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Platelet-Derived Growth Factor C Deficiency in C57BL/6 Mice Leads to Abnormal Cerebral Vascularization, Loss of Neuroependymal Integrity, and Ventricular Abnormalities

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Platelet-derived growth factors (PDGFs) and their tyrosine kinase receptors (PDGFRs) are known to play important roles during development of the lungs, central nervous system (CNS), and skeleton and in several diseases. PDGF-C is a ligand for the tyrosine kinase receptor PDGFRα. Mutations in the gene encoding PDGF-C have been linked to clefts of the lip and/or palate in humans, and ablation of PDGF-C in 129/Sv background mice results in death during the perinatal period. In this study, we report that ablation of PDGF-C in C57BL/6 mice results in a milder phenotype than in 129/Sv mice, and we present a phenotypic characterization of PDGF-C deficiency in the adult murine CNS. Multiple congenital defects were observed in the CNS of PDGF-C–null C57BL/6 mice, including cerebral vascular abnormalities with abnormal vascular smooth muscle cell coverage. *In vivo* imaging of mice deficient in PDGF-C also revealed cerebral ventricular abnormalities, such as asymmetry of the lateral ventricles and hypoplasia of the septum, reminiscent of cavum septum pellucidum in humans. We further noted that PDGF-C–deficient mice displayed a distorted ependymal lining of the lateral ventricles, and we found evidence of misplaced neurons in the ventricular lining. We conclude that PDGF-C plays a critical role in the development of normal cerebral ventricles and neuroependymal integrity as well as in normal cerebral vascularization. *(Am J Pathol 2012, 180:1136–1144; DOI: 10.1016/j.ajpath.2011.12.006)*

Platelet-derived growth factors (PDGFs) and their tyrosine kinase receptors (PDGFRs) are well known to play crucial roles during development and in several diseases.[1] Currently, there are four known PDGFs, PDGF-A through PDGF-D, that signal via two PDGFRs, PDGFRα and PDGFRβ. PDGFRβ signaling has been shown to primarily direct the development of vascular mural cells, whereas PDGFRα signaling has been implicated in numerous developmental processes, including gonad, lung, central nervous system (CNS), and skeletal development.

PDGF-C is secreted as a latent disulphide-linked homodimer (PDGF-CC) that requires proteolytic activation to induce signaling via PDGFRα.[2] We previously showed that tissue plasminogen activator can activate PDGF-CC[3] and that activation in the neurovascular unit leads to increased vascular permeability.[4] PDGF-C/PDGFRα signaling was found to be involved in the loss of blood-brain barrier function during stroke, and pharmacologic inhibi-

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tion of PDGF signaling with the receptor antagonist imatinib or with neutralizing antibodies to PDGF-C improved blood-brain barrier integrity.[4] However, further dissection of this novel role for PDGF signaling using PDGF-C– or PDGFRα–deficient mice was not feasible since PDGFRα and PDGF-C ablation in mice was reported to result in embryonic or perinatal lethality.[5,6] However, the severity of PDGF-C ablation seems to depend on the genetic background of the mice.[7]

In this study, we demonstrate that PDGF-C deficiency on a C57BL/6 background is largely compatible with postnatal life, thus offering the opportunity to study the physiologic and pathophysiologic roles of this protein in the adult mouse. However, these PDGF-C–deficient mutants present numerous congenital defects, including cerebral ventricular malformations with a distorted ependymal lining and abnormal cerebral vascularization. The ventricular defects associated with PDGF-C ablation, such as asymmetry of the lateral ventricles and hypoplastic development of the septum separating the lateral ventricles, were unexpected since this has not been linked to PDGFRα signaling previously.[1] It is likely that these cerebral ventricular abnormalities are masked by the more severe phenotype reported for PDGF-C ablation in 129/Sv background mice and by the embryonic lethality associated with PDGFRα deficiency.[5,6] These findings are of interest because the molecular mechanisms underlying brain ventricle formation remain poorly understood.[8] Taken together, the data presented herein will aid in our understanding of the role of PDGF-C during CNS development, and the description of viable PDGF-C–deficient mice presents an excellent genetic tool for the study of modifier genes of PDGF signaling in mice and for the potential to study the role of PDGF-C in adult mice.

Materials and Methods

Mouse Strains

The generation of PDGF-C–deficient mice has been described previously.[6] These mice were backcrossed onto a C57BL/6J genetic background. For localization of PDGFRα, we used reporter mice heterozygous for green fluorescent protein (GFP) expression in the nucleus of PDGFRα-positive cells (*Pdgfra:GFP*).[9] All mice used were age- and sex-matched littermate controls from heterozygous breedings. The animal experiments were approved by the local committees for animal experiments at the University of Michigan, Ann Arbor, Michigan, and the Karolinska Institutet, Stockholm, Sweden.

Tissue Preparation, Histologic Analysis, and Immunostaining

Tissue preparation for sectioning and immunostaining was conducted using standard protocols. All images were captured using a Nikon Eclipse TE2000 inverted microscope (Nikon Instruments Inc., Melville, NY) or, where stated, a Zeiss LSM 510 confocal microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany) and are representative of the respective stainings. Mice were anesthetized with chloral hydrate (450 mg/kg i.p.). After transcardial perfusion fixation with 4% paraformaldehyde in PBS, the brains were removed. For paraffin embedding, the brains were postfixed in 4% paraformaldehyde at 4°C overnight, and then were processed for paraffin embedding and sectioning using standard protocols. For frozen and sliding microtome sections, brains were postfixed in 4% paraformaldehyde for 1 hour at room temperature and then were kept in 30% sucrose at 4°C overnight.

Paraffin-embedded sections (6 μm) were rehydrated, followed by quenching of endogenous peroxidase activity using 3% H_2O_2 for 30 minutes at room temperature and blocking of nonspecific binding in TNB blocking buffer for 30 minutes at room temperature [TSA biotin system (NEL700A001KT); Perkin Elmer, Waltham, MA]. The primary antibodies used were affinity-purified rabbit antibodies directed against human PDGF-C (10 μg/mL; in-house #615, prepared as reported by Li et al[2]). Biotin conjugate secondary antibodies (dilution 1:200) (BA-1000; Vector Laboratories, Burlingame, CA) were applied and signal amplified using the Vectastain ABC system (PK-4001; Vector Laboratories) according to the manufacturer's instructions. The signal was developed using SIGMA*FAST* 3,3′-diaminobenzidine (D4418; Sigma-Aldrich, St. Louis, MO). As negative controls, the sections were incubated with secondary Ig or preimmune rabbit IgG only, and in both cases, only background staining was observed (data not shown).

For immunofluorescence, frozen sections (14 μm) were air-dried, whereas sliding microtome sections (30 μm) were stained free floating in 24-well plates before being mounted on Superfrost Plus slides (Thermo Fisher Scientific Inc., Waltham, MA). Sections were fixed in 4% paraformaldehyde for 10 minutes at room temperature and were permeabilized in 0.5% Triton X-100 (Roche Diagnostics GmbH, Mannheim, Germany) in PBS for 20 to 60 minutes at room temperature (depending on section thickness). The primary antibodies used were podocalyxin (1:100) (AF1556; R&D Systems, Minneapolis, MN), α-smooth muscle actin–Cy3 (1:300) (C6198; Sigma-Aldrich), glial fibrillary acidic protein (GFAP; 1:1000) (Z0334; Dako Denmark A/S, Glostrup, Denmark), S100B (rabbit polyclonal, 1:100) (Z0311, antigen retrieval was performed by boiling 20 minutes in retrieval solution, S1700, Dako), NeuN (1:500) (MAB377; Millipore, Billerica, MA), Alexa Fluor 555 phalloidin (1:50) (A34055; Invitrogen, Carlsbad, CA), glucose transporter 1 (GLUT1; 1:100) (07–1401; Millipore), CDP (CUX1; 1:10) (sc-6327; Santa Cruz Biotechnology, Santa Cruz, CA), Ctip2 (1:500) (ab18465; Abcam Plc, Cambridge, UK), NG2 (1:200) (AB5320; Millipore), and Olig2 (1:100) (MABN50; Millipore). Appropriate Alexa Fluor–conjugated secondary antibodies (Invitrogen) were used, and the sections were mounted using media containing DAPI (Vector Laboratories). In all the immunofluorescence stainings, blocking of nonspecific binding was performed using TNB blocking buffer [TSA biotin system (NEL700A001KT); Perkin Elmer]. H&E staining was performed according to standard protocols.

5-Bromo-4-Chloro-3-Indolyl-β-D-Galactoside Analysis of PDGF-C Reporter Expression

Sliding microtome sections (free floating) or 1-mm brain slices were permeabilized with 2 mmol/L $MgCl_2$, 0.01% sodium deoxycholate, and 0.02% Nonidet P-40 in PBS for 30 minutes at room temperature. *LacZ* reporter gene expression was visualized by staining with 5 mmol/L potassium ferricyanide, 5 mmol/L potassium ferrocyanide, and 1 mg/mL of 5-bromo-4-chloro-3-indolyl-β-D-galactoside in permeabilization solution at 37°C overnight in the dark.

Magnetic Resonance Imaging

All magnetic resonance imaging (MRI) was conducted at the Center for Molecular Imaging, Department of Radiology, University of Michigan. During the imaging procedure, the mice were anesthetized with isoflurane, and the body temperature was maintained using forced heated air. MRI was performed using a 7.0T Varian MRI scanner (183-mm horizontal bore; Varian Medical Systems Inc., Palo Alto, CA). A double-tuned volume radiofrequency coil was used to scan the head region of the mice. Coronal T2-weighted images were acquired using a fast spin-echo sequence with the following parameters: repetition time/effective echo time, 4000/60 ms; echo spacing, 15 ms; number of echoes, 8; field of view, 20 × 20 mm; matrix, 256 × 128; slice thickness, 0.5 mm; number of slices, 25; and number of scans, 1 (total scan time, ~2 minutes). The Matlab development environment (The MathWorks Inc., Natick, MA) was used to assess differences in ventricular volume, and the analysis was conducted blinded.

Statistical Analysis

All quantifications were made using ImageJ if not stated otherwise. All measurements are depicted as mean ± SEM from the number of observations stated in the figure legends. The individual observations are based on analysis of two to five fields of vision from comparable anatomical positions. For analysis of the Kaplan-Meier survival plot, a log-rank test was used; for all other analyses, a Wilcoxon two-sample test was used, with statistical significance defined as $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, and $^{***}P \leq 0.001$.

Results

Expression of PDGF-C in the Murine Brain

Because our previous findings indicated that PDGF-C/PDGFRα signaling was involved in the regulation of blood-brain barrier function, we were interested in obtaining a better understanding of the role of this signaling cascade in the adult murine CNS. For our initial analysis, we first mapped the expression pattern of PDGF-C in adult brain by immunohistochemical staining. These studies revealed high expression of PDGF-C protein in wild-type adult murine brain, especially in limbic structures, such as the septal nucleus (septum) separating the lateral ventricles, cerebral cortex, hippocampus, and amygdala (Figure 1A). This expression pattern was largely confirmed in *Pdgfc*$^{+/-}$ and *Pdgfc*$^{-/-}$ C57BL/6 mice by expression of the *lacZ* reporter targeted to the *Pdgfc* locus (Figure 1B).[6]

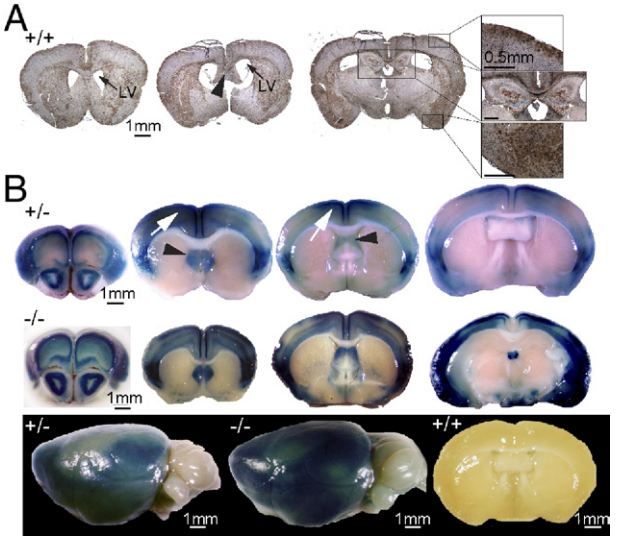

Figure 1. Expression of PDGF-C in the murine brain. **A:** Immunostainings for PDGF-CC protein in the adult murine brain ($n = 4$). High immunoreactivity was seen in limbic areas such as the cerebral cortex (**right panel, top box**), hippocampus (**right panel, middle box**), and amygdala (**right panel, lower box**) of wild-type mice (boxed areas enlarged). High immunoreactivity was also detected in the septum (**arrowhead**, **middle panel**), a limbic region that separates the lateral ventricles (LVs). **B:** *LacZ* reporter gene expression analysis in 1-mm-thick sections of adult *Pdgfc*$^{+/-}$ and *Pdgfc*$^{-/-}$ brains ($n = 5$). 5-Bromo-4-chloro-3-indolyl-β-D-galactoside staining (blue) represents *Pdgfc* gene activity. High *Pdgfc* gene activity was recorded in limbic areas such as the cerebral cortex (**arrows**) and in the septum (**arrowheads**). Wild-type brains were used as negative controls ($n = 4$).

Postnatal Survival of PDGF-C–Deficient Mice on a C57BL/6 Background

It has previously been reported that PDGF-C–deficient mice on a 129/Sv background (*Pdgfc*:129/Sv) all die before weaning, possibly due to respiratory and feeding problems caused by a complete cleft of the secondary palate.[6] When backcrossed onto a C57BL/6 background, *Pdgfc* ablation (herein referred to as *Pdgfc*$^{-/-}$ mice) resulted in a much less severe phenotype, thus opening the possibility of studies in adult PDGF-C–deficient mice. We found that a large proportion, ~20% (expected, 25%), of the *Pdgfc*$^{-/-}$ mice survived the perinatal period (Figure 2A). However, we noted a sex-specific survival where significantly fewer *Pdgfc*$^{-/-}$ males, ~8.5% (expected, 12.5%), were viable at weaning. Some spontaneous deaths were also recorded in the *Pdgfc*$^{-/-}$ mice after weaning (three females and two males; Figure 2B). Postnatal surviving *Pdgfc*$^{-/-}$ mice, both females and males, looked normal overall except for being ~20% smaller than wild-type controls (Figure 2C). This runted phenotype was recorded at all ages checked (4 to 15 months). *Pdgfc*$^{-/-}$ mice also presented with spina bifida occulta, an incomplete closure of one or a few vertebrae in the spinal column (data not shown), a skeletal defect previously observed in *Pdgfc*:129/Sv–deficient mice.[6] In addi-

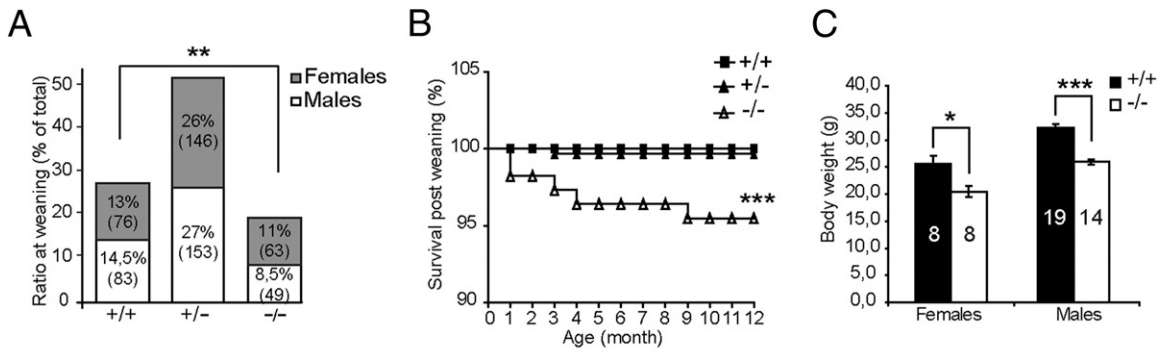

Figure 2. Postnatal survival and weight of *Pdgfc*$^{-/-}$ mice. **A:** Distribution of genotypes at weaning from heterozygous breedings (n = 78 litters). Significantly fewer *Pdgfc*$^{-/-}$ mice were alive at weaning owing to a loss of *Pdgfc*$^{-/-}$ males. **B:** Kaplan-Meier survival plot. Significantly more spontaneous deaths were recorded for *Pdgfc*$^{-/-}$ mice after weaning than for wild-type littermate controls (log-rank test). **C:** Weight of *Pdgfc*$^{-/-}$ and wild-type littermate controls at 7 months of age. *Pdgfc*$^{-/-}$ mice were significantly smaller than their wild-type littermate controls at all recorded ages (4 to 15 months). Numbers of animals are displayed on the respective bars. *$P \leq 0.05$, **$P \leq 0.01$, and ***$P \leq 0.001$. Error bars represent SEM.

tion, *Pdgfc*$^{-/-}$ mice were poor breeders and produced fewer and smaller litters (data not shown).

Abnormal Vascular Bed in PDGF-C–Deficient Murine Brain

Pdgfc: 129/Sv-deficient mice were reported to experience subcutaneous edema formation and hemorrhage of extracranial vessels, which are signs of vascular defects and abnormal vascular permeability.[6] Immunofluorescent stainings using podocalyxin antibodies, a marker for vascular endothelial cells, and α-smooth muscle actin antibodies, a marker for vascular smooth muscle cells (vSMCs), also revealed abnormal vascularization in the brains of adult *Pdgfc*$^{-/-}$ mice compared with wild-type controls (Figure 3, A–D). In wild-type brains, the vascular bed appeared uniform, with equally sized vessels evenly distributed throughout the entire area analyzed, whereas in *Pdgfc*$^{-/-}$ brains, the vascular bed was more variable, with an apparent increase in larger-diameter vessels relative to wild-type mice. This was confirmed by quantification of the podocalyxin stainings, showing a significant overall increase in vessel density (Figure 3E) and diameter (Figure 3F) in *Pdgfc*$^{-/-}$ mice compared with wild-type littermate controls. Staining and quantification with CD31 antibodies (platelet-endothelial cell adhesion molecule 1) (data not shown) achieved similar results. This is consistent with previous studies of murine tumor models illustrating that overexpression of PDGF-C causes a decrease in vessel diameter,[10,11] whereas down-regulation of PDGF-C expression causes a shift to larger-diameter vessels compared with controls.[11] Quantification of the α-smooth muscle actin–positive staining in *Pdgfc*$^{-/-}$ mice demonstrated significantly more vSMC coverage compared with wild-type controls (Figure 3G). This finding was unexpected as increased vSMC coverage is suggestive of a more mature vascular network. However, the vSMCs did not seem to show normal organization around the vessels in *Pdgfc*$^{-/-}$ mice; instead of forming a tight and uniform superficial layer around the endothelium as seen in wild-type mice, the vSMCs in *Pdgfc*$^{-/-}$ mice were partially dissociated from the abluminal endothelial cell surface (Figure 3, C and D). In contrast, the microvascular capillaries in the adult murine brain of *Pdgfc*$^{-/-}$ mice seemed to have normal coverage of pericytes as visualized by immunostainings with PDGFRβ and CD13 antibodies (data not shown).

Asymmetry of the Cerebral Lateral Ventricles in PDGF-C–Deficient Mice

During the characterization of *Pdgfc*$^{-/-}$ mice, we noted that these mice presented with asymmetrical lateral ventricles. Histologic analysis of *Pdgfc*$^{-/-}$ brains by H&E staining revealed that the asymmetrical lateral ventricles seemed to coincide with displacement of the septum toward the side of the smaller ventricle (Figure 4A). The septum, equivalent to the septum pellucidum in humans, is the anatomical structure that separates the lateral ventricles, and its displacement in *Pdgfc*$^{-/-}$ mice made the smaller ventricle appear compressed. To ensure that the asymmetry was not an artifact of tissue preparation, *in vivo* MRI in live mice was used (Figure 4B). Quantification of the extent of ventricular asymmetry was determined from the H&E stainings (Figure 4C) and the MRIs (Figure 4D). For the H&E quantifications, the average size of the respective lateral ventricle was determined using three identical anatomical positions for each mouse and is expressed as the ratio of the smallest to the largest ventricular area (Figure 4C). This analysis suggested (P = 0.1) that the smaller of the lateral ventricles in *Pdgfc*$^{-/-}$ mice was approximately half the size (mean ± SEM, 53% ± 6%) of the larger ventricle. In wild-type controls, the ventricles were evenly sized (the smaller being a mean ± SEM 96% ± 1% of the larger). Comparing the total volume of the smallest to the largest ventricle from the MRIs (Figure 4D) revealed significant asymmetry of the lateral ventricles in *Pdgfc*$^{-/-}$ mice (mean ± SEM, 58% ± 5%) compared with in wild-type controls (mean ± SEM, 95% ± 2%), thus confirming the results of histologic staining. In *Pdgfc*$^{+/-}$ mice, asymmetry was intermediate (mean ± SEM, 81% ± 6%) compared with that in *Pdgfc*$^{-/-}$ mice and wild-type mice, indicating a gene dosage effect of PDGF-C in the control of ventricular symmetry. Three examples of *Pdgfc*$^{-/-}$ adult mice with a hypoplastic septum were

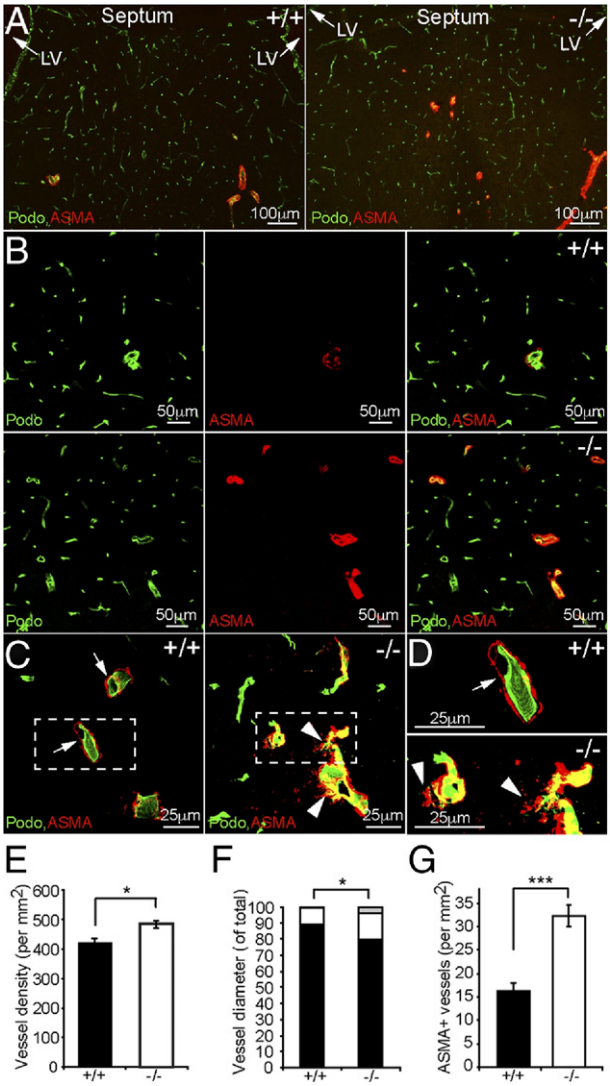

Figure 3. Abnormal vascularization in the brains of *Pdgfc*$^{-/-}$ mice. **A–D:** Immunofluorescent co-stainings of vessels using the endothelial cell marker podocalyxin (Podo; green) and the vSMC marker α-smooth muscle actin (ASMA; red) revealed that the vascular bed was disorganized and displayed more ASMA-positive, large-diameter vessels in adult *Pdgfc*$^{-/-}$ mouse brains compared with wild-type littermate controls (**A** and **B**). The pictures in **C** and **D** are two-dimensional renderings of the three-dimensional pictures made from confocal Z-stacks and illustrate that vSMC coverage appears thicker, with defect investment around the endothelial cells in *Pdgfc*$^{-/-}$ mice (**arrowheads**), which was not seen in wild-type controls (**arrows**). Quantification of vessel density (**E**), vessel diameter (**F**), and number of ASMA-positive vessels (**G**) shows significantly more and larger vessels present in *Pdgfc*$^{-/-}$ mice compared with in wild-type controls ($n = 9$). **F:** Gray bar, >10 μm; white bar, 5–10 μm; black bar, <5 μm. *$P \leq 0.05$, ***$P \leq 0.001$. Error bars represent SEM. The pictures in **B** are single-plane confocal images. LV, lateral ventricle.

also observed by MRI (one female and two males). Figure 4E shows a montage from MRIs of an adult male illustrating this defect. No similar defect was noted in any wild-type animals, and *Pdgfc*$^{-/-}$ mice with a hypoplastic septum were excluded from this analysis of ventricular symmetry.

Abnormal Ventricular Lining in PDGF-C–Deficient Mice

Based on the present findings of lateral ventricle abnormalities in *Pdgfc*$^{-/-}$ mice and a previous study showing high expression of PDGF-C in cells surrounding the ventricles in the developing murine brain,[12] we next examined the integrity of the ependymal cell layer lining the lateral ventricles in adult PDGF-C mutant mice.

In *Pdgfc*$^{-/-}$ mice, we noted that the ependymal lining, herein visualized by the ependymal marker S100,[13] was poorly formed. It appeared convoluted and pleomorphic (Figure 5A) and seemed to easily separate from the underlying subependymal cell layer (data not shown). Also, areas of ependymal denudation were seen in *Pdgfc*$^{-/-}$ brains (Figure 5A). These stainings further revealed that the normal cuboidal shape of ependymal cells was often lost in *Pdgfc*$^{-/-}$ animals (Figure 5A). Immunostaining with phalloidin antibodies against F-actin revealed that the strong apical border staining seen in wild-type ependyma was abnormal and only weakly present in *Pdgfc*$^{-/-}$ mutant ependyma (Figure 5B). Furthermore, staining with antibodies against GLUT1 a marker for mature ependyma,[14] showed a decrease in GLUT1 expression in *Pdgfc*$^{-/-}$ ependyma (Figure 5C) compared with wild-type controls. Taken together, these results demonstrate that ablation of PDGF-C signaling affects ependymal differentiation and integrity.

In addition to ependymal abnormalities, staining of subventricular cells with GFAP antibodies revealed that the subventricular lining of the lateral ventricles was highly irregular in *Pdgfc*$^{-/-}$ mice (Figure 5D). Discontinuous GFAP staining was observed in most (~90%) of the

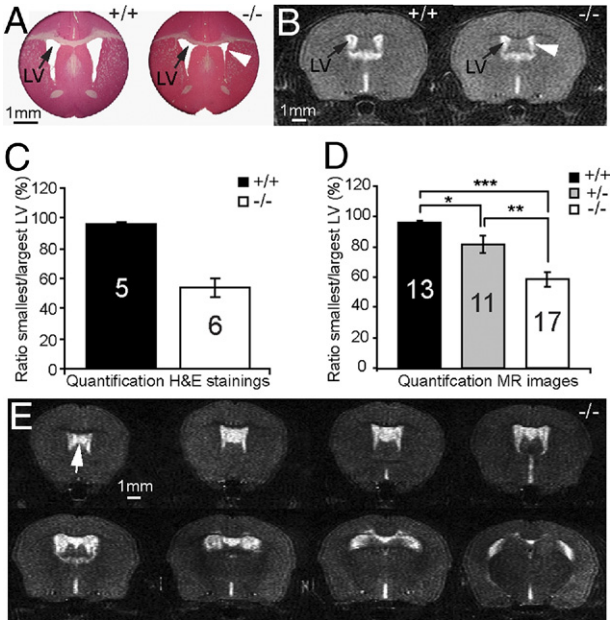

Figure 4. Lateral ventricular defects in the brains of *Pdgfc*$^{-/-}$ mice. *Ex vivo* H&E stainings (**A**) and *in vivo* MRIs (**B**) of *Pdgfc*$^{-/-}$ and wild-type adult brains. The septum separating the lateral ventricles (LVs; **arrows**) seemed displaced in *Pdgfc*$^{-/-}$ mice (**arrowheads**). Quantification of the ratio between the smallest to the largest ventricular size from the H&E stainings (**C**) and the MRIs (**D**) confirmed that *Pdgfc*$^{-/-}$ mice displayed significant asymmetry in LV size compared with wild-type littermate controls. Numbers of analyzed animals are displayed on the respective bars. *$P \leq 0.05$, **$P \leq 0.01$, and ***$P \leq 0.001$. Error bars represent SEM. **E:** Montage from MRIs of a *Pdgfc*$^{-/-}$ male mouse illustrating hypoplasia of the septum (**arrow**). The quantifications were made in **C** using ImageJ to trace the area of the right and left ventricles from three anatomically similar fields of vision and in **D** using Matlab to trace the entire volume for each LV.

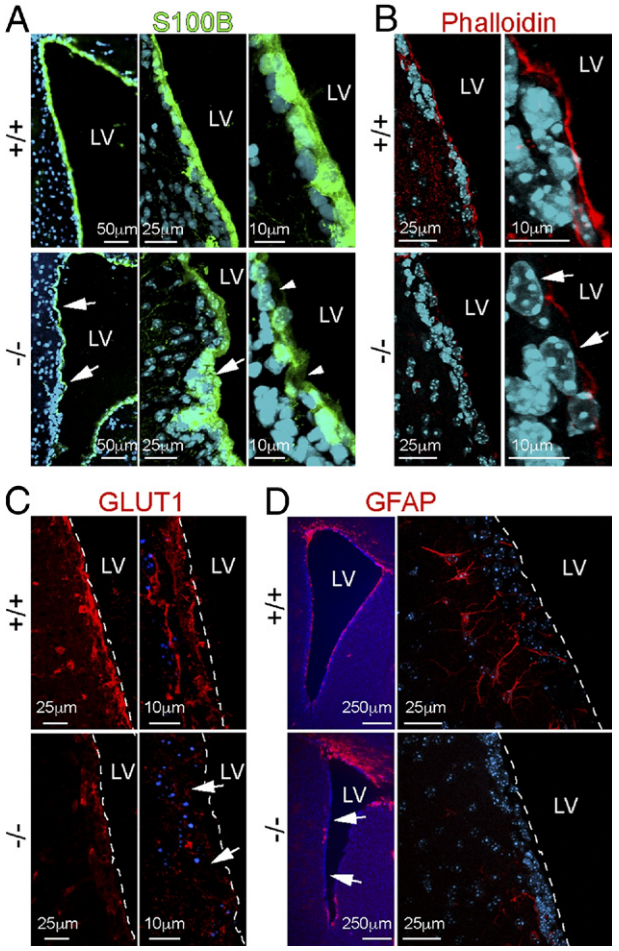

Figure 5. Abnormal ependymal lining of the lateral ventricles (LVs) in *Pdgfc*$^{-/-}$ mice. **A–C:** Immunofluorescent stainings of adult control and *Pdgfc*$^{-/-}$ ependymal cell linings of the lateral wall of the LVs using S100B (**A**), phalloidin (**B**), and GLUT1 (**C**) antibodies. **A:** Note the tortuous ependymal lining (**arrows**) and the occasional gaps in the ependyma (**arrowheads**) of *Pdgfc*$^{-/-}$ mutant mice. Each picture is a representative from different individuals. **B** and **C** also note the low levels of apical phalloidin staining (**arrows; B**) and GLUT1 staining (**arrows; C**) in *Pdgfc*$^{-/-}$ mice compared with littermate controls. **D:** Immunofluorescent stainings of subependymal astrocytes with antibodies against GFAP show highly irregular staining in *Pdgfc*$^{-/-}$ mice around the LV (**arrows**) compared with wild-type littermate controls. Low-magnification images in **A** and **D** are single-plane confocal micrographs, whereas the other pictures are two-dimensional renderings of the three-dimensional pictures made from confocal Z-stacks (n = 8). Cell nuclei were visualized by using DAPI (blue).

Pdgfc$^{-/-}$ animals compared with wild-type controls (~20%). These GFAP-positive subventricular zone (SVZ) astrocytes, also referred to as type B cells, are believed to be the neural stem cells of the adult mammalian brain.[15] Note that these type B cells have been reported to express PDGFRα in the adult SVZ.[16]

Cortical Lamination in PDGF-C–Deficient Murine Brain

A recent study has linked PDGF-C signaling to neuronal survival *in vitro,*[17] prompting us to study the effect of PDGF-C ablation on the neuronal population in *Pdgfc*$^{-/-}$ mice. Staining for NeuN, a marker for mature neurons, indicated that the distribution of NeuN-positive cells in *Pdgfc*$^{-/-}$ mice was similar to that in wild-type mice (Figure 6A). These NeuN stainings revealed areas around the lateral ventricles in *Pdgfc*$^{-/-}$ mice where NeuN-positive neurons disrupted the ependymal cell lining (Figure 6, A and B). This disruption was not seen in wild-type controls. The boxed areas in Figure 6A are magnified in Figure 6B. Such misplaced neurons are suggestive of a neuronal migration defect since it is possible that these ectopic neurons have failed to migrate to their proper position in the cerebral cortex. To determine whether PDGF-C deficiency results in defects in neuronal migration, we analyzed the cortical lamination in adult *Pdgfc*$^{-/-}$ mice using various layer-specific neuronal markers (Figure 6C). Staining for CUX-1 (layers II to IV) and Ctip2 (layer V) showed an overall normal-looking cortex in *Pdgfc*$^{-/-}$ mice, although layer V appeared modestly, but not significantly, increased in *Pdgfc*$^{-/-}$ mice (Figure 6D).

Target Cells of PDGF-C Signaling in the Adult Murine Brain

Expression of PDGFRα in the CNS, the receptor for PDGF-C,[2] has been extensively studied, and it is well established that PDGFRα signaling is essential for glial cell development.[18,19] However, some studies have suggested that PDGFRα is also expressed by mature neurons in the adult mammalian CNS, and it has been proposed that PDGF-C/PDGFRα signaling may be critical for neuronal survival *in vitro.*[17,20] Owing to these discrepancies regarding the expression and role of PDGFRα in the CNS, we next examined where PDGFRα is expressed in the adult murine brain to see potential target cells of PDGF-C signaling. For this analysis, we used *Pdgfrα:GFP* reporter mice.[9] Staining of brain sections from adult *Pdgfrα:GFP* mice with the general neuronal maker NeuN, expressed in the nucleus of mature neurons, revealed no evidence of expression of PDGFRα in mature neurons *in vivo* (Figure 7A). In fact, we did not encounter any nuclei with overlapping expression of PDGFRα reporter (green) and NeuN (red); however, the red and green nuclei often appeared in close proximity to each other, supporting the idea of close communication between neurons and PDGFRα-expressing cells. These results, indicating no expression of PDGFRα in mature neurons in the adult CNS, are consistent with the normal appearance of cortical neuronal layering in the brains of *Pdgfc*$^{-/-}$ mice (Figure 6). Co-stainings of brain sections from adult PDGFRα reporter mice with various glial markers revealed that the PDGFRα reporter gene was expressed in glial cells such as arteriole-associated astrocytes (Figure 7B), oligodendrocytes (Figure 7C), and NG2-positive oligodendrocyte progenitors (Figure 7D). Together, these data suggest that *in vivo* PDGF-C is not critically required for neuronal survival through PDGFRα signaling on neurons. Instead, the present data are consistent with studies suggesting that PDGFRα may be important for glial cell development and survival (reviewed by Andrae et al[1]).

Ablation of PDGF-A, the other known ligand for PDGFRα, has been reported to decrease the number of

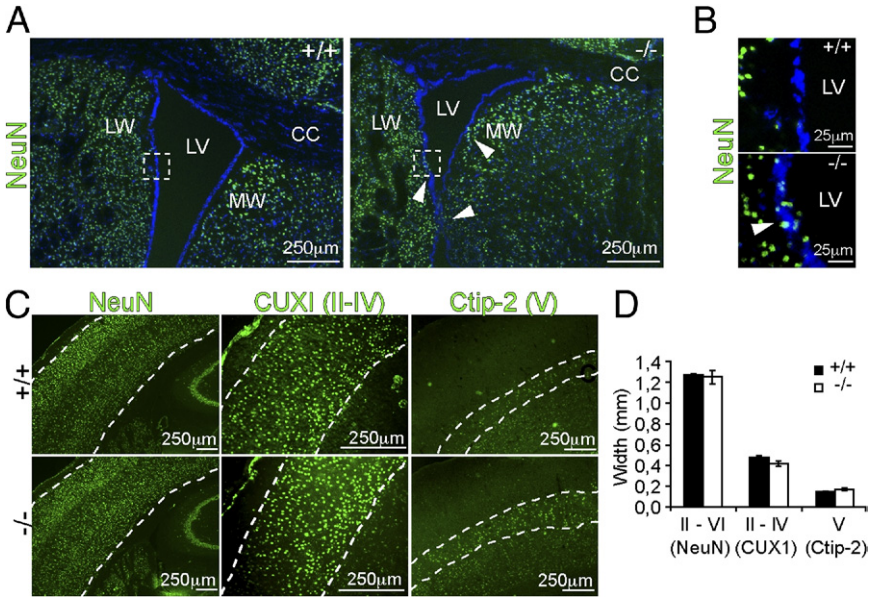

Figure 6. Ectopic neurons in the ventricular lining but normal cortical lamination in *Pdgfc*$^{-/-}$ mice. **A–C:** Immunofluorescent stainings for mature neurons with NeuN antibodies illustrated overall normal distribution of NeuN-positive cells in *Pdgfc*$^{-/-}$ mice; however, misplaced NeuN-positive neurons were noted in the ependymal cell lining of *Pdgfc*$^{-/-}$ mice (**arrowheads; A** and **B**) but not of wild-type controls. Boxed areas in **A** are magnified in **B**. **C:** Stainings for layer II to IV neurons with CUX-1 antibodies and layer V neurons with Ctip2 antibodies showed that *Pdgfc*$^{-/-}$ mice displayed a cerebral cortical lamination comparable with that of wild-type littermate controls. The width of the respective layers was measured as the distance between the dashed lines in **C** and is quantified in **D** (n = 7). A slight, although nonsignificant, increase in the width of layer V was noted. Cell nuclei were visualized by using DAPI (blue). Error bars represent SEM. LV, lateral ventricle.

oligodendrocytes and myelination in the perinatal murine brain.[19] However, PDGF-A ablation has only intermediate effects on the number of oligodendrocytes in the cerebral cortex. Since PDGF-C is highly expressed in the adult cerebral cortex (Figure 1, A and B) and PDGFRα is expressed in oligodendrocytes and NG-2 progenitors (Figure 7, C and D), we investigated whether ablation of PDGF-C decreased the number of oligodendrocyte progenitors in this area of the brain. We found that the number of NG-2 positive glia in the cortex of adult *Pdgfc*$^{-/-}$ mice was similar to that of wild-type controls (Figure 7E). It is, however, still possible that PDGF-C functions in parallel with PDGF-A during embryonic and perinatal oligodendrogenesis in the cerebral cortex but that this is not apparent in the surviving adult *Pdgfc*$^{-/-}$ mice studied herein.

Discussion

This study shows that loss of PDGF-C in C57BL/6 mice is compatible with postnatal survival, offering the opportunity to study the physiologic and pathophysiologic roles of this protein in the adult mouse. However, despite postnatal survival, these data demonstrate the presence of significant congenital defects in the CNS of *Pdgfc*$^{-/-}$ mice, such as spina bifida occulta, cerebral vascular abnormalities, and asymmetrical lateral ventricles with distorted ependymal lining. The milder phenotype of PDGF-C ablation in C57BL/6 mice will facilitate the study of developmental roles of PDGF-C, obscured by the more severe phenotype seen in *Pdgfc*:129/Sv mice.[6] The observation that phenotype severity depends on genetic background also offers an opportunity to study modifier genes of PDGF signaling in mice.

The unexpected finding that PDGF-C ablation in C57BL/6 mice is associated with cerebral ventricular defects was especially intriguing, first because this has not been linked to PDGFRα signaling previously[1] and second because the molecular mechanisms underlying brain ventricle formation still remain poorly understood.[8] The smaller ventricle in *Pdgfc*$^{-/-}$ mice seemed to arise from displacement of the septum, ie, the structure that separates the lateral ventricles. In some *Pdgfc*$^{-/-}$ mice, we even noted incomplete development of the septum, which is reminiscent of cavum septum pellucidum seen in humans. A cavum of the septum is often present at birth but is normally eliminated by age 3 to 6 months as the septal leaves fuse postnatally in a posterior-to-anterior manner.[21] Preservation of a cavum septum pellucidum in adults is a marker for fetal neural maldevelopment, although the cause is largely unknown.[22] However, it has been suggested that it is likely due to underdevelopment of limbic structures, such as the hippocampus, amygdalae, and septal nuclei,[23,24] ie, structures where we report high expression of PDGF-C. A persistent cavum septum pellucidum in the adult population has also been associated with craniofacial malformations, such as clefts of the lip and/or palate.[25] As mentioned previously herein, PDGF-C ablation in 129/Sv mice results in a complete cleft of the secondary palate,[6] and mutations in the human *PDGFC* gene have recently been shown to strongly associate with clefts of the lip and/or palate.[26,27]

The cerebral ventricles in adult mammals are normally lined with an uninterrupted single layer of specialized epithelial cells, the ependymal cells, that are important for normal brain function.[28] The present study shows that PDGF-C ablation causes defects in the ependymal lining of the lateral ventricles and in the underlying SVZ. These defects are characterized by the loss of the normal cuboidal ependymal cell shape and by ependymal denudation and loss of GFAP-positive cells in the SVZ, indicating that PDGF-C/PDGFRα signaling regulates the formation of normal lateral ventricle lining. These observations are of particular interest since the SVZ is believed to be the source of neuronal and oligodendrocyte stem cells in the adult brain and the molecular mechanism guiding ependymal differentiation is not yet fully understood.[15]

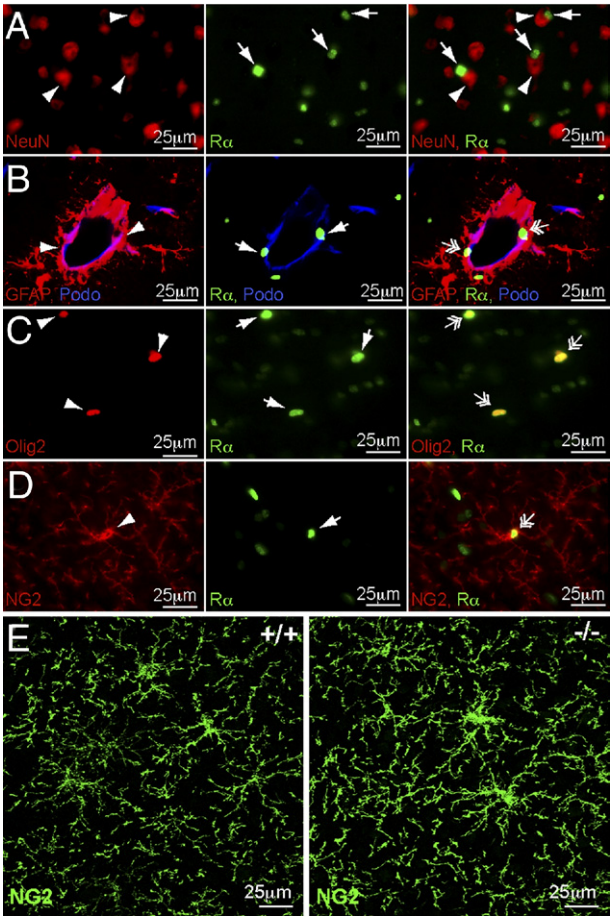

Figure 7. Expression of *Pdgfrα* in the adult murine brain. Adult murine brain sections from *Pdgfrα*:GFP reporter mice were stained with NeuN (**A**), GFAP (**B**), Olig2 (**C**), and NG2 (**D**) antibodies. Pdgfrα reporter (GFP) (**arrows**) was not expressed by mature NeuN-positive neurons (**arrowheads**; **A**) but was found to be expressed by various macroglial cells, including vessel-associated astrocytes (**arrowheads**; **B**), oligodendrocytes (**arrowheads**; **C**), and NG2-glia (**arrowheads**; **D**). Co-expression is visualized as yellow in the overlay pictures (**double-arrows**; **B–D**). **B:** Podocalyxin (blue) was used to visualize vessels. **E:** Immunostainings for NG2-glia in *Pdgfc*$^{-/-}$ and wild-type littermate controls revealed a normal distribution in adult *Pdgfc*$^{-/-}$ mutant brains (n = 5). The pictures are two-dimensional renderings of the three-dimensional pictures made from confocal Z-stacks.

Although, the present work does not establish whether loss of PDGF-C disrupts the important ependymal layer directly, or indirectly by affecting the stem cell population in the SVZ, it is tempting to speculate that loss of PDGF-C during development affects the pool of radial glia which are giving rise to both ependymal as well as GFAP/PDGFRα-positive cells in the SVZ.[16,29,30] Note that this subset of radial glia is derived from the neuroepithelium, which is known to display high expression of PDGFRα as early as embryonic day 8.5 (E8.5),[31] and that the first evidence of ependymal differentiation is seen in the floor plate of the neural tube, a structure in which high expression of PDGF-C has been reported.[12] It will be interesting to use the PDGF-C mutants characterized herein to further establish the molecular mechanism of how PDGF signaling guides ependymal/SVZ development.

Ependymal malformations and loss of neuroependymal integrity have been linked to hydrocephalus and neural tube defects and to neuronal migrational defects.[32,33] In the lateral ventricular wall of *Pdgfc*$^{-/-}$ mice, we found misplaced neurons disrupting the ependymal lining, a feature seen in patients diagnosed as having the neuronal migrational disorder periventricular nodular heterotopia[34] and in victims of sudden infant death syndrome.[35] In addition, we also found one *Pdgfc*$^{-/-}$ male mouse (sacrificed at 7 months of age) with extreme hydrocephalus, manifested as an abnormal accumulation of cerebrospinal fluid and enlargement of the lateral ventricles. Whether these defects are due to ependymal abnormalities associated with PDGF-C ablation remains to be determined.

Based on our earlier findings, we suggested that PDGF-C and its receptor PDGFRα are "drugable" targets in the management of ischemic stroke in mice,[4] and a clinical trial has been initiated to evaluate the safety and feasibility of inhibiting the PDGF-C/PDGFRα signaling pathway in patients with stroke (I-STROKE Trial of the European Stroke Network). The rationale is that targeting this signaling pathway will extend the relatively short treatment window of thrombolytic therapy by protecting the integrity of the blood-brain barrier. However, for this to succeed, it is important to know that PDGF-C is also expressed in the neurovascular unit in human brains. In studies performed in parallel with those reported herein, we find that the expression pattern of PDGF-C in the human brain seems to be conserved, with high expression in limbic regions, such as in the cortical gray matter and around cerebrovascular arterioles (data not shown). This similar pattern of distribution to that seen in mice, including localization around the arterioles, supports a role for PDGF-C in the neurovascular unit in humans.

A recent study has suggested that PDGF-C is a potent neuroprotective factor and cautioned against inhibition of the PDGF-C/PDGFRα signaling pathway in clinical neuropathology.[17] However, the present data do not support a role for PDGF-C as a neuronal survival factor *in vivo* because in this study, *Pdgfc*$^{-/-}$ mice displayed apparent normal distribution of neurons in the brain despite complete ablation of PDGF-C and, importantly, because we found no evidence of PDGFRα expression on mature neurons *in vivo*. The present results are also consistent with those of studies that have shown that PDGFRα is critical for oligodendrogenesis but not for neurogenesis.[1,16] In contrast to a neuronal phenotype, the present data demonstrate the presence of significant nonneuronal congenital defects in the CNS of *Pdgfc*$^{-/-}$ mice, including cerebral vascular and ventricular abnormalities. These developmental defects were not noted in the study by Tang and colleagues[17] and substantially complicate the interpretation of their experimental data obtained with *Pdgfc*$^{-/-}$ mice. We suggest that these congenital abnormalities need to be considered when using *Pdgfc*$^{-/-}$ mice as a genetic tool in the investigation of pathophysiologic roles of PDGF-C and PDGFRα in the adult CNS. Finally, an independent study has confirmed our previous observation that blocking PDGFRα signaling *in vivo* protects the integrity of the blood-brain barrier.[36]

In conclusion, these data demonstrate that PDGF-C plays an important role in the formation of normal cerebral vascularization and normal cerebral ventricles and in

neuroependymal integrity. The *Pdgfc*$^{-/-}$ mice characterized herein present an excellent genetic tool for further unraveling aspects and underlying molecular mechanisms of cerebral ventricle and ependymal development and differentiation. Also, it will be interesting to use these postnatally surviving *Pdgfc*$^{-/-}$ mice to study the link between PDGF signaling, ependymal/ventricular anomalies, and neuropathologic conditions, such as cavum septum pellucidum and hydrocephalus, and to gain further insight into the stem cell compartment in the SVZ.

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