Microglia shape corpus callosum axon tract fasciculation: functional impact of prenatal inflammation

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Abstract

Microglia colonise the brain parenchyma at early stages of development and accumulate in specific regions where they participate in cell death, angiogenesis, neurogenesis and synapse elimination. A recurring feature of embryonic microglial is their association with developing axon tracts, which, together with *in vitro* data, supports the idea of a physiological role for microglia in neurite development. Yet the demonstration of this role of microglia is lacking. Here, we have studied the consequences of microglial dysfunction on the formation of the corpus callosum, the largest commissure of the mammalian brain, which shows consistent microglial accumulation during development. We studied two models of microglial dysfunction: the loss-of-function of DAP12, a key microglial-specific signalling molecule, and a model of maternal inflammation by peritoneal injection of lipopolysaccharide at embryonic day (E) 15.5. We also took advantage of the $Pu.1^{-/-}$ mouse line, which is devoid of microglia. We performed transcriptional profiling of maternally inflamed and *Dap12*-mutant microglia at E17.5. The two treatments principally down-regulated genes involved in nervous system development and function, particularly in neurite formation. We then analysed the developmental consequences of these microglial dysfunctions on the formation of the corpus callosum. We show that all three models of altered microglial activity resulted in the defasciculation of dorsal callosal axons. Our study demonstrates that microglia display a neurite-development-promoting function and are genuine actors of corpus callosum development. It further shows that microglial activation impinges on this function, thereby revealing that prenatal inflammation impairs neuronal development through a loss of trophic support.

Introduction

Microglia differentiate from primitive myeloid progenitors produced by the yolk sac that migrate into the developing brain parenchyma (Herbomel *et al.*, 2001; Ginhoux *et al.*, 2010). This colonisation occurs early in central nervous system embryogenesis and in a highly stereotyped manner in all species examined, supporting the notion that microglia have important physiological roles in development. During development microglia can be found throughout the brain but tend to reside preferentially at specific locations where they actively contribute to such processes as cell death, angiogenesis, synapse elimination and neurogenesis (references in Pont-Lezica *et al.*, 2011). A consistent feature of embryonic microglia distribution is their close association with developing axon tracts. Such recurring association has been found in rats (Ling, 1976), birds (Cuadros *et al.*, 1993), fish (Herbomel *et al.*, 2001) and humans (Verney *et al.*, 2010), and raised the question of whether microglia might contribute to tract formation. Under physiological conditions, it has been

proposed that in agreement with their phagocytic function, microglia clear a path for developing axons (Valentino & Jones, 1982) or eliminate transient axonal projections (Innocenti *et al.*, 1983). Yet this contrasts with the morphological observation of microglia associated with non-transient axons (Cuadros *et al.*, 1993) and with *in vitro* studies suggesting a trophic role in neurite extension (David *et al.*, 1990; Chamak *et al.*, 1994). Despite these compelling *in vitro* data and circumstantial *in vivo* evidence, there has been no *in vivo* proof of a definitive role for microglia in neurite development. In addition, microglia might be involved in developmental white matter diseases given that prenatal inflammation and mutations affecting the function of DAP12, a microglial-specific signalling molecule, result in adult white matter alterations including lesions and hypomyelination (Paloneva *et al.*, 2001; Dean *et al.*, 2011).

The corpus callosum (CC) is the largest brain commissural structure between the cerebral hemispheres. CC formation occurs towards the end of embryogenesis and involves multiple steps including midline crossing, axon guidance and fasciculation, and the elimination of exuberant axons (Fame *et al.*, 2011). The abundant presence of microglia during callogenesis and the tract's susceptibility to prenatal inflammation make it a relevant system to decipher the role of microglia in shaping developing tracts. To study the functional

relationship between microglia and CC development, we changed their activity pharmacologically and genetically. We then combined transcriptome and morphological analyses to conclude that microglia are actively involved in the fasciculation of CC axons.

Materials and methods

Mouse lines and treatments

The experiments were carried out in accordance with the European Community's Council Directive 2010/63EU of 22 September 2010 on the protection of animals used for scientific purposes and our protocols were approved by the Charles Darwin Committee on Animal Experiments (Ce5/2012/017). $Cx3cr1^{+/gfp}$ (Jung *et al.*, 2000), $Dap12^{-/-}$ (Tomasello *et al.*, 2000) and $Pu.1^{+/-}$ mice (Back *et al.*, 2004) were maintained on a C57Bl/6j background. C57Bl/6j or heterozygous embryos were used as controls. The day of vaginal plug formation was considered as embryonic day (E)0.5. Pregnant dams were given lipopolysaccharide [LPS, 0.12 μg/g in phosphate-buffered saline (PBS); In-VivoGen, Toulouse, France] by a single intraperitoneal injection at E15.5. Injection of sterile PBS and needle prick were used as controls.

Immunohistochemistry, image acquisition and statistical analysis

Immunostaining was carried out on C57Bl/6j, $Dap12^{-/-}$ and $Pu.1^{-/-}$ embryos as described (Deck *et al.*, 2013). Primary antibodies: rat CD68 (AbD Serotec, Colmar, France); chicken GFP (Aves); goat nrp1 (R&D Systems, Minneapolis, MN, USA), rat L1 (Millipore, Billerica, MA, USA) and rabbit Iba1 (Wako, Richmond, VA, USA). Secondary antibodies were from Jackson ImmunoResearch (West Grove, PA, USA). Hoechst (Invitrogen, Carlsbad, CA, USA) was used for nuclear staining. Images were acquired with Leica microscopes (MZ16F and TCS SP5). Image analysis was carried out using IMAGEJ software. Briefly, a stack of ten images per section was Z-projected and the ratio of the Nrp1-positive tract to the total CC width (L1-positive tract) was calculated. GRAPHPAD (GraphPad Software, San Diego, CA, USA), R (R Project for Statistical Computing, http://www.r-project.org) and SPSS (IBM Corp., Armonk, NY, USA) statistical software were used for statistical analysis.

Data were analysed using Grubb's test to identify outliers (Graphpad), and either non-directional Student's *t*-test, or one-way analysis of variance (ANOVA) followed by Tukey or Games–Howell *post hoc* tests in the event of significant interaction (R v.2.15.2 or SPSS v.20 statistical software). Differences were considered statistically significant at $P < 0.05$. Results are given as mean ± 2SEM.

Microglia isolation and transcriptome analysis

Medial cortical regions including the hippocampus, the surrounding fibre tracts (fimbria, CC) and the overlying prospective cingulate cortex were isolated from $Cx3cr1^{+/gfp}$ and $Dap12^{-/-}$ (on $Cx3cr1^{+/gfp}$ background) E17.5 embryos, and dissociated by incubation with 0.25% trypsin, then DNaseI (10 mg/mL). Microglia were sorted on a FACSAria II (Becton Dickinson, Franklin Lakes, NJ, USA) directly into QIAzol (Qiagen, Valencia, CA, USA) for RNA isolation. We analysed four PBS-injected mice (7.8 ± 1.0 embryos per litter; 27 800 ± 9600 GFP+ cells per litter), four LPS-injected mice (7.5 ± 0.6 embryos per litter; 28 300 ± 12 100 GFP+ cells per litter), four DAP12-KO/$Cx3cr1^{+/gfp}$ mice (6.5 ± 1.7 embryos per litter; 11 600 ± 4200 GFP+ cells per litter) and five untreated $Cx3cr1^{+/gfp}$ (7.0 ± 1.6 embryos per litter; 14 300 ± 3700 GFP+

cells per litter). Flow cytometric analysis was used to phenotype the CX3CR1-GFP+ cells, by labelling them with antibodies against the CD11b (Becton Dickinson) and CD45 (eBioscience, San Diego, CA, USA). The expression of these cell surface makers was analysed with Flowjo software (Tree Star, Ashland, OR, USA). RNA was isolated with the miRNeasy Mini Kit according to the manufacturer's protocol. The RNA was processed with the Ovation Pico WTA v2 and Encore Biotin Module (NuGEN Technologies, San Carlos, CA, USA) and hybridised on Mouse Genome 430 2.0 Arrays (Affymetrix, Santa Clara, CA, USA) according to the manufacturer's protocols. The gene expression data are accessible through GEO Series accession number GSE49079.

The .CEL files were processed with the Partek genomics suite and gene expression data normalised using Robust Multichip Average with GC background correction (Wu & Irizarry, 2004). Differentially expressed genes were identified using an ANOVA linear contrasts model with multiple testing corrections using the false discovery rate method (Benjamini & Hochberg, 1995) with a $P < 0.05$ cut off. Ingenuity pathway analysis (Ingenuity Systems, Redwood, CA, USA) was used for mapping of differentially expressed genes to biological functions.

Results

Alteration of microglial function impairs expression of neurite-growth-related genes

To investigate possible roles for microglial during brain development, we challenged microglial function by two complementary approaches. First, we induced maternal inflammation by peritoneal injection of LPS into pregnant dams. Next, we analysed the consequences of a loss of function of DAP12, a microglia-specific membrane protein necessary for normal phagocytosis (Takahashi *et al.*, 2005) and signalling (Roumier *et al.*, 2008). We compared the gene expression profiles of microglia from control, maternally inflamed (MI) by LPS, and $Dap12$-mutated embryos. Microglia were purified by cell-sorting from the medial cortical region of $Cx3cr1^{+/gfp}$ E17.5 mouse embryos (Jung *et al.*, 2000). This led to a > 99% pure microglial population (Fig. 1A) according to the expression of CD45 and CD11b (Ginhoux *et al.*, 2010). Microglial genome-wide gene expression profiles were obtained using Affymetrix microarrays and analysed using INGENUITY software.

Maternal inflammation and DAP12 mutation induced the differential expression of 3906 and 628 genes, respectively (Fig. 1B; Table S1). In microglia from MI embryos, we found an up-regulation of immune genes such as IL1β (+3.075-fold; $P = 1.19 \times 10^{-2}$), CCL4 (+2.632-fold; $P = 4.54 \times 10^{-3}$), CCL5 (+6.186-fold; $P = 2.28 \times 10^{-2}$), NFkB (+2.097-fold; $P = 7.90 \times 10^{-3}$) and STAT5B (+2.062-fold; $P = 1.60 \times 10^{-3}$), as anticipated for an inflammatory condition (Tambuyzer *et al.*, 2009). Within the Ingenuity database, genes are associated with known functions, which are themselves grouped into categories (Fig. 1C). We identified functional groupings of differentially expressed genes based on the statistical association with these functions and categories (Table S2). Within the category "Haematological system development and function", the function "Development of mononuclear leukocytes" was significantly affected by prenatal inflammation [$P = 3.34 \times 10^{-5}$; 154 differentially expressed genes (DEGs) with 64.9% of them being up-regulated]. The functions "Proliferation of mononuclear leukocytes" ($P = 3.80 \times 10^{-4}$; 194 DEGs; 70.6% up-regulated) or "Engulfment of cells" ($P = 1.82 \times 10^{-4}$; 91 DEGs, 68.1% up-regulated) were similarly altered. In $Dap12$-mutated microglia,
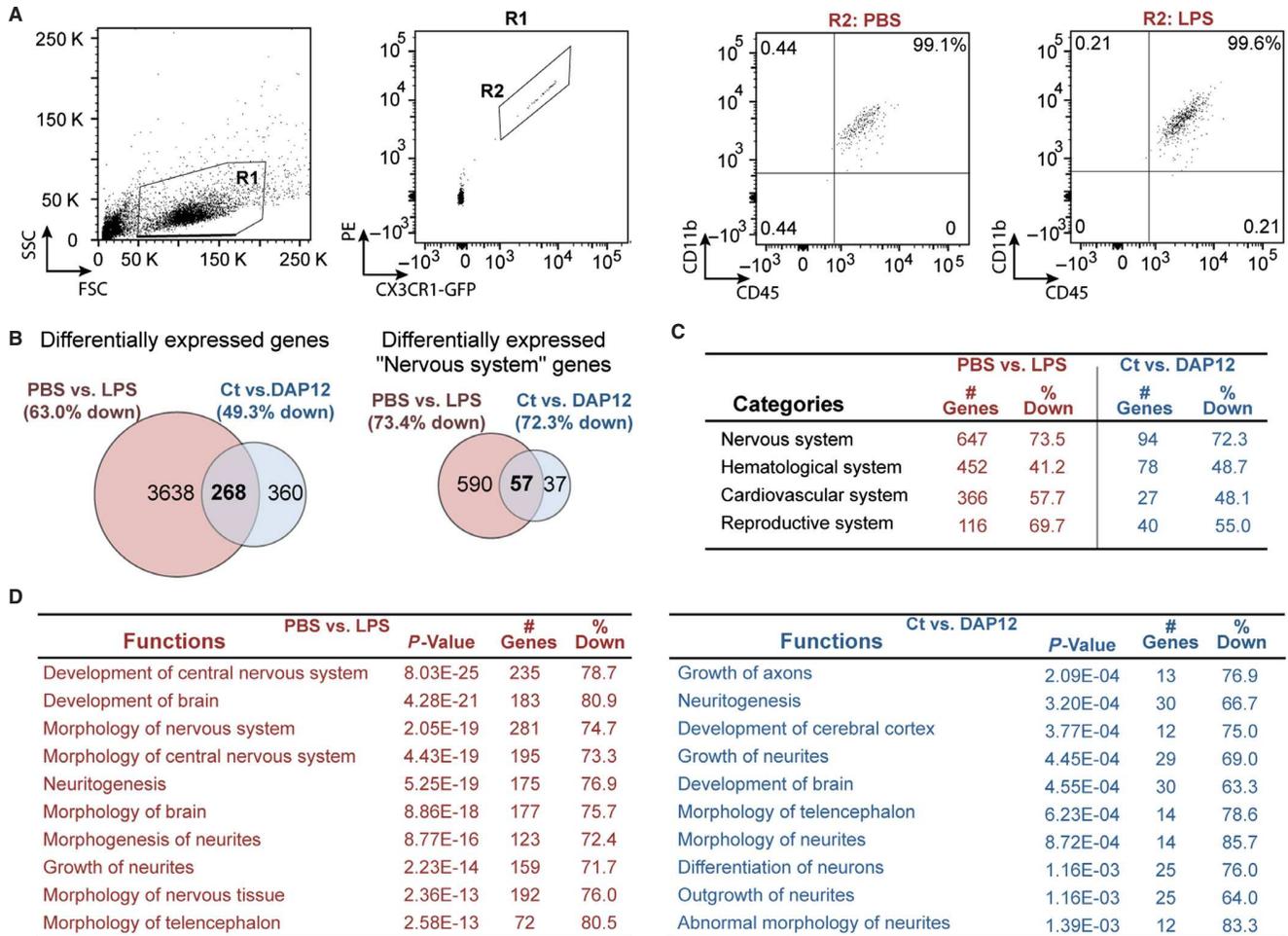
FIG. 1. Transcriptome analysis of E17.5-sorted microglia. (A) Gating strategy of microglia dissected from cortical areas; gate-out cellular debris in R1, gate R2 selects CX3CR1-GFP$^+$ microglia cells. Flow cytometric analysis indicated that > 99% of all CX3CR1-GFP$^+$ cells in gate R2 expressed the microglia markers CD11b and CD45 in both control (PBS) as well as the MI (LPS) mice. Representative figure of four experiments for both PBS- and LPS-injected mice. (B) Venn diagrams showing the number of shared and unique differentially expressed genes upon MI or *Dap12* mutation for all genes (left) and for genes belonging to the "Nervous system development and function" category (right). (C) Top four ingenuity functional categories affected by MI or *Dap12* mutation. The columns show the number of differentially expressed genes and the percentage of genes down-regulated. (D) Top ten functions altered by MI (left in red) or *Dap12* mutation (right in blue). The columns show the *P*-value calculated using the right-tailed Fisher Exact Test, the number of differentially expressed genes and the percentage of genes down-regulated.

DEGs were associated with functions such as "Development of mononuclear leukocytes" ($P = 4.48 \times 10^{-3}$; 31 DEGs; 61.3% up-regulated), "Apoptosis" ($P = 7.84 \times 10^{-8}$; 140 DEGs; 55% up-regulated) and, to a lesser extent, "Phagocytosis by macrophage" ($P = 1.02 \times 10^{-2}$; 7 DEGs). Thus, our data correlate well with previous reports of the functions altered by prenatal inflammation (Boksa, 2010) and mutation of the *Dap12* gene (Hamerman *et al.*, 2005; Takahashi *et al.*, 2005; Wakselman *et al.*, 2008).

The "Nervous system development and function" category displayed the highest number of DEGs in both MI- and DAP12-deficient microglia (Fig. 1C). Among the > 1200 functions of this category, Fig. 1D shows the top ten functions significantly regulated by prenatal inflammation or upon *Dap12* mutation. It is noteworthy that in both conditions, the most significantly regulated functions were related directly to the formation of neurites (Fig. 1D). Remarkably, in both models, the vast majority of differentially expressed "Nervous system" genes were down-regulated (73.5% in MI; 72.3% in DAP12, Fig. 1B). Several genes known to be involved in neuritogenesis are down-regulated both by prenatal inflammation and by

Dap12 mutation: *Sema3C* (LPS: $-2.7\times$, $P = 0.028$; *Dap12*$^{-/-}$: $-2.9\times$, $P = 6.09 \times 10^{-3}$), *Vcan* (LPS: $^-3.1\times$, $P = 1.6 \times 10^{-4}$; *Dap12*$^{-/-}$: $-4.1\times$, $P = 2.9 \times 10^{-2}$), *PlxnA2* (LPS: $-4.1\times$, $P = 4.2 \times 10^{-2}$; *Dap12*$^{-/-}$: $-5.4\times$, $P = 3.08 \times 10^{-2}$) or *NFIA* (LPS: -3.8, $P = 1.1 \times 10^{-3}$; *Dap12*$^{-/-}$: $-2.6\times$, $P = 1.8 \times 10^{-2}$).

Thus, altering microglial function during embryonic development by inducing an inflammatory response or genetic mutation leads to the up-regulation of immune genes, but it also alters, primarily by down-regulation, the expression of genes related to neuritogenesis. These results raised the provocative hypothesis that prenatal inflammation would lead to the loss of a neurite development function in microglia. This led us to examine more closely the relationship between microglia and the developing axon tracts.

Alteration of microglial function impairs the fasciculation of CC dorsal tract

The CC is the largest commissure of the brain. Callogenesis in the mouse begins at E15.5 and the first callosal axons cross the midline
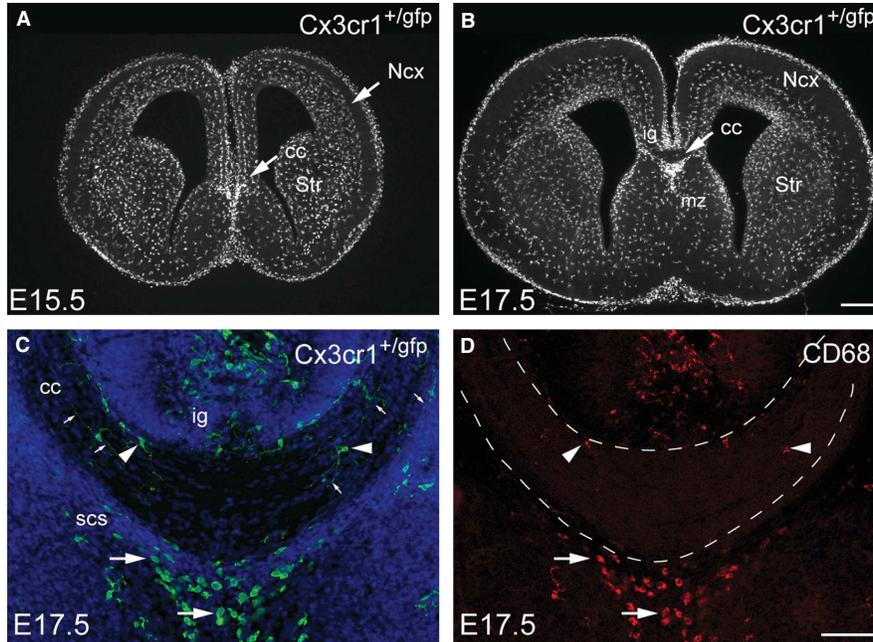
at E16.5 (Ozaki & Wahlsten, 1998). The coincidence of the early stages of CC development with the microglial expression of genes involved in neurite formation led us to study the relationship between microglia and callogenesis. Investigation of the localisation of microglia at E15.5 shows that microglia accumulate at the site of the future CC (Fig. 2A). At E17.5, microglia can be seen at high density in the midline zipper, the subcallosal sling (SCS) and in the induseum griseum (IG) (Fig. 2B). Closer examination at E17.5 shows two morphological populations of microglia (Fig. 2C and D): round microglia with high expression of CD68 are accumulated mostly outside the tracts in the ventral region. By contrast, ramified microglia, with low expression of CD68, are located within the callosal tract, their processes lining up parallel to the fibres (Fig. 2C). The accumulation of microglia ventral to the CC suggests that they may contribute to callogenesis. We therefore compared callogenesis in controls, MI and $Dap12^{-/-}$ mutant embryos. As we were interested in putative loss of microglial function, we also analysed CC development in the absence of microglia using $Pu.1^{-/-}$ embryos, in which inactivation of the transcription factor PU.1 disrupts the generation of all myeloid cells, including microglia (Back et al., 2004).

Microglia in the CC of MI and $Dap12^{-/-}$ embryos displayed the same morphology and distribution as in control embryos (Fig. 3), with round CD68-expressing microglia located ventrally to the tract and ramified low CD68-expressing cells within the axon tract. We first studied the organisation of callosal axons by analysing the expression of L1-CAM, an adhesion protein expressed by all callosal fibres (Piper et al., 2009). The overall organisation of the CC was not altered at E17.5 in any of the conditions examined, with callosal axons crossing the midline. These results show that microglia are not necessary for axon midline crossing during callogenesis. CC width was similar under all conditions (Fig. 3).

We next analysed the fasciculation of the callosal axons by measuring the width of the Neuropilin 1 (Nrp1)-positive dorsal tract of

the CC (Piper et al., 2009) as compared with the width of the L1-positive fibres. In MI embryos, the ratio Nrp1/L1 showed a 12% increase relative to controls (Fig. 3). The increase was even more striking in the $Dap12^{-/-}$ and $Pu.1^{-/-}$ mutants with an average 40% and 70% thickening of the Nrp1 tract, respectively (Fig. 3). In some mutant animals, the Nrp1+ tract was not dorsally restricted but spread out over the whole width of the CC ($Dap12^{-/-}$, $n = 3/15$; $Pu.1^{-/-}$, $n = 3/7$); no such events were observed in controls. This increase in the width of the Nrp1+ tract shows that in MI animals and in those with altered microglial function, there is a defasciculation of the dorsal tract within the CC.

Discussion

In this study, we show that alteration of prenatal microglial preferentially alters genes involved in neurite formation, and induces a defasciculation of axonal tracts within the CC.

To address the potential roles of microglia during late central nervous system development we specifically isolated and carried out transcriptional profiling on cortical microglia in two models of altered microglial function, maternal inflammation by LPS (MI) and $Dap12$ mutation. Microglia purification by cell-sorting combines all microglial phenotypes. The transcriptional profile of sorted microglia is thus probably biased toward the most abundant populations and functions. The microarray data show that in microglia from both the MI and $Dap12$-mutant embryos, immune function is strongly affected, with expression of the majority of immune genes being up-regulated. These results are in accordance with previous findings that demonstrated an activation of the fetal immune system in response to maternal inflammation (Wang et al., 2006; Boksa, 2010). Similarly, $Dap12$-mutant microglia were shown to secrete higher levels of pro-inflammatory cytokines and to have impaired phagocytosis (Takahashi et al., 2005; Roumier et al., 2008). The
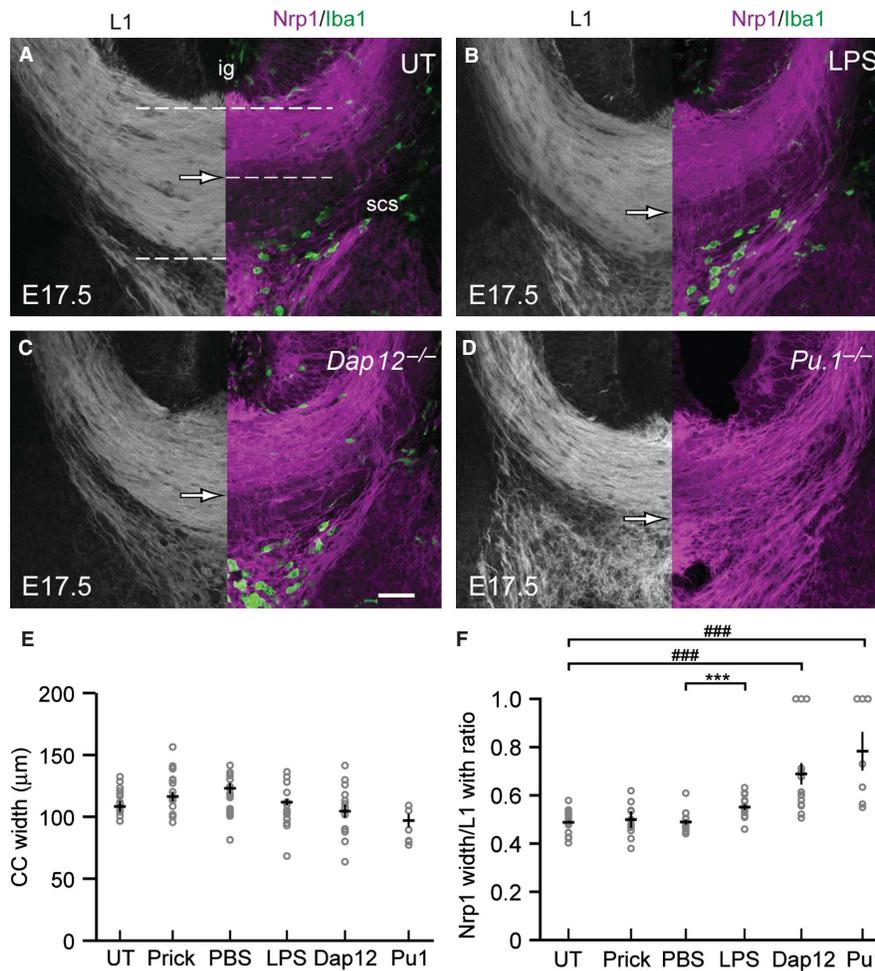
FIG. 3. Defasciculation of the CC upon alteration of microglial function. Coronal sections of E17.5 brains from (A) untreated (UT), (B) maternally inflamed (LPS), (C) $Dap12^{-/-}$ and (D) $Pu.1^{-/-}$ mice immunostained for L1-CAM (left), Nrp1 (right; purple) and Iba1 (right, green). Abbreviations: ig, induseum griseum; scs, subcallosal sling. Scale bar = 25 μm. (E) Quantification of the L1 width in untreated (UT, $n = 18$), needle prick (Prick, $n = 16$), PBS-injected (PBS, $n = 16$), MI (LPS, $n = 15$), $Dap12^{-/-}$ ($n = 15$) and $Pu.1^{-/-}$ ($n = 7$) E17.5 embryos. No significant differences (PBS; Prick; UT: $F_{2,47} = 2.39$, $P = 0.1$. PBS; LPS: $t_{31} = -1.39$, $P = 0.17$. $Dap12^{-/-}$; $Pu.1^{-/-}$; UT: $F_{2,36} = 2.56$, $P = 0.09$). (F) Quantification of the Nrp1/L1 ratio. No significant difference in the Nrp1/L1 ratio was found between PBS-injected, needle prick and untreated controls ($F_{2,47} = 0.17$, $P = 0.846$). PBS vs. LPS, $t_{29} = 3.88$, $P = 0.00056$ (***). $Dap12^{-/-}$; $Pu.1^{-/-}$; UT: $F_{2,37} = 14.58$, $P < 0.0001$ (###), Games-Howell post-hoc test: UT vs. $Dap12^{-/-}$ $P = 0.001$; UT vs. $Pu.1^{-/-}$ $P = 0.023$; $Dap12^{-/-}$ vs. $Pu.1^{-/-}$ $P = 0.578$). Shown are means ± 2SEM.

transcriptional profiling of E17.5 microglia revealed that the highest proportion of DEGs in MI and $Dap12$-mutant microglia belonged to the "Nervous system development and function" category, and more specifically to genes involved in the formation of neurites. Microglia are known to secrete molecules involved in axon growth (Chamak et al., 1994) as well as neurotrophic factors (Kim & Vellis, 2005). Our microarray data show that altering microglia function by pharmacological or genetic means during late embryonic development leads to the anticipated induction of a microglial immune response, but also the unexpected down-regulation of a neurite development function.

The abundance of microglia at the level of the CC is striking. Neither MI nor $Dap12$ mutation altered microglia morphology or distribution in the region. Moreover, CC formation was largely normal in all three models (MI, $Dap12^{-/-}$ and $Pu.1^{-/-}$ mice), with axons effectively crossing the midline and total CC width being similar all around. Closer inspection, however, showed that Nrp1-positive axons are no longer restricted to the dorsal part of the CC but defasciculate and in some cases spread out over the whole struc-

ture. It is of note that the severity of the defasciculation phenotype varies between models, with MI embryos being the least affected and $Dap12$ then $Pu.1$ mutants being progressively more severe. The varied penetration of the phenotype may be due to the timing of the altered microglial function. Indeed, maternal inflammation was induced at E15.5 and the consequences were observed at E17.5, affecting developmental processes in a short time frame. By contrast, $Dap12$ mutation probably affects microglial function from an earlier time point (Thrash et al., 2009), and so might result in a greater developmental effect. Along the same continuum, $Pu.1^{-/-}$ embryos have the most severe callosal phenotype and they lack microglial function from the onset of embryogenesis (Back et al., 2004). There appears to be a discrepancy between the severity of the callosal tract phenotype in MI and $Dap12$-mutant embryos, and the number of differentially expressed genes identified in each case (3906 and 628, respectively). In the case of MI, microglia respond to a variety of signals produced by maternal inflammation (Wang et al., 2006; Boksa, 2010). In $Dap12$ mutants, by contrast, the number of affected pathways is probably restricted, thus altering

fewer genes. The difference between phenotype severity and number of differentially expressed genes suggests that only a small number of key genes and pathways are responsible for the observed defasciculation.

The best described function of microglia during development is phagocytosis of progenitors (Cunningham *et al.*, 2013) and neurons (Wakselman *et al.*, 2008). Microglia located at meeting points of major white matter tracts are thought to contribute to axon growth by utilising their phagocytic capacity to clear a path for developing axons (Valentino & Jones, 1982; David *et al.*, 1990), as well as eliminating exuberant and transient axons (Innocenti *et al.*, 1983). Microglia also associate with developing white matter tracts in the absence of neuronal cell death or axon degeneration (Cuadros *et al.*, 1993). Such an association, in conjunction with their ability to stimulate neuronal differentiation and neurite growth *in vitro*, led to the hypothesis that microglia might contribute to axon growth and guidance via the release of trophic factors (Kim & Vellis, 2005; Deverman & Patterson, 2009; references in Pont-Lezica *et al.*, 2011). We find the same defasciculation phenotype in animals with immune activation, in which microglial phagocytosis is increased, as in $Dap12^{-/-}$ animals, in which it is impaired (Takahashi *et al.*, 2005), and in $Pu.1^{-/-}$ animals that lack microglia. The fact that the defasciculation phenotype does not correlate with the changes in phagocytosis suggests that defasciculation is not purely phagocytosis-dependent. The transcriptional profiling of microglia revealed that they express genes involved in neurite formation and that these are down-regulated in microglia from MI and *Dap12*-mutant animals. Moreover, the mild defasciculation seen in MI embryos is comparable with that observed in mice lacking Syb2, a protein required for Sema3A signalling (Zylbersztejn *et al.*, 2012), supporting the notion that impairment of microglial function may affect trophic support. We thus hypothesise that microglia participate in axon growth and guidance via the modulation of neurite formation pathways. At this stage, however, we cannot exclude that microglia indirectly control the development of CC axonal tracts via engulfment of guidepost glia and neurons, or by endocytosis of guidance molecules themselves.

Prenatal inflammation is a known risk factor for the development of psychiatric conditions, such as schizophrenia and bipolar disorder, which often feature abnormal connectivity, hypomyelination and atrophy of axon tracts (Beumer *et al.*, 2012). DAP12 mutations in humans induce Nasu–Hakola disease, which is characterised by presenile dementia and leukodystrophy (Paloneva *et al.*, 2001). Both MI and *Dap12* mutation lead to changes in microglial activity during central nervous system embryogenesis and our study sheds new light on the pathophysiological mechanisms by which microglia influence tract development and contribute to the development of mood disorders.

Acknowledgements

Abbreviations

CC, corpus callosum; E, embryonic day; IG, induseum griseum; LPS, lipopolysaccharide; MI, maternally inflamed; Nrp1, Neuropilin 1; PBS, phosphate-buffered saline; SCS, subcallosal sling.

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