Angiopoietin-2 Regulates Cortical Neurogenesis in the Developing Telencephalon

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Vascular-specific growth factor angiopoietin-2 (Ang2) is mainly involved during vascular network setup. Recently, Ang2 was suggested to play a role in adult neurogenesis, affecting migration and differentiation of adult neuroblasts in vitro. However, to date, no data have reported an effect of Ang2 on neurogenesis during embryonic development. As we detected Ang2 expression in the developing cerebral cortex at embryonic day E14.5 and E16.5, we used in utero electroporation to knock down Ang2 expression in neural progenitors located in the cortical ventricular zone (VZ) to examine the role of Ang2 in cortical embryonic neurogenesis. Using this strategy, we showed that radial migration from the VZ toward the cortical plate of Ang2-knocked down neurons is altered as well as their morphology. In parallel, we observed a perturbation of intermediate progenitor population and the surrounding vasculature. Taken together, our results show for the first time that, in addition to its role during brain vasculature setup, Ang2 is also involved in embryonic cortical neurogenesis and especially in the radial migration of projection neurons.

Keywords: angiopoietin-2, embryonic neurogenesis, intermediate progenitors, radial migration, Tbr2

Introduction

Neurovascular niches are defined as supportive vascular environment for neurogenesis (Palmer et al. 2000; Riquelme et al. 2008). These neurovascular niches have been first described in the neurogenic zones of the adult brain (Palmer et al. 2000), and more recently, some studies suggested their existence in the developing brain too (Javaherian and Kriegstein 2009; Stubbs et al. 2009). Moreover, over the past few years, a notion of cross talk between neurogenesis and angiogenesis has emerged. Indeed, Vasaudevan et al. (2008) reported that, in the brain, blood vessel development follows the same intrinsic program than neuronal development, and these blood vessels are important for proper patterning of neurogenesis (Vasaudevan et al. 2008; Javaherian and Kriegstein 2009). Shen et al. (2004) also showed that both embryonic and adult neural stem cells respond to factors secreted by endothelial cells (Shen et al. 2004).

It is now well documented that some factors previously described as specific neurogenic or angiogenic growth factors can affect both vascular and nervous systems (Carmeliet and Tessier-Lavigne 2005; Raab and Plate 2007). These factors can be grouped in a family called “angiocurins” (Zachcigna et al. 2008). Among these factors, it has been shown that the main angiogenic factor, the vascular endothelial growth factor (VEGF) is able to regulate neurogenesis (for review: Galvan et al. 2006). During angiogenesis, it has been shown that the vascular-specific growth factor angiopoietin-2 (Ang2) also plays a key role in new blood vessel formation by acting in coordination with VEGF. Interestingly, some studies suggested that Ang2 might be also involved in several steps of adult neurogenesis (Ward and Lamanna 2004; Androutsellis-Theotokis et al. 2009; Liu et al. 2009). In vitro, Ang2 stimulates the proliferation and migration of neuronal precursor cells isolated from the adult brain (Liu et al. 2009; Androutsellis-Theotokis et al. 2010). In vivo, stroke upregulates Ang2 expression in subventricular zone (SVZ) neural progenitor cells (Liu et al. 2009), and an administration of Ang2 stimulates the proliferation of neural precursor cells in the healthy brain (Androutsellis-Theotokis et al. 2009). Nevertheless, no data have reported a role of Ang2 in neural progenitors in the embryonic brain where both vascular and nervous systems set up simultaneously. As we detected the expression of Ang2 in the developing dorsal telencephalon, we hypothesized that this angiogenic factor could be also involved in embryonic cortical neurogenesis. Global and long-term genetic manipulation of Ang2 could affect both neural and vascular development and involves compensation mechanisms, making difficult to determine its role in each process. Thus, to circumvent these phenomena, the aim of this study was to evaluate the role of Ang2 during embryonic cortical neurogenesis, using in utero electroporation as a transient knockdown approach to specifically target neural progenitors in the ventricular zone (VZ) of the cerebral cortex. The results obtained in this study demonstrate that endogenous Ang2 influences neurogenesis and especially neuronal migration in the developing dorsal telencephalon.

Materials and Methods

Animals

Parkes mice were housed, bred, and treated according to the guidelines approved by the UK home office under the Animals (Scientific Procedures) Act 1986.

Plasmids

Ang2 short hairpin RNA (shRNA) and Ang2 expression constructs were purchased from Origene. To generate Ang2 shRNA, the following hairpin was cloned into pGFP-V-RS vector (Origene): GFS81922 5'-ACAGGCAAGGCTCAACAACCTGGCTCCTTTCA-3'. The non-targeting shRNA pGFP-V-RS plasmid served as control (Origene). To generate Ang2 expression construct, the mouse Ang2 cDNA (NM_009640) was cloned into pCMV6-Kan/Neo (Origene). To generate expression construct harboring silent point mutations in the sequence recognized by Ang2 shRNA, site-directed mutagenesis was performed using QuickChange II Site-Directed Mutagenesis Kit (Stratagene) using the following primers: sense, 5'-GCTGGTACGTCACGGTGAACACTGCTCTCTTGAGGCGAGCAGG-3'
cortical plate (CP) based on cell density as previously described (Nguyen et al. 2006). In all experiments, slices from at least 3 independent experiments were processed, and for each sample, 2 sections were analyzed by confocal microscopy, and 10× magnified fields zoomed 1.5× were acquired. The proportion of GFP+ neurons in each subregion was counted. Eight sections were analyzed for each condition from at least 3 embryos from 2 or 3 litters obtained in parallel experiments. Results are indicated as mean ± standard error of the mean (SEM). A statistical analysis was performed using 1-way analysis of variance (ANOVA) followed by a Fisher protected least significant difference (PLSD) post hoc test.

Cell Culture and Western Blot
Mouse embryocarcinoma cell line P19 was grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 2 mM glutamine, and 1% penicillin/streptomycin and cotransfected with mouse Ang2 cDNA cloned in pCMV6/Kan/Neo (Origene) and Ang2 shRNA using lipofectamine and according to the manufacturer’s protocol. After protein extraction, western blotting was performed using standard protocols (Pacary et al. 2007). The following primary antibody was used: goat anti-Ang2 (2 mg/mL; Santa Cruz) and secondary antibody rabbit anti-goat horseradish peroxidase conjugate (Biorad). Signal was revealed using enhanced chemiluminescent detection reagents according to the manufacturer’s protocol (Amersham Biosciences).

Vessel Analysis
Analysis of blood vessels was performed automatically using NIH ImageJ software, version 1.41 for Windows (http://rsb.info.nih.gov/ij/) as previously described (Valable et al. 2009). Cryosections of 50 μm immunostained for the vessel marker PECAM were photographed in the electroporated area. The photographs of blood vessels were binarized by local thresholding at the average between minimum and maximum intensity of the neighborhood, thus segmenting vessels close to half height. Vessel area was computed as the surface occupied by vessels, and the quantification of median distance of parenchymal pixels to the closest vessel also called vessel domain was determined using distance maps. Eight slices obtained from the brain of 4 embryos belonging to different litters were analyzed. Results are indicated as mean ± SEM. A statistical analysis was performed using 1-way ANOVA followed by a Fisher PLSD post hoc test.

Results
To start addressing the role of Ang2 in embryonic neurogenesis, we studied its expression in the developing cortex at both messenger RNA (mRNA) and protein levels. By in situ hybridization on coronal brain sections, we showed that Ang2 is expressed in the VZ and SVZ at embryonic day (E) 14.5 (Fig. 1A). As shown in Fig. 1A and B, antisense probe revealed a specific signal compared with sense probe. Immunostaining for Ang2 revealed a similar pattern of expression at E14.5 (Fig. 1C), sustaining our hypothesis of a role of Ang2 in cortical neurogenesis. Negative control for the primary antibody showed no specific signal (Fig. 1D). Similar results were obtained on brain sections at E16.5, revealing an expression of Ang2 in the cortex at both mRNA and protein levels (Fig. 1E and F).

Ang2 angiogenic effects have been mainly relayed by its tyrosine kinase receptor Tie2 (Maisonpierre et al. 1997). More recently, β1monomer-containing integrins have also been involved not only in Ang2 angiogenic effects (Carlson et al. 2001; Hu et al. 2006; Imanishi et al. 2007) but also in neurogenesis (Loulier et al. 2009; Marchetti et al. 2010). Accordingly, we showed that, at E14.5, Tie2 and β1 integrins are expressed on blood vessels (Figs. 2A, B, D, and E). According to Loulier et al. (2009), we observed that radial glial cells lining the ventricle also expressed β1 integrins (Fig. 2D and F).

In Utero Electroperoration
In utero electroporation was performed as previously described (Saito and Nakatsuji 2001; Nguyen et al. 2006). Uteri of anesthetized timed-pregnant mothers (14 days) with isoflurane in oxygen carrier were exposed through a 2-cm incision in the ventral peritoneum. Embryos were carefully lifted using ring forceps through the incision and placed on humidified gauze pads. DNA (1 μg/μL, prepared using Endo Free plasmid purification kit, Qiagen) mixed with 0.05% Fast Green (Sigma) was injected through the uterine wall into the telencephalic vesicle using pulled borosilicate needles and a Femtojet microinjector (VWR). Five electrical pulses were applied at 70 V (50 ms duration) across the uterine wall using a cryostat (Leica). The uterine horns were then replaced using pulled borosilicate needles and a Femtojet microinjector (VWR). The embryos were then allowed to develop for 2 days on warmed heating pads and then processed for tissue analyses.

Immunohistochemistry
Embryonic brains were fixed in 4% paraformaldehyde overnight and placed in 20% sucrose/PBS for 48 h. Brains were then embedded in optimum cutting temperature mounting medium and frozen before sectioning at 14 or 50 μm using a cryostat (Leica). The following primary antibodies were used: chicken anti-green fluorescent protein (GFP; 1.4 mg/mL; Chemicon), rabbit anti-Tbr2 (1.2 μg/mL; Abcam), goat anti-Ang2 (2 mg/mL; Santa Cruz; Lee et al. 2006), rat anti-Platelet/endothelial cell adhesion molecule (PECAM) (1.6 μg/mL; BD Pharmingen), rabbit anti-Pax6 (20 μg/mL; Covance), mouse anti-Ki67 (5 μg/mL; BD Pharmingen), rabbit anti-Caspase-3 (0.5 μg/mL; R&D systems), mouse anti-nestin (100 ng/mL; Millipore), rabbit anti-Tie2 (1 μg/mL; Santa Cruz), rat anti-β1 integrin (10 mg/mL; Chemicon), and goat anti-collagen IV (40 μg/mL; SouthernBiotech). Sections were then incubated with appropriate secondary antibodies (Invitrogen). Negative control was realized using an isotype-matched primary antibody giving no specific signal in the brain.

Cell Counting and Statistics
Nuclear cell staining with Toto-3-iodide was used to define different subregions of the cerebral cortex (SVZ/VZ, intermediate zone [IZ], and cortical plate [CP]) based on cell density as previously described (Marteau et al. 2004).
However, no Tie2 immunostaining was detected in these cells (Fig. 2A and C).

As Ang2 and its receptors are expressed in the VZ at the time of radial migration of cortical projection neurons, we wondered whether it had a role in this process by performing shRNA-mediated knockdown. We selected a construct (GI581922) that efficiently knocked down Ang2 expression (Fig. 3A and Supplementary Fig. 1). This Ang2 shRNA was then electroporated in utero together with an enhanced GFP expression construct into the mouse cerebral cortex at E14.5. Nontargeting shRNA construct served as control. Effect on neuron radial migration was assessed 3 days later by determining the proportion of GFP+ cells in the different regions of the cortex: SVZ/VZ, IZ, and CP. As showed in Fig. 3B and C, Ang2 knockdown led to defects in neuron radial migration. Indeed, after Ang2 silencing, the number of electroporated cells reaching the CP was significantly decreased (control: 62.3 ± 4.1% and Ang2 shRNA: 46.4 ± 3.1%), whereas more cells remained in the IZ compared with control condition (control: 23.4 ± 1.6% and Ang2 shRNA: 36.8 ± 3.0%). In order to verify that this result was not due to an off-target effect of the shRNA, we coelectroporated Ang2 shRNA with an Ang2 expression construct (pCMV-Ang2) harboring 4 silent point mutations in the sequence targeted by the shRNA (Fig. 3A). The
overexpression of Ang2 rescued the radial migration defects (Fig. 3B and C) induced by Ang2 shRNA alone (CP: 63.5 ± 2.2% and IZ: 24.2 ± 1.6%), demonstrating that these defects are due to a specific knockdown of Ang2 expression.

This reduced migration of Ang2-silenced neurons could be an indirect consequence of an increase of cell proliferation or cell death. To test these possibilities, we performed immunostaining for the cell proliferation marker Ki67 and counted the proportion of GFP and Ki67 double-labeled cells. No effect was observed on cell proliferation (Supplementary Fig. 2A and B). In addition, only few cells were immunostained by the cell death marker caspase-3, and no difference was seen between control and Ang2 shRNA (data not shown). Together, these results confirm that Ang2 specifically affects the regulation of cortical neuron migration.

In addition to migration defects, Ang2-silenced neurons also displayed abnormal morphologies. While migrating toward the CP, neurons are normally subjected to morphological changes. The cells exhibit a uni/bipolar morphology in the VZ and the CP and adopt a transient multipolar phenotype when they migrate through the SVZ and IZ. We observed that Ang2-silenced neurons in the VZ had already adopted a multipolar morphology with an irregular shape compared with control (Fig. 4A). In the IZ, electroporated neurons also exhibited more processes than in control condition (Fig. 4B). Moreover, in the CP, 3 days after Ang2 knockdown, the proportion of neurons showing more than 2 primary processes was significantly increased compared with control neurons. This effect was observed (Fig. 4C and D) throughout the CP from the lower to the upper CP (lower cortical plate, control: 46.8 ± 5.3%, Ang2 shRNA: 75.6 ± 4.0%; medial cortical plate, control: 26.4 ± 1.4%, Ang2 shRNA: 61.7 ± 2.6%; upper cortical plate, and...
control: 24.4 ± 4.1%. Ang2 shRNA: 47.6 ± 3.4%). Altogether, these data indicate that Ang2 regulates the shape of migrating cortical neurons.

A subset of cortical neuron precursors, the intermediate progenitor cells (IPC), have been shown to be closely associated with blood vessels and thus might be particularly sensitive to vascular cues and vice versa (Javaherian and Kriegstein 2009). We wondered whether Ang2 knockdown in VZ cells acts on these IPC known to express specifically the T-box containing protein T-brain-2 (Tbr2) (Englund et al. 2005). In cortices electroporated with Ang2 shRNA, we observed (Fig. 5A and B) a significant decrease in the number of GFP+/Tbr2+ cells compared with control (control: 26.1 ± 2.9%; Ang2 shRNA: 8.2 ± 0.73%). During embryonic cortical neurogenesis, IPC are normally present in the VZ and SVZ at nonsurface locations and more scattered in the IZ. As can be observed in Fig. 5C and D, we found that Ang2 knockdown induced an abnormal localization of these Tbr2+ cells. Indeed, IPC were found directly at ventricular surface (green arrow, Fig. 5C), and fewer cells were observed in the IZ than in the control (red arrow, Fig 5C). Conversely, no effect was observed on either expression or localization of Pax6 expressing cells (Supplementary Fig. 3A and B). In addition, no morphological defects of the Ang2-silenced radial glial cells were detected using an immunostaining for nestin (data not shown). Moreover, in parallel, we observed that Ang2 knockdown alters the vascular network in the IZ where the proportion of GFP+/Tbr2+ cells is reduced compared with control (Fig. 6A). A quantification of this disturbance showed that Ang2 silencing leads to a significant decrease of the total vascular surface in the electroporated area (control: 225 245 ± 8790 μm² and Ang2 shRNA: 164 060 ± 10 449 μm²) along with a significant increase of the microvascular domains, which reflect the distance between vessels (control: 17.25 ± 0.34 μm and Ang2 shRNA: 23.5 ± 0.53 μm) (Fig. 6B).

Discussion

The central nervous system vasculature and the neuronal network have been described to develop concomitantly following the same compartment-specific cell autonomous patterning signals involving common transcription factors, cytokines, and/or their corresponding receptors (Vasudevan et al. 2008). Although shared mechanisms regulating vascular and neuronal network have been well identified in adult (Riquelme et al. 2008), they are less documented in the developing brain. The lack of data about the cross talk between neurogenesis and angiogenesis during embryogenesis is mainly due to the early embryonic lethality of angiogenic factor-null embryos, which until now have excluded phenotypical studies in the developing nervous system. For example, homozygous or heterozygous mutants for the VEGF gene die by embryonic day 10.5 or E11.5 (Carmeliet et al. 1996). Similarly, mice lacking the receptor Tie2 or its ligands Ang1 or Ang2 die at E9.5 and E12.5 or at postnatal stage (P14), respectively, due to vascular alterations (Suri et al. 1996). In this study, we used a gene-targeting approach based on in utero electroporation of neural progenitors at E14.5. This approach allows to overcome direct vascular system defects and compensation mechanisms, which
might set up in response to a long-term loss of a gene (Devenport and Brown 2004), and allows to examine the role of Ang2 in embryonic neurogenesis. The present study provides, for the first time, evidences that downregulation of the angiogenic factor Ang2, in the developing murine cerebral cortex, impairs neuronal migration. To date, only one study reported that VEGF overexpression influences the spatial distribution of IPC (Tbr2+) by influencing the developing vasculature of the embryonic cortex (Javaherian and Kriegstein 2009). However, there is no data regarding the angiopoietins, although these factors are well recognized to act in cooperation with VEGF in the vascular system setup. We combined an shRNA approach and in utero electroporation to specifically silence the expression of Ang2 in cortical neural progenitors lining the lateral ventricle of the embryonic brain. The silencing of Ang2 expression in the developing cerebral cortex led to a blockade of neuronal migration toward the CP, showing that endogenous Ang2, known as a specific vascular growth factor, is also involved in cortical embryonic neurogenesis. Moreover, Ang2 knockdown altered the morphology of migrating neurons, from the VZ to the CP, with cells exhibiting irregular shapes and more processes.

Ang2 is a secreted factor and can bind to either its tyrosine kinase receptor Tie2 (Maisonnierre et al. 1997) or β1 monomer-containing integrins (Carlson et al. 2001). Our results, in accordance with other authors (Loulie et al. 2009), showed that, at E14.5, β1 integrin, but not Tie2, is expressed on radial glial cells, suggesting that β1 integrin might mediate Ang2 function in neural progenitors.

Interestingly, a similar role has also been suggested for this angiogenic factor during adult stroke-induced neurogenesis as Ang2 affects SVZ cell migration in an in vitro neurosphere migration assay, an effect that does not require its receptor Tie2 but rather depends on integrins (Liu et al. 2009). β integrins, notably β1 monomer-containing integrins, have been involved both in the angiogenic effects of Ang2 (Carlson et al. 2001; Hu et al. 2006; Imanishi et al. 2007) and in the neurogenesis. Indeed, β1 integrin is expressed by radial glia at the ventricular surface during neurogenesis (Loulie et al. 2009). Interestingly, downregulation of the α5β1 integrin in the developing murine cerebral cortex leads to defects similar to what we observed when we knocked down Ang2 expression, suggesting an involvement of β1 integrin in Ang2 function. Indeed, targeting α5β1 integrin impairs both radial neuronal

Figure 6. Ang2 silencing in neural progenitors alters the surrounding vascular network. (A) Representative picture of the effect of Ang2 silencing on vascular network (indicated by the white arrow) visualized by PECAM staining and on Tbr2 localization in the SVZ/VZ and IZ at E17.5, 3 days after the electroporation. Scale bar, 200 μm. (B) Quantification of the effect of Ang2 silencing on the vascular surface and intervessel distance (median distance of parenchymal pixels to the closest vessel). Graph plots are mean ± SEM, and 8 sections were analyzed for each condition from at least 3 embryos from 2 or 3 litters obtained in parallel experiments. *P < 0.05 vs. control and **P < 0.01 vs. control after a significant ANOVA followed by a Fisher PLSD post hoc test.
migration (Marchetti et al. 2010) and morphology, with an enhancement of the proportion of multipolar cells. Taken together, our results are in favor of a role of Ang2 in neuronal migration during cortical development.

The downregulation of Ang2 expression also affected IPC. IPC belongs to a subpopulation of neuronal progenitors, which are the most closely associated with developing blood vessels during embryonic neurogenesis (Stubbs et al. 2009). As intermediate progenitors mimic the pattern of capillaries, it has been suggested that angiogenesis and neurogenesis patterns are coordinated during development (Stubbs et al. 2009). At the molecular level, our study sustains this hypothesis as we found that Ang2 silencing disrupts normal localization of IPC along with an alteration of the surrounding vascular network. These results might be compared with those obtained with VEGF and reinforce the concept of a cross talk between the neuronal and vascular networks during development. Indeed, a VEGF overexpression leads to an alteration of IPC distribution due to its effects on vasculature (Javaherian and Kriegstein 2009). Like for adult stem cells, there is now recent evidences for a role of a vascular niche in embryonic neurogenesis, specifically in the IZ, where the vasculature provides a neurogenic niche within IPC can proliferate suggesting that blood vessels are important for proper patterning of neurogenesis (Javaherian and Kriegstein 2009; Stubbs et al. 2009).

With in utero electroporation, we only target neural progenitors lining the lateral ventricles (Hatanaka and Murakami 2002). Consequently, the vascular phenotype observed might be due to a non-cell autonomous effect of Ang2 knockdown. As Ang2 receptors, both Tie2 and β1 integrin, are expressed by blood vessels, we can hypothesize that these vascular changes were caused by a reduction of Ang2 secretion by the neighboring neural population. Moreover, according to Javaherian and Kriegstein (2009) study, these vascular changes might be responsible for intermediate progenitors mislocalization. So the phenotype of intermediate progenitors after Ang2 silencing might also result, at least in part, from a non-cell autonomous effect. This hypothesis is reinforced by the fact that a part of mislocalized Tbr2 positive cells after Ang2 knockdown are not electroporated GFP positive cells.

Together, our data reinforce the hypothesis of the existence of a neurovascular niche supporting the development of the embryonic cortex in which neuronal precursors and the developing vasculature respond to identical growth factors. Here, we demonstrated the importance of the angiogenic growth factor Ang2 in this microenvironment and showed that this angiogenic factor also has a crucial role in the developmental processes of neurogenesis and neuronal migration.

Supplementary Material
Supplementary material can be found at: http://www.cercor.oxfordjournals.org/.

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References


