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Netrin 1 contributes to vascular remodeling in the subventricular zone and promotes progenitor emigration after demyelination

Myriam Cayre^{1,2,*}, Sandrine Courtès^{1,2}, Fanny Martineau^{1,2}, Marilyn Giordano^{1,2}, Karen Arnaud^{1,2}, Amandine Zamaron^{1,2} and Pascale Durbec^{1,2}

SUMMARY

Neural stem cells are maintained in the adult brain, sustaining structural and functional plasticity and to some extent participating in brain repair. A thorough understanding of the mechanisms and factors involved in endogenous stem/progenitor cell mobilization is a major challenge in the promotion of spontaneous brain repair. The main neural stem cell niche in the adult brain is the subventricular zone (SVZ). Following demyelination insults, SVZ-derived progenitors act in concert with oligodendrocyte precursors to repopulate the lesion and replace lost oligodendrocytes. Here, we showed robust vascular reactivity within the SVZ after focal demyelination of the corpus callosum in adult mice, together with a remarkable physical association between these vessels and neural progenitors exiting from their niche. Endogenous progenitor cell recruitment towards the lesion was significantly reduced by inhibiting post-lesional angiogenesis in the SVZ using anti-VEGF blocking antibody injections, suggesting a facilitating role of blood vessels for progenitor cell migration towards the lesion. We identified netrin 1 (NTN1) as a key factor upregulated within the SVZ after demyelination and involved in local angiogenesis and progenitor cell migration. Blocking NTN1 expression using a neutralizing antibody inhibited both lesion-induced vascular reactivity and progenitor cell recruitment at the lesion site. We propose a model in which SVZ progenitors respond to a demyelination lesion by NTN1 secretion that both directly promotes cell emigration and contributes to local angiogenesis, which in turn indirectly facilitates progenitor cell emigration from the niche.

KEY WORDS: Migration, Blood vessels, Netrin-1, Mouse

INTRODUCTION

Adult brain does not provide a permissive environment for cell migration, except in the rostral migratory stream (RMS) in which neural progenitors migrate from the subventricular zone (SVZ) to the olfactory bulb (Lois and Alvarez-Buylla, 1994), exhibiting homophilic chain migration. Progenitors follow blood vessels and are surrounded by an astrocytic tube (Lois et al., 1996; Peretto et al., 1997; Snayyan et al., 2009; Whitman et al., 2009). When progenitors reach the olfactory bulb they change to a radial migration and are often still associated with the vasculature (Bovetti et al., 2007).

In a variety of brain pathologies, some SVZ-derived progenitors are rerouted from the RMS and migrate ectopically towards the lesion site in spontaneous attempts at brain repair (for a review, see Cayre et al., 2009). Identifying mechanisms underlying endogenous progenitor cell mobilization is a necessary step toward promoting the regenerative process. Blood vessels have been proposed as a scaffold for guiding progenitor cell migration after stroke. SVZ-derived doublecortin-positive (DCX⁺) neuronal progenitors are then observed in the striatum in close association with astrocytic processes and blood vessels (Yamashita et al., 2006; Thored et al., 2007). Blood vessels secrete BDNF, which activates neural progenitor migration (Grade et al., 2013). However, such association between ectopic migrating SVZ-derived progenitors and blood vessels has not been reported in other types of brain

pathologies, and little is known about the mechanisms controlling the recruitment of endogenous progenitors.

In this study we investigated the mechanisms involved in SVZ-derived progenitor recruitment in the lysolecithin (LPC)-induced focal demyelination of the corpus callosum (CC) model. Although parenchymal oligodendrocyte precursor cells (OPCs) play a major role in spontaneous remyelination (Gensert and Goldman, 1997), SVZ-derived progenitors also participate in the repair process by providing an unlimited source of progenitors for chronic pathologies such as multiple sclerosis. A small population of OPCs present in the SVZ can be activated to proliferate and migrate towards the demyelinated CC (Menn et al., 2006). In addition, SVZ-derived neuronal progenitors (DCX⁺) are able to migrate to the lesion site and differentiate into myelinating oligodendrocytes after LPC-induced demyelination (Jablonska et al., 2010; Etxeberria et al., 2010). SVZ progenitors grafted in the CC can extensively migrate along fiber tracts (Cayre et al., 2006), suggesting that migration within the CC is not the limiting step for their recruitment to the demyelination lesion. We thus focused on the first mobilization step: the extraction of progenitors from the SVZ, a process that we call 'emigration'.

In the present study we showed that a demyelination lesion in the CC triggers a striking vascular reactivity within the SVZ niche via netrin 1 (NTN1) secretion by neural progenitors. We also revealed that this vascular remodeling regulates ectopic endogenous progenitor cell migration. Although NTN1 is well characterized in development, its function in adult brain remains elusive. Our present data on NTN1 expression and function in the adult SVZ after CC demyelination suggest a central role in post-lesional vascular remodeling and progenitor cell recruitment towards the lesion after demyelination, identifying NTN1 as a key factor at the crossroads

¹Aix-Marseille Université, IBDM-UMR7288, 13288 Marseille, France. ²CNRS, IBDM-UMR7288, 13288 Marseille, France.

*Author for correspondence (myriam.cayre@univ-amu.fr)

between cell migration and angiogenesis in the adult pathological brain.

MATERIALS AND METHODS

Animals

All experimental and surgical protocols were approved by the Direction Départementale des Services Vétérinaires (ID number E-13-055-21).

Eight- to ten-week-old wild-type (CD1 and C57BL/6 from Janvier) or transgenic mice were used. Mice expressing GFP fused to the proteolipid protein (which is largely present in myelin) were used to observe demyelination lesions (plpGFP mice). To analyze NTN1 expression, we used a mouse line carrying a gene trap vector (pGT1.8TM) encoding a chimeric NTN1- β -gal fusion protein regulated by the endogenous *Ntn1* promoter (Skarnes et al., 1995; Serafini et al., 1996).

Surgeries and perfusions were performed under ketamine (100 mg/kg)/xylazine (10 mg/kg) anesthesia.

Demyelination lesions

LPC-induced focal demyelination was performed as described previously (Magalon et al., 2007). Briefly, mice were anesthetized and placed in a stereotaxic frame (Kopf). Then, 0.7 μ l of a solution of 1% LPC (Sigma) in 0.9% NaCl was injected unilaterally into the CC (1.5 mm anterior, 1 mm lateral to bregma, 2 mm deep from cortical surface). When used, blocking antibodies [anti-mouse VEGF mAb 20-4.1.1, 16 mg/ml (mouse IgG2a; Genentech); and anti-mouse NTN1 antibody, 4 mg/ml (goat IgG; R&D Systems)] were injected intracerebrally consecutively to LPC at the same coordinates, as well as 2 and 4 days after surgery via intraperitoneal injection (5 mg/kg). Control mice were injected with non-specific mouse and goat IgGs.

Biochemistry

Three days after demyelination lesion, SVZ and peri-lesional structures (CC, cortex, striatum) and their counterparts in the contralateral hemisphere were dissected in cold Hank's Balanced Salt Solution (HBSS). For SVZ samples, owing to the very small size of the structure, the SVZ of four to five animals were pooled. Sample preparation, western blot and semi-quantitative analysis were performed as described (Courtès et al., 2011). The following antibodies were used: chicken anti-netrin (1/500; Abcam), goat anti-DCC (1/1000; R&D Systems), goat anti-neogenin (1/1000; R&D Systems), goat anti-UNC5 (1/500; Santa Cruz); and mouse anti- α -tubulin (1/30,000; Sigma).

SVZ-derived cell tracing

The thymidine analog 5-bromo-2'-deoxyuridine (BrdU) was used to trace SVZ-derived cells as described (Magalon et al., 2007). One day before surgery, the animals received four BrdU injections (100 mg/kg intraperitoneally; Sigma) at 2-hour intervals. Using this protocol, cells incorporating BrdU at the time of injection (i.e. before any lesion had been performed) are restricted to the SVZ and RMS (supplementary material Fig. S1). Therefore, 7 days after lesion, labeled cells detected at the lesion site are assumed to originate from the SVZ or the RMS (Picard-Riera et al., 2002). This protocol allowed us to strongly label a large proportion of SVZ progenitors without reaching toxic doses and without staining of dying cells (Magalon et al., 2007; Cameron and McKay, 2001).

For analysis of the relationship between ectopically migrating SVZ-derived progenitors and blood vessels, sections were immunolabeled for DCX and laminin 4 days after LPC injection. DCX labeling allows visualization of the whole neuronal progenitor morphology (cell body and processes), which is a prerequisite for an analysis of the close interactions with blood vessels. Importantly, progenitors migrating within SVZ/RMS were excluded. Only ectopically migrating progenitors were analyzed, either while they were in the process of leaving their niche (identified by a cell body outside the SVZ/RMS but an extension still in contact with the niche) or when they had already reached adjacent structures (striatum and CC). They were classified according to their type of association with blood vessels: tangential association, simple contact or no contact (see Fig. 2D). Results are expressed as the percentage of ectopic progenitors of each specified type of association among the total number of ectopic progenitors in the structure.

Immunohistochemistry

For whole-mount immunolabeling, the lateral wall of the ventricle was dissected and processed as described (Mirzadeh et al., 2010). Paraformaldehyde-perfused brains were sectioned at 50 μ m using a Leica vibratome. The following primary antibodies were used: rat anti-BrdU (1/500; AbCys); mouse IgG1 anti-MBP (1/500; Euromedex) for myelin; rabbit anti-laminin (1/1000; Interchim) for blood vessels; rat anti-CD31 (1/50; BD Pharmingen) for endothelial cells; goat anti-DCX (1/250; Santa Cruz) or mouse IgM anti-PSA-NCAM (our laboratory) for migrating neuronal precursors; rabbit anti-phospho-histone H3 (pH3) (1/250; Upstate) or mouse anti-Ki67 (1/200; BD Pharmingen) for mitotic cells; chick anti-NTN1 (1/100; AbCam) or rabbit anti-NTN1 (1/100; Santa Cruz); goat anti-DCC and goat anti-neogenin (1/50, R&D Systems). Secondary antibodies coupled to Cy3 or Cy5 (1/200 in PBS with 0.1% Triton X-100; Interchim Jackson) or to Alexa 488, 555 or 647 (1/500; Invitrogen Molecular Probes) were used. For NTN1, DCC and neogenin immunofluorescence, an amplification step was performed using biotinylated secondary antibodies and streptavidin coupled to Alexa 488. For BrdU immunofluorescence, acid unmasking was performed (2 N HCl for 20 minutes at 37°C). Negative controls were performed by omitting primary antibodies. Sections were then counterstained with Hoechst 33342 (1/1000; Sigma).

X-Gal histology was performed as described (Durbec and Rougon, 2001).

Image analysis and quantification

Confocal imaging was performed with the Apotome system (Zeiss). The area of the structures of interest (SVZ, demyelinated lesions) was measured using ImageJ (NIH). For quantification of blood vessel density in the SVZ, a constant exposure time was applied for image acquisition of all sections and all whole-mounts, and the percentage of SVZ area occupied by CD31⁺ or laminin⁺ labeling was quantified using ImageJ. Branching complexity was analyzed using SVZ whole-mount preparations immunolabeled for laminin. The number of blood vessel branches was counted manually and expressed relative to the total SVZ area.

For SVZ-derived cell recruitment at the lesion site, BrdU⁺ cells were counted in every fourth section throughout the whole demyelinated lesion.

Primary cultures of SVZ explants

SVZ explants were obtained and cultured in defined medium as described (Chazal et al., 2000) from 8- to 9-week-old mice. Explants were mixed with Matrigel (BD Biosciences) diluted with HBSS in a 2:1 ratio. An anti-NTN1 blocking antibody (R&D Systems; 2 μ g/ml) or a non-specific IgG (Jackson ImmunoResearch; 2 μ g/ml) was added as a control when appropriate. The specificity of the NTN1 blocking antibody was checked on neonate SVZ explants: it abolished NTN1-induced migration of neural progenitors (not shown). In some experiments, cell proliferation was checked by adding 10 μ M BrdU to the culture medium 4 hours before fixation (in methanol with 1% acetic acid for 10 minutes at -20°C) and immunocytochemistry.

Explants were analyzed (Colibri microscope, Zeiss) 4 and 7 days after plating. Calcein (4 μ g/ml, 1 hour at 37°C) was used to visualize all migrating cells and to assess viability. The total number of cells that migrated outside the explant as well as the repartition of these cells according to their distance from the edge of the explant (0-100 μ m, 100-200 μ m, 200-300 μ m, or more than 300 μ m away; see Fig. 5A) were determined using ImageJ. The data shown are from two independent experiments with a total of 46-61 explants analyzed per group.

Tube formation assay

Mouse brain endothelial cells (bEnd3 cell line, ATCC) were cultured in DMEM with 10% fetal calf serum (FCS), 1% sodium pyruvate and 1% penicillin/streptomycin (hereafter called complete culture medium), and maintained at 37°C, 7% CO₂. For the tube formation assay, endothelial cells (60,000 cells/cm²) were seeded in DMEM alone and left to adhere on polymerized Matrigel for 45 minutes, and then cultured in complete culture medium with 0.5% FCS. NTN1 (50 or 100 ng/ml; R&D Systems) was added to test its effect on angiogenesis. VEGF (35 ng/ml; Sigma) was used as a positive control.

Angiogenesis was estimated after 9 hours in culture by quantifying total endothelial tube length/mm² using ImageJ.

For co-culture experiments, endothelial cells were first seeded (75,000 cells/mm²) on Matrigel in bEnd3 cell medium. Two hours later, SVZ progenitors from actin-GFP mice (44,000 cells/mm²) were added. Co-cultures were analyzed after 5-7 hours incubation at 37°C, 5% CO₂.

Gene expression profile of demyelinated versus healthy mouse-derived SVZ progenitors

The SVZ from eight mice induced for experimental autoimmune encephalomyelitis (EAE mice) at the peak of paralytic symptoms and from eight adult healthy mice as controls were micro-dissected and pooled. SVZ were exposed for 2 minutes to trypsin (2.5 mg/ml) and dissociated into single cells. Neuronal (PSA-NCAM⁺) and glial (NG2⁺) SVZ progenitors were separated using magnetic cell sorting (Miltenyi Biotec). This experiment was replicated in an independent similar experiment. We thus obtained eight samples: healthy NG2⁺ replicate 1; healthy PSA⁺ replicate 1; EAE NG2⁺ replicate 1; EAE PSA⁺ replicate 1; healthy NG2⁺ replicate 2; healthy PSA⁺ replicate 2; EAE NG2⁺ replicate 2; EAE PSA⁺ replicate 2.

Cell samples were lysed using SuperAmp Lysis Buffer and stored according to the instructions of the SuperAmp Preparation Kit (Miltenyi Biotec). SuperAmp RNA amplification was performed according to the manufacturer's instructions (Miltenyi Biotec). Briefly, the amplification is based on a global PCR protocol using mRNA-derived cDNA. mRNA was isolated via magnetic bead technology. Amplified cDNA samples were quantified using an ND-1000 spectrophotometer (NanoDrop Technologies). The integrity of the cDNA was checked using the 2100 Bioanalyzer platform (Agilent Technologies). The cDNAs (250 ng) were used as template for Cy3 and Cy5 labeling according to the manufacturer's instructions (Miltenyi Biotec). The Cy3- and Cy5-labeled cDNAs were combined and hybridized overnight (17 hours, 65°C) to Agilent Whole Mouse Genome Oligo Microarrays 4×44K using Agilent's recommended hybridization chamber and oven. Typically, control samples are labeled with Cy3 and experimental samples with Cy5. Microarrays were washed once with 6× SSPE buffer containing 0.005% N-lauroylsarcosine for 1 minute at room temperature followed by a second wash with pre-heated 0.06× SSPE buffer (37°C) containing 0.005% N-lauroylsarcosine for 1 minute. A final washing step was performed with acetonitrile for 30 seconds. Fluorescence signals of the hybridized microarrays were detected using a DNA

microarray scanner (Agilent Technologies). Agilent Feature Extraction Software (FES) was used to readout and process the microarray image files. The software determines feature intensities and ratios (including background subtraction and normalization), rejects outliers and calculates statistical confidences (*P*-values). For determination of differential gene expression, FES-derived output data files were further analyzed using the Rosetta Resolver gene expression data analysis system (Rosetta Biosoftware). We obtained a gene list with all normalized Cy5/Cy3 log10 ratios, Cy5/Cy3 fold changes, sequence description and *P*-values. Microarray data are available at GEO with accession number GSE47486.

Statistics

Data are given as mean ± s.e.m. For comparison of two independent groups, the non-parametric Mann-Whitney test was used. For multiple independent group comparison, ANOVA was performed followed by Kruskal-Wallis two-group comparison. For population repartition comparison, χ^2 test was applied.

RESULTS

Focal demyelination in the CC induces vascular reactivity in the SVZ

Mice were injected with LPC in order to trigger a focal demyelination lesion in the CC. In plpGFP mice, demyelination was clearly visible as soon as 3 days post-lesion (dpl) by the lack of GFP in the CC (Fig. 1A). We examined the vasculature using laminin (a major basement membrane glycoprotein of the blood vessel) and CD31 (also known as PECAM1; specific for endothelial cells).

We observed strong vascular reactivity that was particularly pronounced at the lesion site at 3 dpl (Fig. 1A-B'). More surprisingly considering the more distant location, vascular reactivity was also present within the ipsilateral SVZ (Fig. 1A,C',D'). Indeed, vessels appeared more numerous and of enlarged diameter in the SVZ ipsilateral to the lesion side compared with the contralateral SVZ (Fig. 1C-D'). As a result, blood vessel density was significantly higher in the ipsilateral SVZ (Fig. 1E;

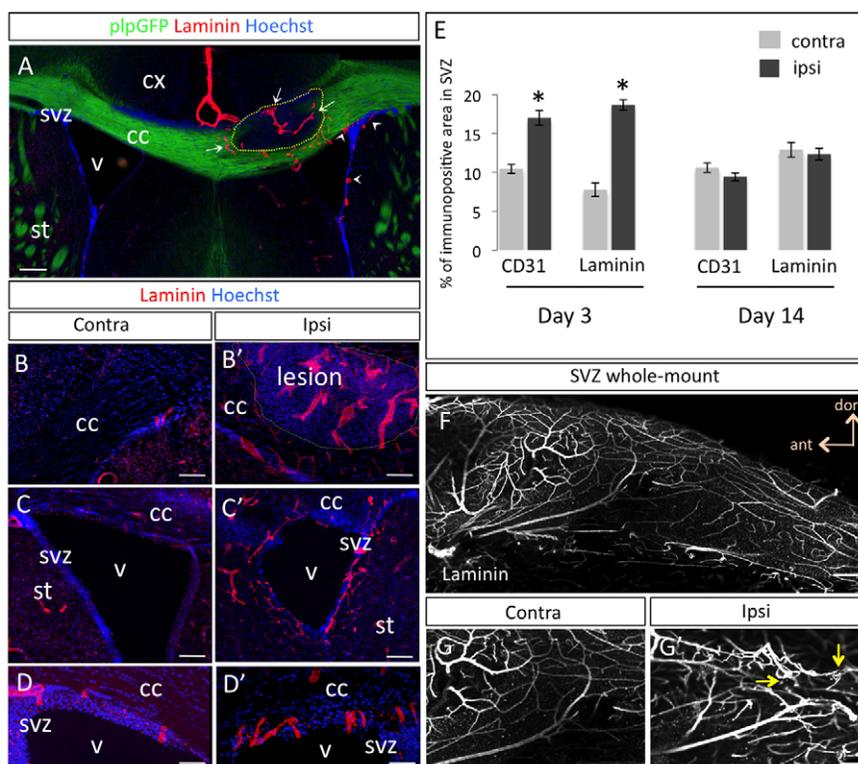


Fig. 1. Vascular remodeling after LPC-induced demyelination of the CC. (A) An LPC-induced demyelinated lesion is clearly detectable in the CC by the absence of GFP expression (delineated by a dotted line) in plpGFP transgenic mice 3 days after injection. Strong vascular reactivity (laminin, red) is observed within the lesion (arrows) but also in the ipsilateral SVZ (arrowheads). (B-D') Blood vessel labeling at the lesion site (B) and in ipsilateral SVZ (C',D') compared with corresponding areas on the contralateral side (B,C,D). (D,D') High magnification of the dorsal SVZ showing numerous blood vessels orientated perpendicularly to the ventricle toward the CC in the ipsilateral (D') but not contralateral (D) side. (E) Quantification of blood vessel density in SVZ contralateral and ipsilateral to the lesion side, 3 and 14 days post-lesion (dpl), using CD31 as a marker of endothelial cells and laminin as a marker of the blood vessel surface. **P*<0.05. Error bars indicate s.e.m. *n*=5 mice per group. (F) SVZ whole-mount preparation from an LPC-injected mouse immunostained with laminin antibody (SVZ contralateral to the lesion). (G,G') Higher magnification of SVZ whole-mounts comparing the vascular tree in the SVZ. Arrows (G') indicate complex ramifications and twisted branches. CC, corpus callosum; CX, cortex; st, striatum; SVZ, subventricular zone; v, ventricle. Scale bars: 200 μ m in A,F; 100 μ m in B-C',G,G'; 50 μ m in D,D'.

$P=0.02$ with both markers). Interestingly, in the roof of the ipsilateral SVZ (at the edge between ventricle and CC), numerous blood vessels were orientated perpendicular to the ventricle, as a route towards the CC (Fig. 1D'). In order to better visualize the whole vascular tree we performed whole-mount dissection of the ventricular wall (Fig. 1F). Increased blood vessel density was confirmed, with a 71% increase in ipsilateral SVZ compared with the contralateral side ($26.0\pm 1.4\%$ versus $15.2\pm 2.1\%$ laminin⁺ area, respectively; $P=0.05$) and structural modifications of the vascular tree became obvious, with more complex ramifications and twisted branches (Fig. 1G,G'). We quantified a $42.2\pm 23.4\%$ increase in the number of branch points in the ipsilateral compared with contralateral SVZ ($n=4$ mice; $P=0.05$).

Fourteen days later, vascular reactivity was still detectable at the lesion site (not shown), whereas blood vessel density had returned to basal levels in the SVZ with no significant difference between contralateral and ipsilateral sides (Fig. 1E). Notably, no significant increase in blood vessel density was observed in the striatum even at 3 dpl ($15.5\pm 0.5\%$ versus $15.3\pm 0.3\%$ laminin⁺ area in contralateral and ipsilateral striatum, respectively; $P=0.34$), suggesting that vascular reactivity was specific to the lesion site and SVZ.

Altogether, these results show a strong and transient vascular remodeling of the SVZ after LPC-induced demyelination in the CC.

Physical association between blood vessels and ectopically migrating neural progenitors after demyelinating lesion of the CC

After LPC-induced demyelination of the CC a number of SVZ-derived neuroblasts (DCX⁺ progenitors) have been shown to escape from their niche (SVZ and RMS), reach the lesion site, differentiate into oligodendrocytes and thus participate in myelin repair (Cantarella et al., 2008; Jablonska et al., 2010; Etxeberria et al., 2010). At 4 dpl we indeed observed numerous DCX⁺ cells escaping the niche, in structures adjacent to the SVZ/RMS, notably in the CC

and striatum (Fig. 2A). These DCX⁺ ectopic progenitors were very rarely observed in unlesioned mice (not shown), and were 4-fold more numerous in the hemisphere ipsilateral to the lesion compared with the contralateral side (65.7 ± 6.3 versus 16.9 ± 2.1 cells/section, respectively; $P=0.0007$).

We then questioned whether the vascular reactivity within the stem cell niche could contribute to the emigration of SVZ progenitors after lesion. We examined the physical relationships between blood vessels and DCX⁺ cells in their escape from the niche. We focused on three zones of emigration (Fig. 2B): the fringes between the wall of the SVZ and the striatum (region 1a); between the roof of the SVZ and CC (region 1b); and between SVZ/RMS junction and CC (region 1c). For comparison, we also analyzed ectopic DCX⁺ progenitors that had already emigrated from the niche and reached the striatum (region 2) or CC (region 3). Ectopically migrating progenitors were classified according to their relationship with the vasculature. We defined three types of association with blood vessels: progenitors with no contact; those in simple contact; or those in tangential contact (see Fig. 2C,D). Of the progenitors in the process of exiting from the niche (regions 1a-c), a large majority were in association (either simple or tangential contact) with blood vessels ($67.3\pm 3.4\%$, mean of regions 1a, 1b and 1c). Among these, $62.0\pm 3.1\%$ were in tangential contact, suggesting migration along the vessels ($n=8$ mice; $N=904$ cells) (Fig. 2E,F). By contrast, in the CC a majority of progenitors migrated parallel to the fiber tracts, with no contact with blood vessels ($58.9\pm 2.6\%$; Fig. 2G,I) and only a few ($16.1\pm 1.8\%$) were tangentially associated ($n=8$ mice; $N=1314$ cells). Fewer DCX⁺ cells were found in the striatum ($n=8$ mice; $N=256$ cells). These showed more complex branched morphology (Fig. 2H) and were found mostly in contact with blood vessels ($86.6\pm 2.3\%$); however, the mode of association (simple contact versus tangential association) was equally distributed (52.0% and 48.0% , respectively; Fig. 2I).

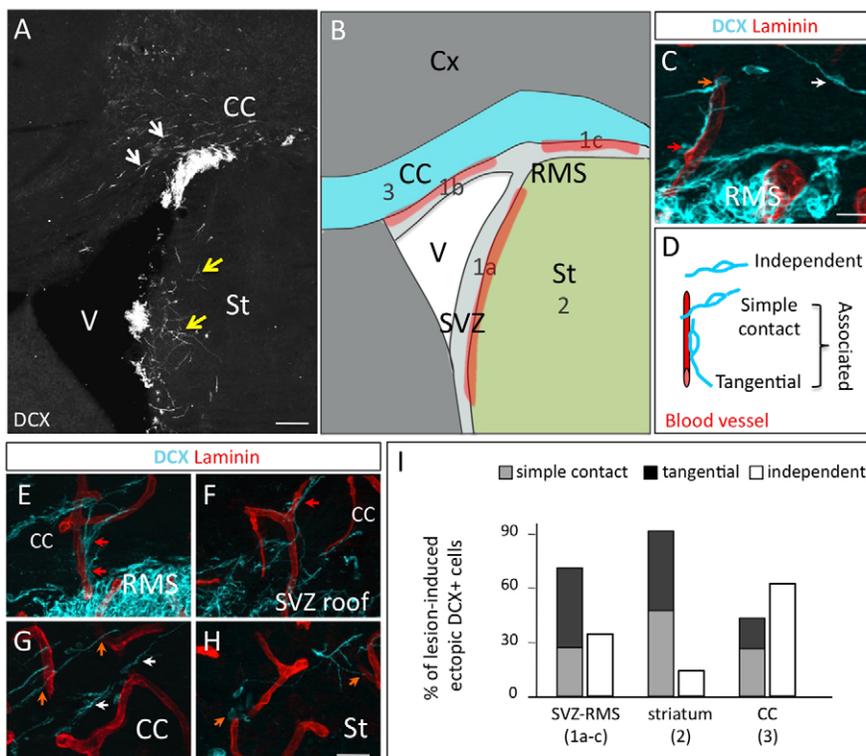


Fig. 2. Analysis of association between ectopically migrating SVZ-derived progenitors and blood vessels. (A) At 4 dpl, numerous ectopic migrating progenitors (DCX⁺) are observed around the ventricle, notably in the striatum (yellow arrows) and the CC (white arrows). (B) Schematic showing the areas chosen for analysis: 1a, SVZ wall-striatum boundary; 1b, SVZ roof-CC boundary; 1c, RMS-CC boundary; 2, striatum; and 3, CC. (C,D) Illustration of the three different types of association between DCX⁺ progenitors and blood vessels that were distinguished: independent (no contact, white arrow); simple contact (orange arrow); and tangential association (red arrow). (E-H) Examples of the association between DCX⁺ progenitors and blood vessels in different locations (RMS, SVZ roof, CC and striatum), using the same color code for arrows as in C. (I) Distribution of ectopic progenitors according to their type of association with blood vessels in each defined area (1a-c, 2 and 3). $n=9$ mice analyzed. CC, corpus callosum; Cx, cortex; RMS, rostral migratory stream; St, striatum; SVZ, subventricular zone; V, ventricle. Scale bars: 150 μ m in A; 25 μ m in C,E-H.

A similar pattern of association between ectopic progenitors and blood vessels was observed in the corresponding structures contralateral to the lesion (supplementary material Fig. S2) but with a restricted number of ectopic cells and fewer blood vessels, as mentioned above. Interestingly, at 14 dpl, when blood vessel density had returned to basal levels, the number of ectopic progenitors dropped to 8.2 ± 2.6 cells/section in the ipsilateral hemisphere (compared with 65.7 ± 6.3 at day 4; $P=0.0003$). Only very rare DCX⁺ progenitors were observed in the process of escaping from their niche (1.1 ± 0.5 cells/section at day 14 versus 33.9 ± 6.4 at day 4; $P=0.0003$), precluding any association analysis.

Altogether, these results suggest that, while escaping from their niche, SVZ-derived progenitors migrate in close association with blood vessels and that the number of ectopic progenitors in adjacent structures is correlated to blood vessel density in the SVZ. The lesion does not change the type of relationship between progenitors and blood vessels, but neovascularization increases the chance of meeting together and deviating neuroblasts.

Reducing post-lesional angiogenesis in the SVZ inhibits progenitor cell recruitment at the lesion site

We blocked reactive angiogenesis to determine whether lesion-induced remodeling of the vasculature within the niche might facilitate neural progenitor cell migration and recruitment at the lesion site. Mice were treated with an anti-mouse VEGF blocking antibody developed by Genentech (mAb 20-4.1.1) concomitant with demyelination induction. Anti-VEGF treatment significantly reduced vessel density both at the lesion site (not shown; 56.1% decrease; $P=0.01$) and within the ipsilateral SVZ (Fig. 3A-C; $P=0.01$) compared with IgG-treated mice; it thus strongly weakened the lesion-induced increase in vascular reactivity (Fig. 3A-C).

Since SVZ-derived progenitors lose DCX expression once at the lesion site, their recruitment within the lesion was estimated using BrdU tracing (see Materials and methods) (supplementary material Fig. S1). At 5 dpl, numerous BrdU-labeled cells were observed within the demyelination lesion (Fig. 3E-E''). Notably, the number of SVZ-derived recruited cells was strongly reduced (41% decrease; $P=0.012$) by anti-VEGF treatment compared with the IgG-injected group (Fig. 3D,F,G), which itself did not differ from a group injected only with LPC ($P=0.30$). By contrast, we did not detect any significant reduction in SVZ cell proliferation (78.5 ± 20.9 versus 132.4 ± 22.2 pH3⁺ cells/mm² in the IgG and anti-VEGF groups, respectively; $P=0.10$). In conclusion, anti-VEGF treatment inhibited post-lesional angiogenesis in the SVZ and concomitantly strongly reduced SVZ-derived cell recruitment at the lesion site, suggesting a correlation between reactive vasculature and ectopic progenitor cell migration after demyelination.

Altogether, these results demonstrate that the induction of a demyelination lesion in the CC is capable of triggering a cascade of events leading to vascular remodeling within the SVZ and show that preventing post-lesional angiogenesis is sufficient to reduce progenitor cell recruitment.

NTN1 is overexpressed in the SVZ of demyelinated mice

To reveal the molecular events taking place in the SVZ of demyelinated mice we performed a microarray analysis to compare gene expression in purified SVZ progenitors of healthy versus demyelinated mice. SVZ cells were sorted using PSA-NCAM (for neuronal progenitors) and NG2 (also known as CSPG4; for glial

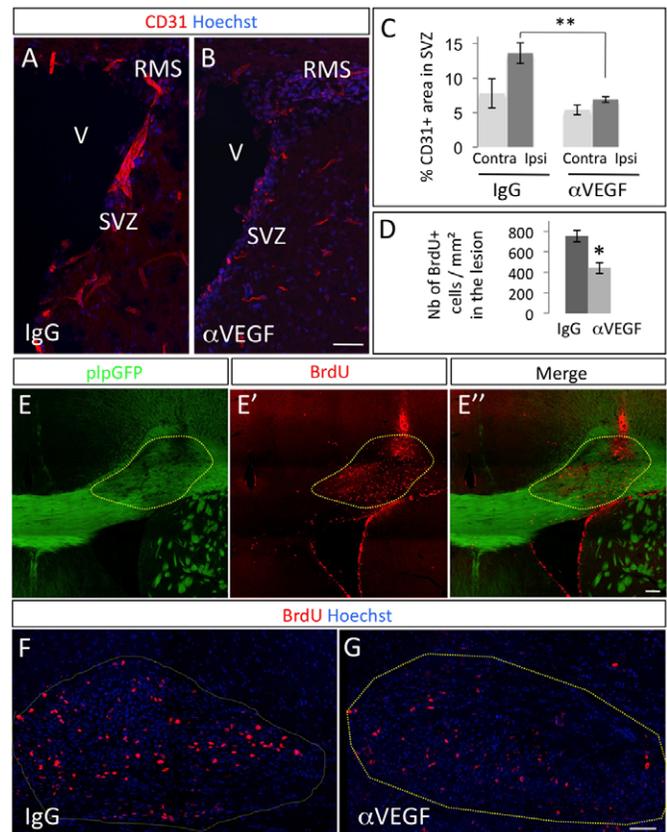


Fig. 3. Effect of anti-VEGF treatment on blood vessel density and SVZ-derived cell recruitment at the lesion site. (A,B) Blood vessel staining by CD31 immunofluorescence in ipsilateral SVZ of LPC-injected mice after IgG (A) or anti-VEGF (B) treatment. (C) Blood vessel density in the SVZ 5 days after LPC injection in mice treated with anti-VEGF blocking antibody compared with control mice that received non-specific IgG. (D) Quantification of SVZ-derived cells within the lesion in control and anti-VEGF-treated mice. * $P<0.05$, ** $P<0.01$. Error bars indicate s.e.m. For C and D, $n=4$ and 6 mice for IgG and anti-VEGF, respectively. (E-E'') SVZ-derived cell recruitment at the lesion site (delineated by a dotted line) in a plpGFP transgenic mouse. SVZ-derived cells were traced using BrdU injection the day before LPC injection (see Materials and methods). (F,G) Images of the lesion site (delineated by the dotted line) in the CC, illustrating the reduced number of BrdU⁺ cells recruited after anti-VEGF treatment. RMS, rostral migratory stream; SVZ, subventricular zone; V, ventricle. Scale bars: 50 μ m in A,B; 200 μ m in E-E''; 100 μ m in F,G.

progenitors). At the crossroads between migration and angiogenesis (according to functional annotations provided by Mouse Genome Informatics), NTN1 was found to be robustly and significantly upregulated (Table 1). The NTN1 upregulation observed in the microarray analysis was confirmed by western blot analysis (Fig. 4A). The expression of its receptors DCC, neogenin and UNC5 was also examined.

Three days after LPC injection, NTN1 and neogenin were upregulated in the SVZ (Fig. 4A) but not in the cortex, striatum or CC (not shown). Semi-quantitative analysis of NTN1 expression in the SVZ at day 3 showed a $63 \pm 27\%$ increase in the lesioned compared with the healthy SVZ (Fig. 4A'); NTN1 expression returned to basal levels after 14 days. By contrast, DCC expression was not modified (Fig. 4A) and UNC5 was almost undetectable in the SVZ (not shown). We then used NTN1-lacZ mice (Serafini et al., 1996) to observe NTN1 expression in brain sections. A previous

Table 1. Data extracted from microarray analyses comparing gene expression in purified SVZ progenitors from healthy versus demyelinated mice

SVZ	Glial progenitors (NG2 ⁺)		Neuronal progenitors (PSA-NCAM ⁺)	
	Experiment 1	Experiment 2	Experiment 1	Experiment 2
Duplicates				
NTN1 (fold increase)	5.3	6.8	4.1	4.5
P-value	1.61×10 ⁻¹⁵	1.31×10 ⁻¹⁶	2.58×10 ⁻¹³	3.04×10 ⁻¹⁴

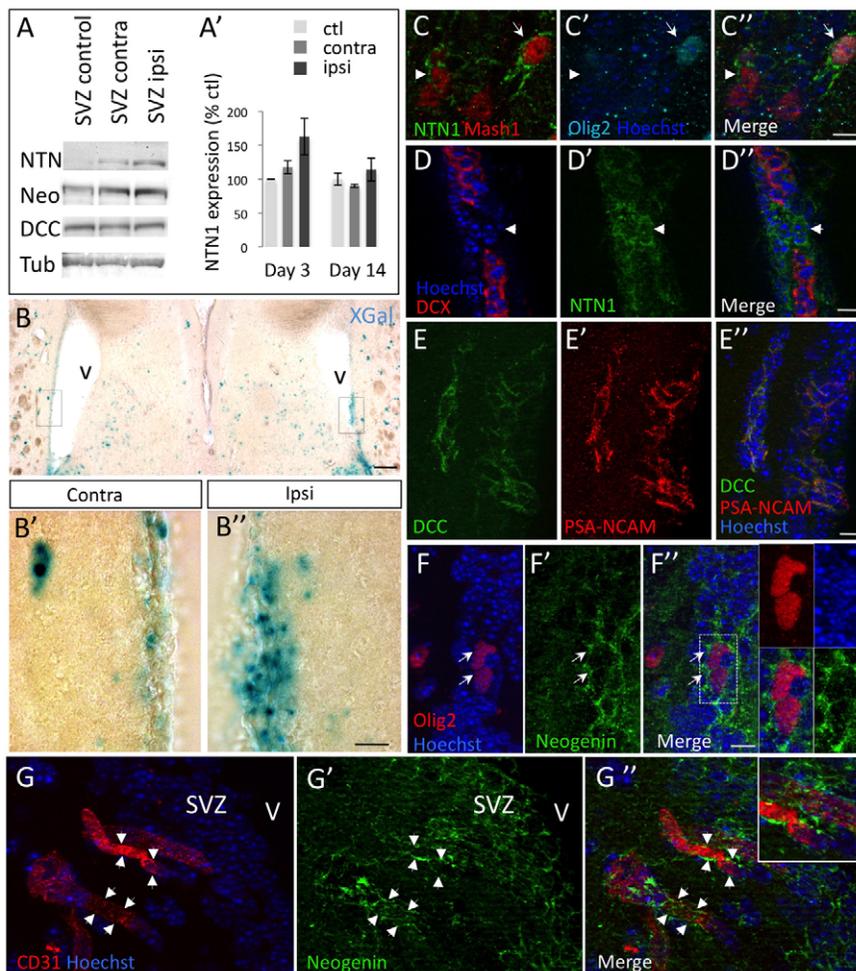
study provided evidence that this gene trap vector accurately reports the pattern of endogenous gene expression in the adult rodent brain (Shatzmiller et al., 2008). We observed an increase in X-Gal staining (Fig. 4B-B'') in the SVZ ipsilateral to the LPC-induced lesion without any increased expression around the lesion site (not shown).

To gain insight into the SVZ cell population expressing NTN1 and its receptors after demyelination, immunolabeling was performed on brain sections. Cells expressing NTN1 were predominately positive for MASH1 (also known as ASCL1) and/or OLIG2 (Fig. 4C-C''). Few GFAP⁺ processes were seen to colocalize with NTN1 labeling (not shown). By contrast, we did not detect DCX⁺ cells expressing NTN1 (Fig. 4D-D''). Thus, it seems that mainly type C cells express NTN1 in the SVZ of demyelinated mice. By contrast, a subpopulation of both neuronal (PSA-NCAM⁺) and glial (OLIG2⁺) SVZ progenitors appeared to express the receptors DCC and neogenin (Fig. 4E-F''). Of note, PSA-NCAM⁺ progenitors exiting the RMS 3 days after LPC injection were often robustly positive for DCC and neogenin (not shown). We also observed neogenin expression in SVZ endothelial cells (Fig. 4G-G'').

These data demonstrate that NTN1 is upregulated in the SVZ of demyelinated mice and that SVZ progenitors and endothelial cells expressing NTN1 receptors could thus respond to this signal. This led us to examine the potential role played by NTN1 in post-lesional angiogenesis and endogenous progenitor cell recruitment to the lesion.

NTN1 promotes progenitor emigration from SVZ explants *in vitro*

The direct effect of NTN1 on cell migration was examined using the SVZ explant migration assay in Matrigel. The SVZ was dissected from healthy adult mice. After 4 days in culture, progenitors began to migrate out of the explants; this process was amplified after 7 days (Fig. 5A-C). Adding NTN1 in the culture medium triggered a significant increase in the number of migrating cells around the explants (49.1% and 31.1% increase after 4 and 7 days, respectively; $P=0.014$ and $P=0.018$; Fig. 5A,B). We ruled out the potential contribution of cell proliferation and/or survival to this increased number of migrating cells (Table 2; supplementary

**Fig. 4. NTN1 is upregulated in the SVZ 3 days after LPC-induced demyelination of the CC.**

(A) Western blot illustrating the expression of NTN1 and its receptors neogenin and DCC in the SVZ of healthy mice or demyelinated mice. Tubulin (Tub) was used as a loading control. (A') Semi-quantitative analysis of NTN1 expression in the SVZ of healthy (ctl) or LPC-injected mice, contralateral and ipsilateral to the lesion side. Error bars indicate s.e.m. Quantification from three independent western blots. (B-B'') X-Gal staining in an NTN1-lacZ transgenic mouse at 3 dpl. (B', B'') Enlarged views of the boxed regions in B. (C-D'') Phenotype of NTN1-expressing cells in the SVZ after demyelination. (C-C'') Triple immunolabeling for NTN1, MASH1 and OLIG2 showing a SVZ oligodendrocyte precursor cell (OPC) (MASH1⁺ OLIG2⁺, arrow) and a type C cell (MASH1⁺ OLIG2⁻, arrowhead) strongly expressing NTN1. (D-D'') DCX⁺ cells in the SVZ do not appear to express NTN1 (arrowhead) after demyelination. (E-F'') Phenotype of SVZ cells expressing NTN1 receptors. (E-E'') Type A cells (PSA-NCAM⁺) expressing the receptor DCC. (F-F'') SVZ OLIG2⁺ cells expressing the receptor neogenin (arrows). (G-G'') Blood vessels (CD31 labeling of endothelial cells in red) expressing neogenin in the SVZ (arrowheads). Inset (G') illustrates the central part of the picture at higher magnification. V, ventricle; SVZ, subventricular zone. Scale bars: 200 μ m in B; 50 μ m in B', B''; 10 μ m in C-G''.

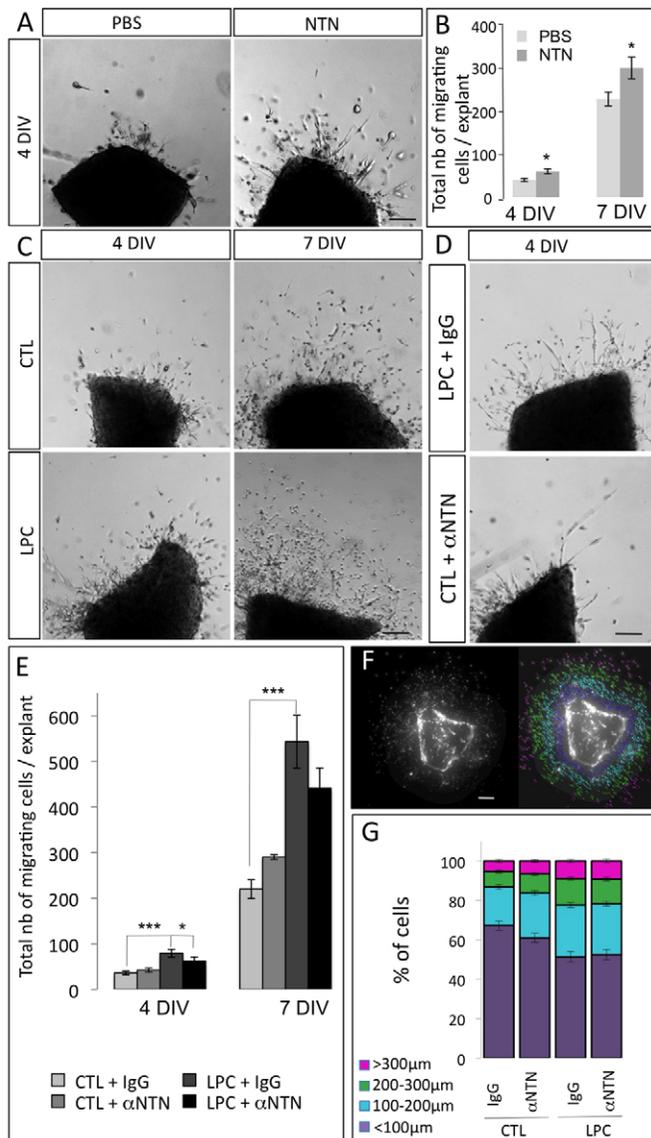


Fig. 5. NTN1 promotes progenitor cell emigration from SVZ explants.

(A) Representative images of SVZ explants from healthy adult mice after 4 days in culture (DIV, days *in vitro*) in Matrigel, with (NTN) or without (PBS) the addition of NTN1 (10 ng/ml) in the medium. (B) Quantitative analysis of the number of emigrating cells at 4 and 7 days after plating in the presence or absence of NTN1. Quantification from two independent cultures, $n=15-35$ explants. (C) Representative images of adult SVZ explants from healthy (CTL) and demyelinated (LPC) mice after 4 and 7 days of culture. (D) Adult SVZ explants from demyelinated mice in the presence of anti-NTN1 blocking antibody (or non-specific IgG control antibody) in the culture medium after 4 days of culture. (E) Total number of cells that had emigrated from the explants after 4 and 7 days of culture. Quantification from two independent cultures, $n=18-24$ explants. (F) Method for the quantitative analysis of migration distance: 100 μm -wide bands were drawn around the explants, and the number of cells present in each ring was counted. (G) Quantitative analysis of cell repartition around the explants according to their migration distance. * $P<0.05$, *** $P<0.001$. Error bars indicate s.e.m. Scale bars: 100 μm .

material Fig. S3). These results suggest that NTN1 could directly contribute to increased cell emigration after lesion.

To confirm this, we examined the SVZ from LPC-injected adult mice. Remarkably, the number of cells outside the explants was

approximately double in the LPC group compared with the control group at both times (Fig. 5C). To examine the contribution of NTN1 to this migration, an NTN1 blocking antibody (or a non-specific IgG control) was added to the culture medium (Fig. 5D,E). Whereas IgG did not prevent LPC-induced cell emigration (79.4 ± 8.4 versus 36.0 ± 4.0 cells in LPC versus control explants, respectively; $P=5.88\times 10^{-6}$; Fig. 5D,E), the anti-NTN1 antibody partly counteracted lesion-induced migration (Fig. 5E; $P=0.026$) without affecting cell proliferation or survival (Table 2; supplementary material Fig. S3); therefore, the difference between control and lesion-derived explants was no longer significant ($P=0.157$). Seven days after plating, the general pattern was similar to that observed on day 4, although the inhibitory effect of the NTN1 blocking antibody on lesion-induced cell migration was no longer significant (Fig. 5E).

By contrast, anti-VEGF blocking antibody in the culture medium had no impact on the number of migrating cells (85.4 ± 6.7 versus 81.8 ± 6.0 cells for IgG and anti-VEGF, respectively; $P=0.523$), suggesting that VEGF does not directly affect cell migration outside the explants after LPC.

To further evaluate cell migration we analyzed the repartition of cells according to their distance from the edge of the explants (Fig. 5F). In lesioned mice, the proportion of cells that migrated most distally from the edge of the explants was significantly increased compared with control mice (Fig. 5G; $P=0.00029$). The addition of NTN1 blocking antibody to the culture medium did not modify this repartition (Fig. 5E; $P=0.805$).

These results indicate that progenitor cell migration *in vitro* is enhanced when SVZ explants are derived from demyelinated mice. Furthermore, these data demonstrate that NTN1 contributes to the promotion of cell exit from SVZ explants but does not influence migration distances.

NTN1 promotes angiogenesis *in vitro* and *in vivo*

The tube formation assay is a well-established *in vitro* assay to examine the angiogenic properties of candidate molecules. bEnd3 mouse brain endothelial cells grown in a three-dimensional Matrigel matrix have the ability to migrate, rearrange and form tubes, thus recapitulating the angiogenic process. Using this assay, we showed that NTN1 addition to the culture medium significantly promotes tube formation (Fig. 6A-D; $P=0.01$). Interestingly, when SVZ progenitors from actin-GFP mice were co-cultured with bEnd3 cells in tube-forming conditions, neural progenitors were found associated with endothelial cells (Fig. 6E). Furthermore, SVZ cells adopted the typical morphology of migrating progenitors along the tubes (Fig. 6F).

To reinforce this finding, NTN1 (or PBS for control) was administered via intracerebroventricular injection in adult mice and blood vessel density was examined 3 days later in the SVZ. NTN1-injected mice exhibited a 30.8% increase in vessel density in the SVZ compared with controls ($14.0\pm 0.9\%$ versus $10.7\pm 0.4\%$ in the NTN1 and PBS groups, respectively; $P=0.025$; Fig. 6G-I). Interestingly, in the context of this analysis progenitor cells emigrating from the SVZ/RMS were more numerous in NTN1-injected compared with PBS-injected mice (423.2 ± 70.0 versus 232.0 ± 82.8 DCX⁺ cells in CC and striatum for NTN1-treated and PBS-treated mice, respectively; $P=0.05$). This effect was not associated with variations in SVZ cell proliferation (633.6 ± 69.3 versus 551.0 ± 74.7 pH3⁺ cells/mm² in PBS-injected and NTN1-injected mice, respectively; $P=0.21$), suggesting a role for NTN1 in SVZ cell emigration.

Table 2. Quantification of cell proliferation and survival in SVZ explant migration experiments

	BrdU ⁺ Hoechst ⁺ cells		BrdU ⁺ Tuj1 ⁺ cells		Calcein ⁺ Hoechst ⁺ cells		Casp3 ⁺ Hoechst ⁺ cells		Casp3 ⁺ Tuj1 ⁺ cells	
PBS	4.0±0.9%		1.8±0.6%		68.1±3.5%		2.6±0.5%		3.4±0.6%	
NTN1	2.9±0.7%	<i>P</i> =0.46	1.6±0.4%	<i>P</i> =0.81	67.5±3.7%	<i>P</i> =0.39	2.9±0.5%	<i>P</i> =0.75	3.9±0.6%	<i>P</i> =0.48
IgG	5.5±0.7%		4.4±0.8%		61.6±5.5%		2.8±0.7%		4.6±1.6%	
Anti-NTN1	5.6±0.8%	<i>P</i> =0.98	4.0±0.7%	<i>P</i> =0.64	66.3±3.0%	<i>P</i> =0.58	2.5±0.3%	<i>P</i> =0.91	3.7±0.4%	<i>P</i> =0.87

Cell proliferation was analyzed using BrdU labeling (BrdU was added in the culture medium for 4 hours) and cell survival was estimated both by the vital dye calcein and by activated caspase 3 (Casp3) immunocytochemistry.

Blocking NTN1 inhibits post-lesional angiogenesis in the SVZ and neural progenitor cell recruitment to the demyelination lesion

Considering the effects of NTN1 on angiogenesis and progenitor cell migration, we postulated that NTN1 upregulation by SVZ progenitors after LPC-induced demyelination might participate in post-lesional reactivity. In order to test this hypothesis we carried out intracerebral injection of anti-NTN1 blocking antibody concomitant with LPC-induced demyelination. Lesion-induced

blood vessel reactivity in the SVZ was abolished by anti-NTN1 blocking antibody treatment (Fig. 7A-C). By contrast, it did not impact blood vessel density at the lesion site ($10.5\pm 0.4\%$ versus $9.6\pm 0.2\%$ laminin⁺ area in IgG-treated and anti-NTN-treated mice, respectively; *P*=0.09).

The type of association between progenitors and blood vessels was not altered (*P*>0.4), but, although it did not reach significance (*P*=0.14), anti-NTN1 treatment led to a 26% decrease in the number of DCX⁺ progenitors that were in the process of emigrating from the niche (not shown). Remarkably, the total number of BrdU-traced SVZ-derived cells present at the lesion site was also significantly diminished, by 31%, by anti-NTN1 treatment (Fig. 7D-F; *P*=0.05), whereas IgG injection did not alter cell recruitment as compared with mice injected with LPC alone (891.4 ± 130.0 and 717.4 ± 76.1 without and with IgG treatment, respectively; *P*=0.48). In addition, NTN1 neutralizing antibody treatment did not significantly affect SVZ cell proliferation (3111.3 ± 530.6 and 4480.4 ± 695.1 Ki67⁺ cells/mm² in IgG-treated and anti-NTN1 treated mice, respectively;

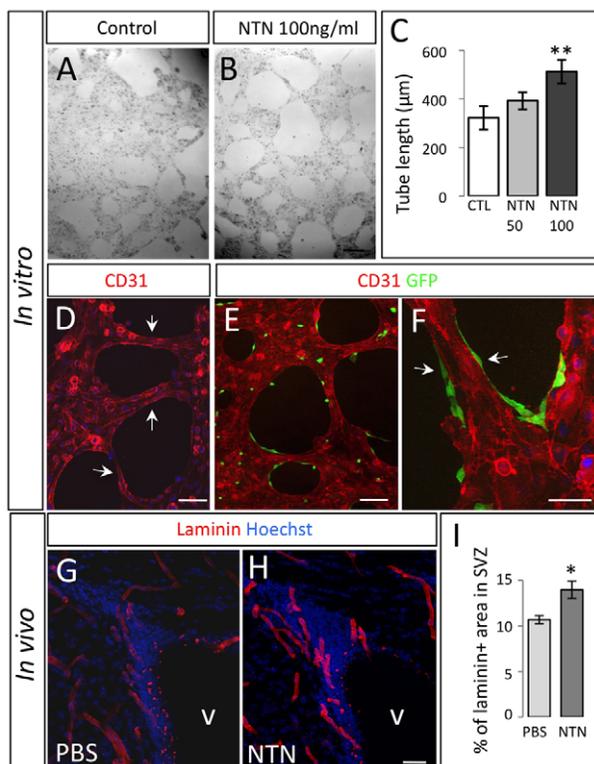


Fig. 6. NTN1 positively regulates brain angiogenesis *in vitro* and *in vivo*. (A-D) Tube formation assay with bEnd3 cells cultured in Matrigel. (A,B) Addition of NTN1 in the culture medium stimulates the formation of tubes. (C) Quantitative analysis of endothelial tube length in the absence (CTL) or presence of NTN1 in the culture medium. Quantification from three independent cultures, *n*=11 dishes. (D) CD31 labeling at the end of the culture showing the organization of endothelial cells in tubes (arrows). (E,F) Co-culture of bEnd3 cells (CD31⁺) and SVZ progenitors (GFP⁺). Most neural progenitors are found associated with endothelial cells and exhibit the typical migratory phenotype along the endothelial tubes. (G,H) Blood vessel labeling (laminin antibody) in the SVZ of mice 3 days after intracerebroventricular injection of PBS (G) or NTN1 (H). (I) Quantitative analysis of blood vessel density in the SVZ 3 days after intracerebroventricular injection of PBS or NTN1. **P*<0.05, ***P*<0.01. Error bars indicate s.e.m. *n*=4 and 5 mice for PBS- and NTN1-injected mice, respectively. V, ventricle. Scale bars: 200 µm in A,B; 50 µm in D-H.

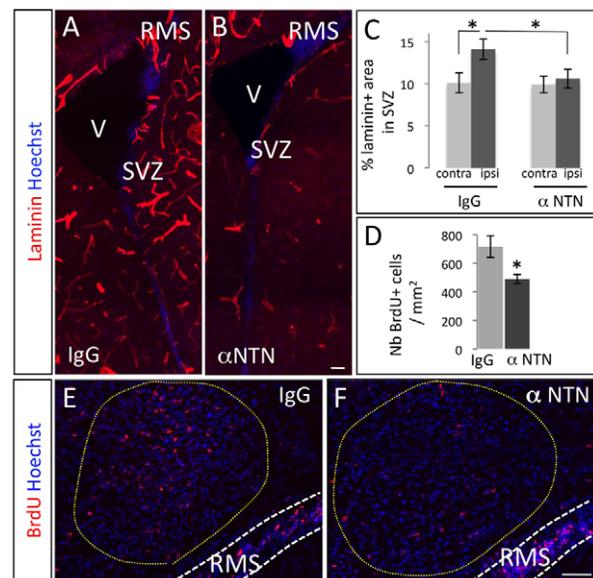


Fig. 7. Blocking NTN1 inhibits post-lesional angiogenesis in the SVZ and reduces SVZ-derived cell recruitment at the lesion site. (A,B) Blood vessel staining (laminin immunofluorescence) in the ipsilateral SVZ of LPC-injected mice after IgG control (A) or anti-NTN1 (B) treatment. (C) Quantitative analysis of blood vessel density in the SVZ at 4 dpl in mice treated with non-specific IgG or with anti-NTN1 blocking antibody. (D) Quantitative analysis of SVZ-derived cell recruitment at the lesion site as assessed by BrdU tracing. **P*<0.05. Error bars indicate s.e.m. For C and D, *n*=5 mice per group. (E,F) Representative images of SVZ-derived cell recruitment in control (E) and anti-NTN1-treated mice (F). BrdU⁺ cells (red) are seen in the RMS (delineated by white dashed lines) and within the lesion (delineated by yellow dotted lines). RMS, rostral migratory stream; SVZ, subventricular zone; V, ventricle. Scale bars: 50 µm.

$P=0.11$), strongly suggesting that the observed effect on progenitor cell recruitment was indeed due to reduced migration from the SVZ/RMS to the lesion.

Altogether, these results suggest that NTN1 expression in the SVZ as a consequence of demyelination of the CC participates in SVZ blood vessel remodeling and in progenitor cell mobilization at the lesion site.

DISCUSSION

In this study we have shown that major vascular remodeling occurs in the SVZ as a consequence of LPC-induced demyelination of the CC. We demonstrate that neural progenitors ectopically migrating outside the SVZ/RMS are tightly associated with blood vessels while escaping from their niche. Further, prevention of post-lesional angiogenesis resulted in reduced SVZ-derived cell recruitment at the lesion site, suggesting a functional role for reactive blood vessels in this emigration process. Finally, we identified NTN1, which is upregulated in the SVZ after demyelination, as one of the key factors involved in this process. NTN1 is able to increase blood vessel density and stimulate neural progenitor cell emigration. Blocking NTN1 signaling suppressed post-lesional angiogenesis in the SVZ and decreased SVZ-derived cell recruitment to the lesion.

Many studies have revealed the impact of brain pathology on stem cell proliferation and migration. However, the impact on blood vessel remodeling within the SVZ has been neglected despite the important relationships between neural stem cells and the vasculature in the niche. The rare studies that examined angiogenesis in the SVZ were conducted in models of cerebral ischemia, as hypoxia triggers the upregulation of VEGF, and showed either no effect (Thored et al., 2007) or a dramatic expansion of the vascular tree in the SVZ (Gotts and Chesselet, 2005; Young et al., 2013). Here, we show in a non-ischemic but demyelinating lesion of the CC that the SVZ vasculature exhibits structural remodeling and increased blood vessel density. Although VEGF participates in this SVZ reactivity, it is clearly not the only factor involved. Indeed, blocking NTN1 also antagonizes demyelination-induced angiogenesis in the SVZ. Interestingly, a previous study suggested that NTN1 and VEGF might act synergistically to induce angiogenesis (Park et al., 2004). The potential relationship between VEGF and NTN1 in our model remains to be clarified.

To note, anti-NTN1 treatment did not impact blood vessel density at the lesion site, a result in accordance with our finding that NTN1 is specifically upregulated in the SVZ but not at the lesion site. These results underline a specific role for NTN1 in lesion-induced SVZ reactivity. Several groups have highlighted the influence of NTN1 on angiogenesis both *in vitro* and during development (Navankasattusas et al., 2008; Park et al., 2004; Wilson et al., 2006; Fan et al., 2008; Castets and Mehlen, 2010). We demonstrate here that NTN1 injection in the lateral ventricle of adult mice is sufficient to trigger angiogenesis in the SVZ. Since we observed expression of the NTN1 receptor neogenin by endothelial cells in the SVZ, this suggests a direct effect of NTN1 on vascular remodeling, a hypothesis that is also supported by our *in vitro* results from the tube formation assay. Beneficial effects of exogenous NTN1 have been described after stroke, which could in part be attributed to a more rapid restoration of cerebral blood flow (Lu et al., 2011), reduced apoptosis (Wu et al., 2008) and increased progenitor cell migration (Sun et al., 2011). In this context, our study provides evidence that, even in the absence of ischemia, NTN1 can contribute to angiogenesis and brain repair.

Although major NTN1 functions (regulation of axon guidance and progenitor cell migration) mainly operate during development, NTN1 is still present in the adult brain, notably in the cerebellum and striatum (Livesey and Hunt, 1997; Shatzmiller et al., 2008). Furthermore, NTN1 and its receptors have recently been detected in postnatal SVZ and RMS (Bradford et al., 2010; Hakanen et al., 2011). Here, we observed by western blot very low NTN1 expression in the SVZ of adult healthy mice but a strong and significant increase in SVZ a few days after CC demyelination. Co-immunostaining experiments indicate that mainly type C cells and SVZ OPCs express NTN1 and that both neuronal and glial SVZ progenitors express the receptors DCC and neogenin, suggesting that they can respond to NTN1. Notably, NTN1 seems to play a role in the development of the olfactory bulb via the regulation of progenitor cell migration from the SVZ to the bulb (Murase and Horwitz, 2002; Hakanen et al., 2011). We showed here that lesion-induced emigration of neuronal progenitors from SVZ explants is significantly reduced by NTN1 blocking antibodies.

The effect of NTN1 on cell emigration could be explained by a chemorepulsive or a detachment effect on SVZ progenitors. We found DCC and neogenin expression in the adult SVZ, in agreement with a previous study (Bradford et al., 2010), but almost undetectable levels of UNC5, which is the main receptor responsible for NTN1 repulsion effects (Bradford et al., 2009). This receptor expression pattern is therefore not favorable to the repulsive hypothesis. It has recently been suggested that NTN1 might function in a cell-autonomous manner to promote the movement/detachment/release of progenitor cells from embryonic forebrain explants (Hakanen et al., 2011). Interestingly, a recent study pointed out a dual effect of NTN1 on glioblastoma progression by promoting both angiogenesis and invasiveness (Shimizu et al., 2012). A detachment effect of NTN1 could therefore contribute to enhancing progenitor cell emigration and recruitment at the lesion site. In line with this hypothesis, we previously showed that a strategy that promotes progenitor cell detachment from the SVZ/RMS is sufficient to enhance the recruitment process (Courtès et al., 2011).

Since we observed a highly prominent tangential association between SVZ-derived progenitors exiting from their niche and blood vessels, we assumed that these new vessels could contribute to progenitor cell recruitment at the lesion site. This hypothesis is strengthened by the positive correlation between the number of ectopic neuroblasts and SVZ vascular reactivity (comparing ipsilateral versus contralateral sides, day 3 versus day 14 after lesion, NTN1 versus PBS injection in healthy mice), and by decreased SVZ-derived cell recruitment when angiogenesis is abolished by anti-VEGF or anti-NTN1 treatment. The type of association between progenitors and blood vessels was not affected in these various conditions but the number of progenitors migrating outside their niche was strongly correlated to blood vessel density. Therefore, we assume that vascular reactivity disrupts the organized blood vessel scaffold that normally guides and maintains neuroblast migration within the SVZ/RMS pathway. Newly generated blood vessels create new 'roads' facilitating the emigration of the progenitors from the SVZ/RMS towards the CC.

Numerous studies have underlined the importance of vasculature for progenitor cell migration in the adult brain, both under physiological conditions (Bovetti et al., 2007; Snappyan et al., 2009; Whitman et al., 2009) and during ectopic migration following an ischemic insult (Thored et al., 2007; Yamashita et al., 2006). Growth factors and matrix metalloproteases secreted by activated endothelial cells have been shown to promote progenitor cell

proliferation and survival, and to facilitate migration, respectively (Wang et al., 2006). In the demyelination model that we used, the lesion is located in the CC. We observed that progenitors are closely associated with blood vessels specifically while they emigrate from their niche, to which they are normally restricted by an astrocytic network. Once they reach the CC they spontaneously migrate along fiber tracts (Cayre et al., 2006) and thus do not need to associate with blood vessels. Chemoattractant cytokines and factors secreted at the lesion site, such as TGF β , FGF2, PDGFA (Hinks and Franklin, 1999), semaphorin 3A and 3F (Williams et al., 2007) or CNTF (Vernerey et al., 2013), then probably contribute in concert to orient cell migration toward the lesion. We thus propose that post-lesional SVZ angiogenesis is an important step to initiate ectopic progenitor cell emigration out of the niche.

We propose a scheme in which, following CC demyelination, SVZ progenitors modify their environment via NTN1 secretion that leads to cell detachment and to local vasculature remodeling, which in turn provides new roads for progenitors to emigrate from their niche.

Altogether, this study highlights the potential utility of the dual and synergistic effect of the vascular and neural properties of NTN1 in the development of new therapeutic strategies for brain repair.

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Competing interests statement

The authors declare no competing financial interests.

Author contributions

M.C. and P.D. conceived the project, designed the experiments and wrote the manuscript; S.C. initiated the work (first observations and analysis); F.M. and M.G. performed progenitor/blood vessels association analysis and *in vitro* migration assays; K.A. performed angiogenesis blocking experiments *in vivo*; and A.Z. analyzed NTN1 effects *in vitro*.

Supplementary material

Supplementary material available online at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.092999/-/DC1>

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