloid precursors colonize the developing murine CNS between embryonic days 7 and 7.5 [36], where they proliferate ex-

tensively during embryonic and early postnatal development, eventually giving rise to mature ramified microglia [29, 37]. Proliferation is rapidly restricted in the first 2 postnatal weeks [29], with adult microglia displaying low turnover [38] and highly limited replacement by peripheral monocytes under non-pathological conditions [39]. Our observation of increased hippocampal microglial cell number and density 48 h after LPS administration is broadly in agreement with previous reports studying the effects of peripheral inflammatory stimulation with *E. coli* in neonatal rodents [27], and with LPS in juvenile and adult [21, 24, 40] rodents. These studies have variously shown increased total numbers of Iba1^{pos}, activated (ED1^{pos} or Iba1^{pos}/morphologically activated) and proliferating (Iba1^{pos}/BrdU^{pos}) microglia in some, or all, hippocampal regions analysed. Whilst our stereological studies did not specifically address microglial activation status, we did observe a greater number of Iba1^{pos} cells exhibiting elongated to rounded cell bodies with thickened processes (fig. 1c, d, 2d, e) consistent with the morphological characteristics of activated microglia [10]. Additionally we found that LPS increased microglial density in P7 animals to a level similar to that observed in saline- and LPS-administered P21 and P74 animals. These observations of similar microglial densities in LPS-administered P7, P21 and P74 animals could speculatively suggest that there may be a target or maximum density in the brain and that control of microglial proliferation may be juxtacrine in nature. Indeed microglia express both Notch-1 receptor and its ligands Jagged-1 and Delta-1 [41], and Notch activation induces expression of the Runx1 (Runt-related) transcription factor [42], which in turn inhibits amoeboid microglial progenitor proliferation and facilitates progression from amoeboid precursor to ramified microglia [37]. Having observed an LPS-mediated acute increase in microglial cell density at P7, we asked whether these additional microglia were derived from endogenous microglia/myeloid precursor cell proliferation or peripheral monocyte recruitment. Experiments utilizing *Lys-EGFP-ki* mice, in which myeloid cells generated by definitive but not primitive haematopoiesis express EGFP [31, 32], indicated a complete lack of peripheral contribution to the LPS-induced increase in microglia number.

Upon stimulation, microglia assume an 'activated' phenotype which has been associated with inhibition of adult neurogenesis [21, 43]. Enhanced scrutiny of activated microglia allows the distinction of populations with both pro- (M1) and anti-inflammatory (M2) phenotypes [14]. Interestingly, in vitro studies indicate that LPS-acti-

vated microglia (M1) reduce neurogenesis, whereas IL4-activated microglia (M2) act to enhance neurogenesis [15, 44]. We employed qRT-PCR to probe hippocampal expression of genes associated with the M1 (*Cd68*, *Il1b*, *iNOS* and *Il6*) and M2 (*Ym1*, *Cd206*, *Arg1* and *Il10*) phenotypes. Our data show regulation of M1- and M2-related target genes 48 h, and of M1-related genes 2 weeks, after LPS administration. At 48 h, *Cd86* and *Ym1* are both upregulated, whilst 14 days later *iNOS* is upregulated and *Il6* downregulated. When comparing the temporal regulation of whole hippocampal gene expression in the present study with data generated by Hu et al. [45], who investigated expression of a larger panel of M1/M2-related genes at numerous time points following transient focal cerebral ischemia in 10- to 12-week-old mice, we see striking concurrence. Comparison of the 6 genes present in both studies reveals a similar postinjury temporal expression pattern with upregulation of *Cd86* and *Ym1* and no change in *iNOS* expression 72 h after middle cerebral artery occlusion, and upregulation of *iNOS* and no change in *Cd86*, *Ym1* and *Arg1* 14 days after the injury.

Specific analysis of gene expression in hippocampal microglia 2 weeks after LPS administration supported the lack of regulation of M2 markers at this time point. Interestingly, we observed a relatively large, but insignificant, percentage regulation of the M1 markers *iNOS* (121.8%) and *Il6* (158.8%). Whilst it is unwise to draw strong conclusions from insignificant data, we can make two minor inferences from the direction of regulation. First, *Il6* is positively regulated in isolated hippocampal microglia, suggesting that another cell type is responsible for the reduced expression of *Il6* quantified in samples derived from whole hippocampi. Secondly, microglia-related *iNOS* expression may contribute to the overall induction of *iNOS* expression in the hippocampus. Together our data indicate a prolonged and evolving hippocampal inflammatory response to neonatal systemic LPS administration, with regulation of both M1- and M2-associated genes in the acute phase, and regulation of only M1-associated genes at P21.

Neurogenesis is a highly plastic process known to be influenced by negative stimuli such as stress [46] and inflammation [15, 21, 43, 47]. In this study, we assessed the main contributors to net neurogenesis, namely proliferation, differentiation and survival, in the hippocampal SGZ and GCL following systemic inflammation in the developing hippocampus. We observed a transient reduction in neuronal commitment (BrdU^{pos}/DCX^{pos}) 16–23 days after LPS administration, an alteration apparently due to effects on type 3 neuronal precursor cells, which

occurred in the absence of effects on hippocampal cell proliferation or survival of BrdU^{pos} cells. This finding is in line with previous reports suggesting that inflammation-mediated effects on cell proliferation and survival are, respectively, limited to cells undergoing mitosis either at [23] or immediately following [27] immune challenge. Our failure to detect any deficit in the number of mature granule cells (BrdU^{pos}/NeuN^{pos}) born in the P22–P25 period (a time at which we observed reduced neuronal commitment) strongly indicates more effective integration of the remaining immature neurons into the developing hippocampal network. Indeed, under normal conditions, many newborn cells die long before maturing to granule cells [48, 49], with survival being linked to network integration and input activity [50]. Thus, the lack of an LPS effect on the absolute number of adult neurons could be due to more efficient network integration and concomitant enhanced survival of the remaining cells.

In conclusion, experimental systemic inflammation occurring during the early postnatal period is transduced to the immature CNS, where it further induces alterations in the developing hippocampal stem cell niche. LPS administration acutely stimulates proliferation of immature microglia, increasing both microglial population size and density, and initiating a prolonged alteration to hippo-

campal inflammatory status. These inflammation-mediated changes occur in conjunction with transient inhibition of neuronal fate commitment among type 3 hippocampal NPCs, an alteration which does not appear to affect the number of mature neurons derived from cells born during this period. Such results suggest that whilst capable of initiating lasting changes within the developing hippocampus, LPS administration at the studied time point and dose is not sufficient to overwhelm the abundant plasticity of the developing hippocampus. However, such changes may reduce the organisms' reserve of hippocampal plasticity, enhancing susceptibility to secondary insult and facilitating the onset of later-life pathologies.

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