A role for PDGF-C/PDGFRα signaling in the formation of the meningeal basement membranes surrounding the cerebral cortex

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ABSTRACT
Platelet-derived growth factor-C (PDGF-C) is one of three known ligands for the tyrosine kinase receptor PDGFRα. Analysis of Pdgfc null mice has demonstrated roles for PDGF-C in palate closure and the formation of cerebral ventricles, but redundancy with other PDGFRα ligands might obscure additional functions. In search of further developmental roles for PDGF-C, we generated mice that were double mutants for Pdgfc−/− and PdgfraGFP/+. These mice display a range of severe phenotypes including spina bifida, lung emphysema, abnormal meninges and neuronal overmigration in the cerebral cortex. We focused our analysis on the glia limitans basement membrane. We also present expression data on Pdgfa, Pdgfc and Pdgfra in the cerebral cortex and microarray data on cerebral meninges.

KEY WORDS: PDGF-C, Meninges, Basement membrane, Cerebrum, PDGFRα

INTRODUCTION
Platelet-derived growth factors (PDGFs) and their receptors (PDGFRs) play pivotal roles in vertebrate development. Analyses of genetically modified mice have shown that three of the four mammalian PDGFs (PDGF-A, -B and -C), and both PDGFRs (PDGFRα and PDGFRβ) are important for a wide range of developmental processes, spanning from gastrulation to epithelial organogenesis, angiogenesis, hematopoiesis and other processes (reviewed by Andrae et al., 2008). For PDGF-D there is currently no published information available about its physiological role. PDGFs are dimeric polypeptides (PDGF-AA, -AB, -BB, -CC and -DD have been demonstrated to date) that act by binding to and inducing dimerization of PDGFRs in the plasma membrane, which in turn triggers receptor signaling. The PDGFRs are receptor tyrosine kinases (RTK) that signal via classical RTK pathways, including Ras-MAPK, PI3K and PLCγ (Heldin et al., 1998).

PDGFRs differentially activate the PDGFRs. PDGF-C and PDGF-A are the principal ligands for PDGFRα in vivo as shown by gene knockout experiments: Pdgfa−/−; Pdgfc−/− double knockout mice phenocopy Pdgfrα−/− mice (Ding et al., 2004; Soriano, 1997), whereas single Pdgfa−/− or Pdgfc−/− knockouts both display substantially milder phenotypes. This suggests that PDGF-C and PDGF-A exert partially overlapping and redundant functions via PDGFRα. Pdgfb−/− mice phenocopy Pdgfrβ−/− mice (Hellung et al., 1999; Leveën et al., 1994; Soriano, 1994), demonstrating that PDGF-B is the principal physiological ligand for PDGFRβ. Despite the fact that PDGF-B can activate also PDGFRα in vitro, no redundancy between PDGF-A/C and PDGF-B has been implicated through comparative analysis of knockout mutants so far. However, PDGF-B may have physiological functions via PDGFRα that have gone unnoticed. Likewise, although PDGF-D can activate PDGFRβ in vitro, the physiological role of this ligand remains to be elucidated. Thus, even though much is known about the developmental roles of PDGFs through the analysis and comparison of individual knockout mice, the early embryonic lethality of some of the PDGF/PDGFR mutants and the likely redundancy between some of the PDGF ligands (in particular PDGF-C and PDGF-A) suggests that developmental functions exist that were not revealed through previous analyses.

The relative importance of different PDGFs for developmental processes may also vary depending on the genetic background, as illustrated by the perinatal lethality of Pdgfc−/− mice on a 129S1 background due to a complete cleft palate (Ding et al., 2004), whereas the same mutants survive into adulthood when bred on C57BL/6J background (Fredriksson et al., 2012). Thus, both redundancy with other PDGFs and genetic background may variably compensate for the loss of PDGF-C in Pdgfc−/− mice. The study of single or multiple Pdgfknockouts in different genetic backgrounds may therefore add further information about the developmental roles for PDGFs. Moreover, a reduction in PDGF receptor expression levels or signaling may sensitize mice to the loss of single PDGF ligand isoforms. Thus, a way to expose hidden roles of PDGF-C signaling via PDGFRα could be to reduce the level of PDGFRα expression in Pdgfc−/− mice.

Here, we adopted this strategy by generating Pdgfc−/−; PdgfraGFP/+ double mutants. Besides being a Pdgfrα null allele, PdgfraGFP offers the additional benefit of reporting cells that express Pdgfrα through the expression of nuclear GFP. These studies demonstrate that complete loss of PDGF-C together with loss of a single functional copy of Pdgfrα unmask previously unrecognized roles of PDGF-C in the brain and its surrounding meninges, the lungs and the vertebral column. These observations demonstrate that PDGF-C plays a role in lung and vertebral development, similar to what has previously been shown for PDGF-A. However, our analysis also reveals phenotypes not previously observed in PDGF/PDGFR mutants, demonstrating that PDGFRα signaling is required for the establishment of certain CNS compartments. Specifically, we noticed neuronal overmigration in the cerebral cortex. We observed that loss of...
PDGF-C signaling compromises the structure of cerebral meninges, which we hypothesize is the primary cause of the observed brain defects, since intact meninges are considered to be critical for normal CNS development (reviewed by Decimo et al., 2012; Siegenthaler and Pleasure, 2011).

RESULTS

\textit{Pdgfc}^{−/−}; \textit{Pdgfra}^{GFP/+} mice die perinatally

\textit{Pdgfc}^{−/−} mice on a 129S1 background die at birth as a consequence of cleft palate (Ding et al., 2004), whereas they are viable and fertile on C57BL/6J background (Fredriksson et al., 2012). We generated \textit{Pdgfc}^{−/−}; \textit{Pdgfra}^{GFP/+} mice on C57BL/6J background, which were born from crosses of \textit{Pdgfc}^{+/−}; \textit{Pdgfra}^{GFP/+} and \textit{Pdgfc}^{+/−} mice with a mendelian distribution [12% expected, 11.5% (19/165) were obtained]. In total we generated 74 \textit{Pdgfc}^{−/−}; \textit{Pdgfra}^{GFP/+} pups, out of which 47 were observed frequently and regularly until they suddenly died or were deemed necessary to euthanize for ethical reasons. The phenotype severity was variable and disease progression rapid, and although the mice were monitored daily, \textit{Pdgfc}^{−/−}; \textit{Pdgfra}^{GFP/+} mice that appeared vital in the evening were often found dead the following morning. During the first two days 72% of the \textit{Pdgfc}^{−/−}; \textit{Pdgfra}^{GFP/+} mice died or were euthanized, and only 14% were still alive at postnatal day (P)15. Two mice were followed until P21-22 (Fig. 1A).

At birth, \textit{Pdgfc}^{−/−}; \textit{Pdgfra}^{GFP/+} mice were easily distinguishable by the presence of a hemorrhagic stripe along the lower spine (Fig. 1B,C). These lesions were covered by an intact skin and, therefore, classified as spina bifida occulta (reviewed by Greene and Copp, 2009) (Fig. 1D). Indeed, we confirmed the presence of dorsally open vertebral arches in the lumbar region of \textit{Pdgfc}^{−/−}; \textit{Pdgfra}^{GFP/+} mice (Fig. 1E,F). This pronounced spina bifida occulta with associated local hemorrhage was exclusively observed in \textit{Pdgfc}^{−/−}; \textit{Pdgfra}^{GFP/+} mice and absent in all other littermates irrespective of their genotypes.

Similar to \textit{Pdgfa} knockout mice (Boström et al., 1996), \textit{Pdgfc}^{−/−}; \textit{Pdgfra}^{GFP/+} mice displayed an emphysema-like phenotype in the lung (Fig. 1G). The average perimeter of the open airways was increased by 50% ($P=0.0014$) in \textit{Pdgfc}^{−/−}; \textit{Pdgfra}^{GFP/+} lungs, compared to \textit{Pdgfc}^{+/−}; \textit{Pdgfra}^{GFP/+} littermate controls. A similar but less severe phenotype was observed in \textit{Pdgfc}^{−/−} mice in comparison.
with Pdgfc\(^{+/+}\). We did not perform a side-by-side comparison to Pdgfa\(^{-/-}\) mice, but on comparison with data reported earlier (Boström et al., 1996), the alveolar defects in Pdgfc\(^{-/-}\) or Pdgfc\(^{-/-}\); Pdgfra\(^{GFP/+}\) lungs appeared substantially milder than those in Pdgfa\(^{-/-}\) mice.

### Cerebral abnormalities

Dissected brains from newborn Pdgfc\(^{-/-}\); Pdgfra\(^{GFP/+}\) mice were clearly distinguishable from littermate control brains (Fig. 2). The cerebral hemispheres displayed an irregular shape and the cerebellum was reduced in size. To provide at least one quantitative measure of the cerebral abnormality, we assessed the interhemispheric fissure (IHF) and the angle (\(\alpha\)) between then IHF and a line drawn from the frontal end of the IHF and the most lateral point of the cerebral hemisphere in newborn mice (Fig. 2A,B). Both were significantly different in Pdgfc\(^{-/-}\); Pdgfra\(^{GFP/+}\) compared to littermate controls; IHF was 35% increased (\(P<0.0001; \) Fig. 2D) and \(\alpha\) was 8% smaller (\(P<0.01; \) Fig. 2E).

Bleedings were frequently observed in Pdgfc\(^{-/-}\); Pdgfra\(^{GFP/+}\) brains, both superficially (Fig. 2C) and deep in the brain parenchyma (Fig. 3A,B). The extent of the hemorrhage and regions involved varied between individuals. In tissue sections of newborn pups, extravasated erythrocytes were observed in the cerebral cortex, corpus callosum, midbrain, colliculus and cerebellum (Fig. 3A,B, circled areas). Other abnormal phenotypes observed included misplaced neurons (Fig. 3C,D) and folds in the cortical surface (Fig. 3E,F). These defects were focal and locations varied between individuals. However, the gross morphology of the cortical cell layers appeared normal for the most part (Fig. 4). Nissl staining of the cerebral cortex revealed that different cortical cell layers were present, correctly positioned in relation to each other,
During late embryonic development, PDGFC was barely expressed in the forebrain, except in the choroid plexus in the lateral ventricles (Fig. 5A,B). PDGFC expression first appeared in the dorsolateral cerebral cortex (Fig. 5B), and was intensified until P3 (Fig. 5A-E). Histological sections showed that expression was mostly confined to neurons in the cortical plate close to the glial border (Fig. 5F-J). The pattern of expression of PDGF-A was similar to that of PDGF-C (Fig. 5K-O). PDGF-C was not expressed in the skull covering the cerebral cortex at birth (data not shown).

Using PdgfraGFP/+ mice, we mapped the expression pattern of PDGFRα-positive cells in the relevant areas of the cerebrum. The observed pattern is consistent with labeling of cerebral oligodendrocyte progenitors (OPCs) (Pringle et al., 1992). Before and early after birth, the numbers of PDGFRα-positive cells were low in the vicinity of the cortical surface, i.e. the regions where cellular abnormalities were observed, however, suggesting that the observed cerebral defects in Pdgfca−/−; PdgfraGFP/+ mice are not a result of defective signaling in OPCs.

**Abnormal cerebral meninges**

Since PDGFRα expression was high in the cerebral meninges (Fig. 6), we hypothesized that PDGFRα-positive meningeal cells could be target cells for PDGFC-expressed in the developing cerebral cortex. To analyze the meninges, we first peeled off meningeal sheets from the dorsal cerebrum (dotted circle in Fig. 7A), and mounted them on slides for en face morphological analyses. We found that Pdgfca−/−; PdgfraGFP/+ meninges were composed of fewer PDGFRα-GFP-positive cells than controls (Fig. 7C,D). Also, they were thin, fragile and more difficult to detach mechanically compared to meninges from control littersmates. In coronal sections we verified that Pdgfca−/−; PdgfraGFP/+ cerebral meninges were indeed thinner and displayed an irregular pattern of extracellular matrix markers collagen IV, fibronectin and laminin α1 (Fig. 7E-J). These analyses revealed quantitative differences; all tested ECM molecules were present in mutant meninges, albeit in lower amounts.

As the superficial bleedings observed in Pdgfca−/−; PdgfraGFP/+ brains (Fig. 2C, Fig. 7B) were likely located within the meninges, we used the endothelial cell marker (CD31) to visualize vessels in meninges from the dorsal cerebrum. Blood vessels in Pdgfca−/−; PdgfraGFP/+ meninges were disorganized and significantly sparser compared to control mice (Fig. 7K-R). Meningeal cells are known to express PDGFRα (Pringle et al., 1992), but it is unclear which meningeal cell type(s) express it. Both CD31-positive endothelial cells and vascular mural cells, identified by their location between the collagen IV-positive vascular basement membrane and the CD31-positive endothelium were uniformly negative for PDGFRα. We conclude that in meningeal sheets from PdgfraGFP/+ mice, the nuclear GFP expression occurs in non-vascular mesenchymal cells (Fig. 7S-V).

**Expression of Pdgfa and Pdgfc in the developing cerebral cortex**

The cerebral defects in Pdgfca−/−; PdgfraGFP/+ mice imply an important role for Pdgfra signaling in the developing brain. We therefore analyzed the expression of PDGFαRs and its main ligands PDGF-A, PDGF-C in the developing cerebral cortex at time points around birth [embryonic day (E)17.5, E18.5, P0, P1, P3]. As the Pdgfa and Pdgfc null alleles express lacZ from their respective promoters, we performed X-gal staining in heterozygous mice to localize sites of expression (Fig. 5).

**Neuronal gene expression in cerebral meninges**

To gain additional insight into the basis for the morphological abnormalities in the meninges of the Pdgfca−/−; PdgfraGFP/+ mice, we isolated RNA from cerebral meninges of newborn mutants (n=4) and littermate controls (n=9) and analyzed the transcriptome using Affymetrix arrays. This analysis revealed extensive differences in gene expression between the two groups, with numerous genes being consistently up- or downregulated in the mutant meninges (Fig. 8A, www.ncbi.nlm.nih.gov/geo, accession number GSE67644). The most unexpected finding was a highly significant upregulation of neuronal genes in cerebral meninges from Pdgfca−/−; PdgfraGFP/+ mice. Both KEGG and GO analysis confirmed high up-regulation of neuron-associated pathways (Table 1). The data was further compared to a transcriptome database for cell type-enriched genes in the central nervous system (Zhang et al., 2014) (Fig. 8B). We could then confirm that the genes upregulated in...
Pdgfc<sup>−/−</sup>; Pdgfra<sup>GFP/+</sup> meninges contained many representative markers for neurons and oligodendrocytes of different state of differentiation (Fig. 8B). In contrast, markers for vasculature, including endothelial and pericyte markers, were downregulated. The latter observation correlated with the decreased density of vasculature in mutant meninges (Fig. 7O,P). We also noticed upregulation of genes implicated in cortical neuronal migration, out of which some are listed in Fig. 8C. These are genes that, when mutated in mice, cause lissencephaly (Reln, Dcx, Vldlr, Dab1; reviewed by Olson and Walsh, 2002) or other abnormalities related to defective cortical neuronal migration during development; Cspg5 (Zhang et al., 2013), Cxcr4 (reviewed by Tiveron and Cremer, 2008); Pak1 (Pan et al., 2015) and Tbr1 (Hevner et al., 2001). Interestingly, we also noticed a strong decrease in expression of lymphatics markers in Pdgfc<sup>−/−</sup>; Pdgfra<sup>GFP/+</sup> meninges, which might implicate a role for PDGFRα in the development of meningeal lymphatics (Aspelund et al., 2015; Louveau et al., 2015).

**Neuronal overmigration into compact meninges with discontinuous basement membrane coverage at the brain surface**

Although recent data show that a small number of neural precursor cells reside within the meninges (Bifari et al., 2015, 2009), the meninges are normally devoid of mature nervous tissue. However, we hypothesized that the meninges from Pdgfc<sup>−/−</sup>; Pdgfra<sup>GFP/+</sup> mice could be contaminated with neurons as a result of neuronal overmigration from the brain parenchyma. Indeed, staining for
neurofilaments provided overt signs of neuronal overmigration. Neurofilament-positive fibers stretched through the marginal zone into the meninges (Fig. 9A-D). A similar pattern was seen for NeuN-positive neuronal nuclei (Fig. 9G,H), indicating the translocation of entire neuronal cells into the meninges. Like the above-mentioned phenotypes, these signs of neuronal overmigration only appeared as focal lesions with variable locations; in most areas the NeuN-positive cell nuclei resided normally positioned within the cortical plate (Fig. 9E,F).

Neuronal overmigration could explain both why $Pdgfc^{-/-}$; $Pdgfra^{GFP/+}$ meninges appeared more adhesive to the cerebrum and easily broke during dissection, as well as the observation of neuronal genes in the meninges. To further verify the neuronal overmigration, we performed transmission electron microscopy (TEM) analyses on meninges from $Pdgfc^{-/-}$; $Pdgfra^{GFP/+}$ and $Pdgfc^{+/-}$; $Pdgfra^{GFP/+}$ mice. We first made coronal vibratome sections from P1 and P3 brains with the skull still attached (Fig. 10A,B). In these preparations, we observed asymmetric enlargements of the lateral ventricles in $Pdgfc^{-/-}$; $Pdgfra^{GFP/+}$ mice, in agreement with a previously reported defect in $Pdgfc^{-/-}$ mice (Fredriksson et al., 2012). The vibratome sections were trimmed in order to allow ultrathin cross sections of the cerebral meninges. TEM analysis showed that control meninges ($Pdgfc^{+/-}$; $Pdgfra^{GFP/+}$) were regularly formed of loosely packed tissue and

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**Fig. 5. Perinatal expression of PDGF-C and PDGF-A in cerebral cortex.** (A-E) Whole mount X-gal staining of $Pdgfc^{+/-}$ brains, visualizing how PDGF-C expression increases with age. (F-O) Coronal sections of whole mount X-gal stained brains counterstained with Nuclear Fast Red. Illustration in upper right corner shows where the photos are taken, and black dotted lines mark the cortical surface. (F-J) Increasing expression of PDGF-C (blue) close to the cortical border. (K-O) PDGF-A expression (blue) largely overlaps with PDGF-C expression. Scale bar: 1 mm in A-E, 150 µm in F-O.
The most conspicuous specific abnormality of the Pdgfc<sup>−/−</sup>; Pdgfra<sup>GFP/+</sup> meninges was an indistinct, and in some areas totally missing, basement membrane (Fig. 10F). In the latter regions neuronal tissue was observed extending from within the meningo into the meningeal arachnoid layer (Fig. 10G-I). These observations were consistent with neuronal overmigration.

**Involvement of other Pdgf genes**

Pdgfc<sup>−/−</sup> mice develop a mild form of spina bifida (Ding et al., 2004), but homozygous Pdgfc knockout alone was not sufficient to induce the severe form of spina bifida occulta associated with hemorrhage that we observed in Pdgfc<sup>−/−</sup>; Pdgfra<sup>GFP/+</sup> mice (Fig. 1B). As an additional loss of one functional copy of the Pdgfra gene was also required to induce severe spina bifida occulta, we hypothesized that PDGF-C acts in concert with (an) other PDGF ligands in the formation of the vertebral arch. To identify this ligand, we generated Pdgfc<sup>−/−</sup> mice that were also heterozygous for Pdgfa, Pdgfb or Pdgfd. The only genotype that resulted in a spina bifida occulta with hemorrhage was Pdgfa<sup>−/−</sup>; Pdgfd<sup>−/−</sup>, confirming recently published observations (Andrae et al., 2013). Neither Pdgfc<sup>−/−</sup>; Pdgfh<sup>−/−</sup> nor Pdgfc<sup>−/−</sup>; Pdgfd<sup>−/−</sup> showed any similar phenotype (Table 2). Tissue plasminogen activator (tPA) activates the latent PDGF-C protein in vitro (Fredriksson et al., 2004) and acts upstream of PDGF-C/PDGFRα in regulation of blood brain barrier integrity in vivo (Su et al., 2008). To investigate if loss of tPA would phenocopy loss of PDGF-C during vertebral formation, we generated tPa<sup>−/−</sup>; Pdgfra<sup>GFP/+</sup>. Also this combination failed to reproduce spina bifida, suggesting that other protease(s) than tPA can activate PDGF-C during mouse development.

**DISCUSSION**

**Study rationale**

Analysis of Pdgfc<sup>−/−</sup> mice has already demonstrated that PDGF-C play roles in palate closure and the formation of CNS ventricles (Ding et al., 2004; Fredriksson et al., 2012). However, redundancy between PDGF-C and other PDGFs that signal via PDGFRα may hide other functions. We reasoned that the deletion of one copy of the Pdgfra gene, which alone has no known phenotypic consequences, could synergize with Pdgfc deficiency and unmask hitherto hidden functions. The Pdgfra<sup>GFP</sup> knock-in allele (Hamilton et al., 2003) is a null allele that expresses GFP from the endogenous Pdgfra promoter. Homozygous Pdgfra<sup>GFP/GFP</sup> mice are embryonically lethal like Pdgfra<sup>−/−</sup> mice (Soriano, 1997). We found that Pdgfc<sup>−/−</sup>; Pdgfra<sup>GFP/+</sup> mice develop multiple abnormalities, some of which confirmed previously reported phenotypes, whereas others were novel and surprising. Both PDGF-C and its receptor PDGFRα are broadly expressed in developing and adult mammals (Aase et al., 2002; Orr-Urtreger and Lonai, 1992). A complex and lethal phenotype was therefore expected, but the range of observed defects (e.g. in brain, spine, lung and vasculature) made it difficult to pinpoint a single cause of death. Phenotype severity and age of lethality was also variable despite a homogenous C57BL/6J background.

**Differential importance of PDGF-C and PDGF-A in vertebral, lung and oligodendrocyte development**

Some of the phenotypes observed in Pdgfc<sup>−/−</sup>; Pdgfra<sup>GFP/+</sup> mice confirm previous observations. One of them, spina bifida occulta, implies that the vertebral arches are not properly closed. Spina bifida is a common developmental defect in humans with numerous underlying causes, as well as a diverse range of consequences,
Vertebral arches are normally formed from the axial mesoderm that migrates dorsally to cover the closed neural tube. In open spina bifida there is also a problem with the closure of the neural tube itself; as a result, the axial mesoderm cannot cover the unclosed area leading to defective formation of the vertebral arches. In this case, the neural tube is either left openly exposed or covered by meninges. Spina bifida occulta (hidden spina bifida) is a milder variant where the problem resides in the axial mesoderm itself. The vertebral arches form abnormally also in this case, but the lesion is covered with skin and sometimes undetectable in mice without invasive examination (i.e. dissection). Severe spina bifida occulta has previously been observed in Pdgfa null and signaling-deficient mouse mutants (Hamilton et al., 2003; Klinghoffer et al., 2002; Soriano, 1997) and in Pdgfa/Pdgfc double knockout mutants (Ding et al., 2004). Spina bifida occulta with variable severity and penetrance has also been observed in Pdgfc−/−, PdgfaΔex6/Δex6, and Pdgfc−/−; Pdgfa+/− mice (Andrae et al., 2013; Ding et al., 2004), but not in single Pdgfa knockouts. These results suggest that PDGF-C plays a critical role in vertebral development that is overlapping and partially redundant with PDGF-A. The severe and fully penetrant spina bifida occulta observed here in Pdgfc−/−; PdgfaGFP+/+ mice suggest that this overlapping function is mediated by PDGFRα.

Fig. 7. Structural changes in cerebral meninges of Pdgfc−/−; PdgfaGFP+/+ mice. (A) Dorsal view of P1 brain from PdgfaGFP+/+ mouse. Dotted circle indicates area for dissection of meninges. (B) Irregular shape and bleedings in brain from P1 Pdgfc−/−; PdgfaGFP+/+ mouse. (C) Meninges peeled off from the dorsal, cerebral hemisphere of newborn PdgfaGFP+/+ mouse, dotted circle in A. (D) Reduced numbers of PDGFRα-positive cells in Pdgfc−/−; PdgfaGFP+/+ meninges. (E-J) Immunofluorescent staining for extracellular matrix proteins Collagen IV (E,F), Fibronectin (G,H) and Laminin α1 (I,J) in coronal sections through the cerebral cortex in P2 pups show an irregular structure of the meninges in Pdgfc−/−; PdgfaGFP+/+ mice. (K-R) Extended view of confocal z-stack of P4 meninges, PdgfaGFP+/+ (left column) and Pdgfc−/−; PdgfaGFP+/+ (right column), (K,L) DAPI, (M,N) Fewer PDGFRα-GFP-positive cells in mutants. (O,P) CD31 reveal variations in the vascular network. (Q,R) Merged view. (S-V) Pdgfrα expression in non-vascular meningeal cells. Cerebral meninges from PdgfaGFP+/+ brain immunostained for CD31 (red) and collagen IV (white). Arrow points at a PDGFRα negative vascular cell. Scale bars: 50 µm in E-J, 40 µm in K-R.
Whereas PDGF-C seems to be the most important PDGFRα ligand in vertebral development, PDGF-A appears to be the most important PDGFRα ligand in lung development. A severe lung emphysema-like phenotype has been reported in Pdgfa−/− mice (Boström et al., 1996; Lindahl et al., 1997) and a similar but milder lung phenotype was observed in PdgfaΔex6/−; PdgfraGFP/+/ mice (Andrae et al., 2013). The emphysema-like phenotype observed here in Pdgfc−/−; PdgfraGFP+/+ mice, shows that PDGF-C indeed plays a role in lung development, but PDGF-C is clearly less important in this organ compared to PDGF-A.

The numbers of PDGFRα-positive oligodendrocyte precursors in the spinal cord depend directly on the concentration of PDGF-A, and Pdgfa−/− mice develop oligodendrocyte hypoplasia and hypomyelination (Andrae et al., 2013; Calver et al., 1998; Fruttiger et al., 1999). Loss of PDGFRα-positive oligodendrocyte progenitors has also been observed in Pdgfra null and signaling deficient mice (Calver et al., 1998; Fruttiger et al., 1999; Klinghoffer et al., 2002). PDGF-C is expressed in the developing CNS, and could therefore be partially redundant with PDGF-A in oligodendrocyte development. However, when we performed a quantitative analysis of PdgfraGFP expressing cells in the E15.5...
spinal cord of Pdgfc−/−; PdgfraGFP/+ and PdgfraGFP/+ embryos, there was no correlation between PDGF-C expression and the number of PDGFRα-positive cells (data not shown). These results suggest that PDGF-C plays no or only a minor role in oligodendrocyte development.

**Neuronal overmigration**

Pdgfc−/−; PdgfraGFP/+ mice displayed neuronal overmigration, verified by immunofluorescent staining for neuronal markers, transmission electron microscopy and transcriptional profiling of meningeal tissues. The results from the microarray analyses were highly consistent, showing multiple neuron-associated genes being upregulated in Pdgfc−/−; PdgfraGFP/+ meninges. Recent publications have suggested that meninges harbor a set of neural precursor cells (Bifari et al., 2009, 2015), expressing, for example, doublecortin (Dcx). Dcx was upregulated also in Pdgfc−/−; PdgfraGFP/+ meninges, which may suggest an increase in neural precursor cells, but the upregulated genes in the Pdgfc−/−; PdgfraGFP/+ meninges included multiple genes normally expressed by mature neurons, such as Tubb3 (Tuj1), which is not expressed by meningeal neural precursor cells (Bifari et al., 2015).

Based on the pattern of expression of PDGF-C and PDGFRα in the developing brain and associated meninges, we suggest that PDGF-C, expressed by neurons in the neocortex, signal to PDGFRα-positive cells in the meninges (Fig. 11). The abnormalities and regional loss of basement membrane integrity at the brain surface offers a plausible explanation for the observed neuronal overmigration in Pdgfc−/−; PdgfraGFP/+ mice. Previous work demonstrates that changes in meningeal gene expression patterns may result in brain abnormalities. Neuronal migration and interaction with the meninges are important for correct cerebral cortex development, and different signaling pathways are involved. Defective CXCL12/CXCR4 signaling (Borrell and Marin, 2006) and hypomorphic Foxc1 (Zarbalis et al., 2012) generate defective forebrain meningeal formation, which, in turn, impairs tangential migration of cortical interneurons and Cajal Retzius cells. The Pdgfc−/−; PdgfraGFP/+ mice and Foxc1 hypomorphic mice (Zarbalis et al., 2007) share phenotypic abnormalities, including defective meninges and neuronal overmigration. The major cortical dysplasias in Foxc1 mice develop the first week after birth, which could not be studied in Pdgfc−/−; PdgfraGFP/+ mice because the most severe cases died perinatally. Interestingly, Pdgfra signaling was recently placed downstream of Foxc1 in zebrafish development (French et al., 2014).

**A primary defect in the meninges**

During CNS development, PDGFRα expression is mainly restricted to glial cells, starting at E12.5 in oligodendrocyte precursors in the ventral spinal cord (Pringle and Richardson, 1993). There are also reports on PDGFRα expression in early neuroepithelial cells and postnatal cerebellar neurons (Andrae et al., 2001; Nait Oumesmar et al., 1997). In the meninges surrounding the brain, we found that PDGFRα expression was abundant at all analyzed stages. The exact identity of the PDGFRα-positive cells in meninges is not known. Generally, PDGFRα expression in different organs is localized to fibroblasts and other types of mesenchymal cells. Here, we confirm PDGFRα expression in meningeal cells that were not vascular-associated, but likely of mesenchymal origin. Yet, additional analyses are needed to assign the PDGFRα-positive cells to any (or several) specific cell type or category. The structural and cellular

**Table 1. Significantly changed KEGG and GO pathways in Pdgfc−/−; PdgfraGFP/+ cerebral meninges, from microarray data**

| KEGG pathways                                         | Count | Fold enrichment | P-value       |
|-------------------------------------------------------|-------|-----------------|---------------|
| Neuroactive ligand-receptor interaction                | 37    | 2.85            | 1.27×10⁻⁸     |
| Axon guidance                                          | 20    | 3.04            | 2.26×10⁻⁵     |
| Cell adhesion molecules                                | 20    | 2.71            | 1.14×10⁻⁴     |
| MAPK signaling pathways                                | 27    | 2.04            | 6.23×10⁻⁴     |
| GO pathways (top four)                                 |       |                 |               |
| Synaptic transmission                                  | 49    | 5.24            | 7.77×10⁻²²     |
| Neuron projection development                          | 54    | 4.73            | 9.09×10⁻²²     |
| Transmission of nerve impulse                          | 54    | 4.54            | 6.85×10⁻²¹     |
| Cell-cell signaling                                    | 59    | 3.87            | 3.26×10⁻₁⁹     |

*Number of differentially expressed genes

![Image of neuronal overmigration](image-url)
composition of the murine meninges is not well elucidated. Most histological analyses on meninges have been done on human material. Grossly, three different layers make up the meninges: (1) the innermost pia mater, a fibrous layer of flat cells separated from the brain by a basement membrane, (2) the arachnoid, a collagen-rich layer that partly forms a trabecular network containing blood vessels and (3) the outermost dura mater, a harder layer of thick connective tissue attached to the skull (Haines et al., 1993). The pia, arachnoid and the intervening sub-arachnoid space containing blood vessels are often referred to as the leptomeninges. Meninges form early in development, in mice already at E9-10 (reviewed by Siegenthaler and Pleasure, 2011).

It is becoming increasingly evident that the meninges and their basement membranes play a crucial role in the patterning of the CNS. Work by Sievers and colleagues suggest that an impaired meningeal layer affects cerebellar foliation (Sievers et al., 1981). Further work suggested that fibroblast-like meningeal cells deposit extracellular matrix proteins involved in the formation of the glia limitans (Sievers et al., 1994). Additional reports describe molecules and signaling pathways that influence formation of the meningeal basement membrane, the disturbance of which negatively influences both cerebellar and cerebral patterning. For example, mice lacking β1 integrin in neuronal- and glial precursor cells die prematurely with defective basement membrane and glial end-feet formation, irregular cerebral cortical lamination, neuronal overmigration, smaller cerebellar folia and distorted laminar organization (Graus-Porta et al., 2001). This study suggested that the neuron/glia interaction with the meningeal basement membrane...
Intrinsic meningeal defects may also explain the vascular abnormalities observed in Pdgfc−/−; PdgfraGFP/+ mice, with superficial as well as intracranial bleedings. Although PDGFRα-positive meningeal cells were not identified as endothelial or mural cells, the meningeal vessels in mutants were uneven and irregular, and the expression of vascular-associated genes was reduced in Pdgfc−/−; PdgfraGFP/+ meninges. These results confirm and extend previously described effects of PDGF-C on vascular development and revascularization (Fredriksson et al., 2012; Moriya et al., 2014). Recently, lymphatics were identified in murine meninges (Aspelund et al., 2015; Louveau et al., 2015). We found that both Lyve1 and VEGFR3 were downregulated in Pdgfc−/−; PdgfraGFP/+ meninges possibly implicating that meningeal lymphatics may be disturbed in these mice. This issue will need further investigation.

The structure and formation of the murine meninges is a relatively unexplored area, and there are many interesting paths to elucidate further. Here, we show that signaling of PDGF-C is necessary to form an intact meningeal layer around the mouse cerebrum, and we propose that this leads to secondary developmental brain defects. What kind of cells that express PDGFRα, their cooperation with other cells and contribution to the extra cellular matrix to meningeal basement membrane assembly are questions that warrant further study.

**Tissue processing**

Embryos and newborn mice were sacrificed by decapitation. Dissected organs were washed in PBS and fixed in 4% paraformaldehyde (PFA) at +4°C overnight. Mice older than one week were perfused through the heart with Hanks’ balanced salt solution (HBSS) and 4% PFA before dissection, and postfixation overnight. Fixed organs were either; dehydrated and embedded in paraﬁn, vibratome sectioned, or soaked in 30% sucrose and embedded in OCT for cryosectioning. Spines for skeletal preparation were embedded in paraffin, vibratome sectioned, or soaked in 30% sucrose and embedded in OCT for cryosectioning. Spines for skeletal preparation were stained with Alizarin Red S, X-gal and immunofluorescence staining for murine skeletal preparations, X-gal and immunofluorescence staining

**Skeletal preparations, X-gal and immunofluorescence staining**

Skeletal preparations of cartilage and bone were stained with Alizarin Red and Alcian Blue, (according to protocol in Behringer et al., 2014). X-gal staining on freely dissected whole brains has been described before (Andrae et al., 2014). Paraffin-embedded tissue was used to obtain a good morphology of X-gal stained tissues. Sections of 7 µm were counterstained with Nuclear Fast Red (N3020, Sigma Aldrich). For immunofluorescence staining, vibratome or cryosections were used, to better preserve the GFP expression. Sections were blocked in 1% BSA/0.5% Triton X-100 in PBS at +4°C overnight, incubated with a primary antibody (1:100) in 0.5% BSA/0.25% Triton X-100 in PBS at +4°C overnight, washed in PBS, incubated with a secondary antibody at +4°C overnight, and washed in PBS before imaging.
washed in PBS and mounted in ProLongGold with DAPI (Invitrogen). Antibodies: rabbit-anti-mouse-collagen IV (AbD Serotec, 2150-1470); rat-anti-mouse-CD31 (Pharmingen, 553730); rabbit-anti-fibronectin (Sigma, F3648); rabbit-anti-laminin α1 (#317, kind gift from Prof. Lydia Sorokin, Münster, Germany); mouse-anti-NeuN (Chemicon, Mab377).

**Gene expression profiling by microarray**

Cerebral meninges covering the brains of newborn pups were freely dissected and stored in RNAlater® (Ambion). RNA was isolated using RNeasy mini kit (Qiagen) and quality checked in a 2100 BioAnalyzer (Agilent Technologies, Santa Clara, CA, USA). The Bioinformatics and Expression analysis core facility, Karolinska Institute, Sweden (http://agt.bez.ki.se) performed transcription profiling with Gene Chip Mouse Gene 1.0ST array. Affymetrix raw data was normalized using PLIER algorithms (Affymetrix Technical Note, Guide to Probe Logarithmic Intensity Error Estimation, http://affymetrix.com/support/technical/technotes/main.affx). We compared Pdgf−/−; PdgfraGFP+ mice with a control group consisting of all other littermates (Pdgf−/−, Pdgf−/−, Pdgf−/−; Pdgf−/−, Pdgf−/−; PdgfraGFP+ and Pdgf−/−; PdgfraGFP+). One litter was used, including four mutants and nine controls. To select significantly differentially expressed genes in mutant mice we set the cut-off for expression fold change >2-fold and t-test P-value <0.05. The entire microarray data set has been deposited in the NCBI Gene Expression Omnibus database (www.ncbi.nlm.nih.gov/geo/, accession number GSE6764).

**Electron microscopy**

P1 and P3 pups were anaesthetized and perfused through the heart with HBSS and EM-fix (2% paraformaldehyde, 2.5% glutaraldehyde, 0.02% sodium azide in PBS). The heads were cut off, the lower jaw and all skin removed, and the brains (still inside the skull) were postfixed in EM-fix overnight at +4°C. Coronal vibratome sections (100 µm) were cut through cerebrum and skull bone, to obtain intact meninges. Photographs of mutant brain slices were analyzed to identify areas of irregular meninges, which were used for the TEM analyses. Corresponding areas in non-mutant brains served as controls. Slices of brains with coverings were post-fixed for 2 h with 1% osmium tetroxide +1% potassium hexacyano-ferrate in 0.1 M sodium cacodylate buffer, pH 7.2. After en bloc contrasting with 0.5% uranyl acetate in water for 1 h specimens were dehydrated in ethanol to acetone and were infiltrated with epoxy resin (Agar 100, London Resins Co.). They were then embedded between Aclar films and cured by heat. The previously selected surface regions were cut in a Leica Ultracut UC6 ultramicrotome (Leica Microsystems, Vienna, Austria) fitted with diamond knives at a section thickness setting of 50-60 nm. Sections collected on copper grids were counterstained with uranyl acetate and lead citrate before examination in a LEO 912AB transmission electron microscope. Digital image files were obtained with a Veleta 2×2 k CCD camera (Olympus-Soft Imaging Solutions, Münster, Germany) using the iTEM software (Olympus-SIS).

**Quantification of lung alveolar density**

Paraffin sections of lungs from 5-6 Pdgf−/−; PdgfraGFP+ and Pdgf−/− littermate controls (P15-P19), and from 7-7 Pdgf−/− and Pdgf−/− littermate controls (P19-P24) were counter stained with heparin-oxylin/eosin. Bright field images from three different areas of each lung were taken in a Nikon Eclipse E800 microscope at 20× magnification. For each lung, the areas with most sparse alveolar network were chosen. The number of open areas and their perimeter was quantified using the NIS-Elements BR2.3 program. Statistics were calculated with a paired Student’s t-test.

**Quantification of measurements in brain**

Measurements were taken from whole mount photos of dissected, fixed brains from 9 Pdgf−/−; PdgfraGFP+ and 14 littermate controls. The interhemispheric fissure (IHf) was defined as the midline distance where the two cerebral hemispheres were in contact with each other. To generate the angle (α) we drew a line from the frontal end of the IHf to the most lateral point of the brain. Statistics were calculated with an un-paired t-test.

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**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**

J.A. and C.B. designed the experiments, analyzed the data and wrote the manuscript. J.A., L.G. and R.G. performed the experiments. B.R.J. performed and analyzed TEM data and wrote corresponding sections in the manuscript. L.H. analyzed microarray data and performed bioinformatics analyses. L.F., I.N. and U.E. contributed with materials and assisted in writing the manuscript.

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**Data availability**

The Affymetrix microarray data set for transcriptome analysis of gene expression in cerebral meninges is available at www.ncbi.nlm.nih.gov/geo/, accession number GSE67644.

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