Development/Plasticity/Repair

The Angiogenic Factor Angiopoietin-1 Is a Proneurogenic Peptide on Subventricular Zone Stem/Progenitor Cells

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In the adult mammalian brain, the subventricular zone (SVZ) hosts stem cells constantly generating new neurons. Angiopoietin-1 (Ang-1) is an endothelial growth factor with a critical role in division, survival, and adhesion of endothelial cells via Tie-2 receptor activity. Expression of Tie-2 in nonendothelial cells, especially neurons and stem cells, suggests that Ang-1 may be involved in neurogenesis. In the present work, we investigated the putative role of Ang-1 on SVZ neurogenesis. Immature cells from SVZ-derived neurospheres express Ang-1 and Tie-2 mRNA, suggesting a role for the Ang-1/Tie-2 system in the neurogenic niche. Moreover, we also found that Tie-2 protein expression is retained on differentiation in neurons and glial cells. Ang-1 triggered proliferation via activation of the ERK1/2 (extracellular signal-regulated kinase 1/2) mitogen-activated protein kinase (MAPK) kinase pathway but did not induce cell death. Accordingly, coincubation with an anti-Tie-2 neutralizing antibody prevented the pro-proliferative effect of Ang-1. Furthermore, Ang-1 increased the number of NeuN (neuronal nuclear protein)-positive neurons in cultures treated for 7 d, as well as the number of functional neurons, as assessed by monitoring $[Ca^{2+}]_i$ rises after application of specific stimuli for neurons and immature cells. The proneurogenic effect of Ang-1 is mediated by Tie-2 activation and subsequent mTOR (mammalian target of rapamycin kinase) mobilization. In agreement, neuronal differentiation significantly decreased after exposure to an anti-Tie-2 neutralizing antibody and to rapamycin. Moreover, Ang-1 elicited the activation of the SAPK (stress-activated protein kinase)/JNK (c-Jun N-terminal kinase) MAPK, involved in axonogenesis. Our work shows a proneurogenic effect of Ang-1, highlighting the relevance of blood vessel/stem cell cross talk in health and disease.

Introduction

In the adult mammalian brain, neurogenesis occurs constitutively in the subventricular zone (SVZ) from glial fibrillary acidic protein (GFAP)-expressing neural stem cells (Lledo et al., 2006; Zhao et al., 2008; Chojnacki et al., 2009).

Neurogenesis is tightly regulated by diffusible factors and some of them are regulators of angiogenesis. In the postnatal and adult brain, angiogenesis occurs by sprouting of new vessels from preexisting ones. Molecular players act in concert to regulate the multistep process of angiogenesis, such as vascular endothelial growth factor (VEGF), which induces mitosis of endothelial cells. Moreover, stabilization and maturation of the sprouts is ensured by angiopoietin-1 (Ang-1), an endothelial growth factor that promotes cellular adhesion of supporting cells and survival (Thurston et al., 1999; Papapetropoulos et al., 2000; Patan, 2000; Yancopoulos et al., 2000). Ang-1 effects are mediated through binding to the Tie-2 tyrosine kinase receptor (Suri et al., 1996).

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Apart from its role in angiogenesis, VEGF is a potent inducer of neurogenesis, increasing proliferation and neuronal differentiation in progenitor cell cultures as well as in the SVZ and subgranular zone *in vivo* (Jin et al., 2002; Schänzer et al., 2004; Sun et al., 2006; Segi-Nishida et al., 2008). Despite the well described involvement of Ang-1 in angiogenesis, little is still known about its effect on neurogenesis. The Tie-2 receptor is found in neurons (Valable et al., 2003; Kosacka et al., 2005) and glial cells such as Schwann cells and glioblastoma cells (Poncet et al., 2003; Lee et al., 2006; Makinde and Agrawal, 2008). In addition, Tie-2 expression is found in stem cells, including embryonic stem cells from human (Parati et al., 2002) and mouse brains (Bai et al., 2009a), rat liver stem cells (Kuroda et al., 2002), and hematopoietic and mesenchymal stem cells (Huang et al., 1999; Yuasa et al., 2002). Expression outside the vascular compartment suggests a wider biological role of the Ang-1/Tie-2 system than that previously thought and Ang-1 may regulate stem cell dynamic. Indeed, exogenous addition of Ang-1 promotes survival of neurons, mesenchymal stem cells, and neural progenitor cells after exposure to serum deprivation or hypoxia (Valable et al., 2003; Bai et al., 2009b). In hematopoietic stem cells, Ang-1/Tie-2 is crucial for the maintenance of the stem cell state (Hirao et al., 2004). Moreover, Ang-1 elicits neuronal differentiation and neurite outgrowth in mouse embryonic cortical and dorsal root ganglion cells (Kosacka et al., 2005; Bai et al., 2009a). After cortical and striatal stroke, angiogenic remodeling is accompanied by neurogenesis in the peri-infarct area and in the SVZ. Stroke elicits an

upregulation of endothelial-derived Ang-1 and the migration of newly born doublecortin (DCX)-positive neuroblasts from the SVZ to the damaged area. These data suggest a positive action of Ang-1 on SVZ neurogenesis (Ohab et al., 2006; Yamashita et al., 2006; Shin et al., 2008).

In the present work, we propose to unravel the role of Ang-1 on SVZ neurogenesis, with a focus on proliferation, differentiation, and axonogenesis.

Materials and Methods

All experiments were performed in accordance with the European Community (86/609/EEC) guidelines for the care and use of laboratory animals.

Subventricular zone cell cultures. SVZ cells were prepared from 1- to 3-d-old C57BL/6 donor mice as described previously (Agasse et al., 2008b). Briefly, mice were killed by decapitation, and the brains were removed and placed in HBSS (Invitrogen) supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen).

Fragments of SVZ were dissected out of 450-μm-thick coronal brain sections, obtained by using a McIlwain tissue chopper, and then digested in 0.025% trypsin (Invitrogen) and 0.265 mM EDTA (Invitrogen) (10 min; 37°C), followed by mechanical dissociation with a P1000 pipette. The resulting cell suspension was diluted in serum-free medium (SFM) composed of DMEM (DMEM/Ham's F-12 medium GlutaMAX-I) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 1% B27 supplement, 10 ng/ml epidermal growth factor, and 5 ng/ml basic fibroblast growth factor-2 (all from Invitrogen). Single cells were then plated on uncoated Petri dishes at a density of 3000 cells/cm^2 and allowed to develop in an incubator with 5% CO_2 and 95% atmospheric air at 37°C. Six to 8 d after plating, the SVZ neurospheres were collected and seeded onto poly-D-lysine (0.1 mg/ml)-coated glass coverslips, placed into 12-well cell culture plates for single-cell calcium imaging (SCCI) experiments or 24-well cell culture plates for immunocytochemistry, and covered with 1 ml or 500 μl, respectively, of SFM devoid of growth factors. Then, SVZ neurospheres were allowed to develop for 2 d with 5% CO_2 and 95% atmospheric air at 37°C before experimental treatments.

Cell proliferation studies. To investigate the effect of Ang-1 on cell proliferation, SVZ cells were treated with 10, 100, and 500 ng/ml Ang-1 (Sigma-Aldrich) for 48 h and then exposed to 10 μM 5-bromo-2′-deoxyuridine (BrdU) (Sigma-Aldrich) for the last 4 h of each Ang-1 treatment. After BrdU incubation, SVZ cells were fixed in 4% paraformaldehyde (PFA) for 30 min and rinsed for 30 min in PBS (containing 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) at room temperature. BrdU was then unmasked after successive passages in 1% Triton X-100 for 30 min at room temperature, ice-cold 0.1 M HCl for 20 min, and finally 2 M HCl for 60 min at 37°C. After neutralization in sodium borate buffer (0.1 M $Na_2B_4O_7 \cdot 10H_2O$, pH 8.5; Sigma-Aldrich) for 15 min at room temperature, cells were rinsed in PBS, and nonspecific binding sites were blocked with 3% bovine serum albumin (BSA) (Sigma-Aldrich) and 1% Triton X-100 in PBS for 30 min at room temperature. SVZ cultures were then incubated for 90 min with the primary mouse Alexa Fluor 594-conjugated monoclonal anti-BrdU antibody (1:100; A21304; Invitrogen) in PBS containing 0.1% Triton X-100 and 0.3% BSA. After a rinse in PBS, SVZ cell nuclei were stained with Hoechst 33342 (Invitrogen) at 2 μg/ml in PBS for 5 min at room temperature. Finally, the preparations were mounted using Dako fluorescent medium (Dako). Fluorescent images were recorded using a LSM 510 Meta confocal microscope (Carl Zeiss) or an Axioskop 2 Plus fluorescent microscope (Carl Zeiss). To disclose whether the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase pathway was involved in the proliferative effect of Ang-1, in another set of experiments SVZ cells were coincubated with 500 ng/ml Ang-1 and 20 μM 1,4-diamino-2,3-dicyano-1,4-bis(*o*-aminophenylmercapto)butadiene monoethanolate (U0126) (Sigma-Aldrich), a highly selective inhibitor of both mitogen-activated protein extracellular signal-regulated kinase 1 (MEK1) and MEK2, a type of MAPK/ERK kinase (Learish et al., 2000). BrdU (Sigma-Aldrich) incubation and immunorevelation followed as described above.

Determination of cell apoptosis by terminal deoxynucleotidyl transferase dUTP nick end labeling. Cell apoptosis in SVZ cells was evaluated by the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). This method is based on the specific activity of terminal deoxynucleotidyl transferase (TdT), which attaches labeled nucleotides (dUTP) to the 3′-OH ends of the DNA generated during apoptotic-induced DNA fragmentation. At the end of each Ang-1 and/or anti-Tie-2 incubation protocol (48 h), SVZ cultures were fixed for 30 min in 4% PFA at room temperature, rinsed in PBS, and permeabilized in 0.25% Triton X-100 (Sigma-Aldrich) for 30 min at room temperature. Thereafter, SVZ cultures were incubated for 20 min in 3% H_2O_2 and reacted for terminal transferase (0.25 U/μl) biotinylated dUTP (6 μM) nick end labeling of fragmented DNA in TdT buffer, pH 7.5 (all from Roche), for 1 h at 37°C in a humidified chamber. The enzymatic reaction was stopped by 15 min of incubation in 300 mM NaCl and 30 mM sodium citrate buffer (both from Sigma-Aldrich). After an additional rinse in PBS, cultures were incubated for 30 min at room temperature with the avidin–biotin–peroxidase complex (1:100; Vector Laboratories). Peroxidase activity was revealed by the DAB (diaminobenzidine) chromogen (0.025%; Sigma-Aldrich) intensified with 0.08% $NiCl_2$ in 30 mM Tris-HCl, pH 7.6, buffer containing 0.003% H_2O_2. The cell preparations were then dehydrated in ethanol (75°, 2 min; 80°, 2 min; 85°, 2 min; 96°, 2 min), cleared in xylene (3 min), and mounted using DEPEX mounting medium (Fluka). Photomicrographs of TUNEL were recorded using a digital camera (Axiocam HRC; Carl Zeiss) adapted to an Axioskop 2 Plus fluorescent microscope (Carl Zeiss).

Single-cell calcium imaging studies. To investigate the influence of Ang-1 on neuronal differentiation, SVZ neurospheres were allowed to develop for 7 d with recombinant human Ang-1 (500 ng/ml) at 37°C. To determine whether the Ang-1 receptor Tie-2 was involved in the putative proneurogenic effect, SVZ cells were treated with 500 ng/ml Ang-1 together with 5 μg/ml anti-Tie-2 neutralizing antibody (Sigma-Aldrich). At the end of these treatments, SCCI experiments were performed according to a method developed in our laboratory. Briefly, to determine the functional differentiation pattern of SVZ cells, the variations of free intracellular calcium levels ($[Ca^{2+}]_i$) were analyzed in single cells after stimulation with 50 mM KCl or 100 μM histamine (Sigma-Aldrich) (Agasse et al., 2008a). KCl depolarization causes an increase in $[Ca^{2+}]_i$ in neurons (Ambrósio et al., 2000), whereas stimulation with histamine leads to an increase in $[Ca^{2+}]_i$ in stem/progenitor cells (Tran et al., 2004). SVZ cultures were loaded for 45 min with 5 μM fura-2 AM (Invitrogen), 0.1% fatty acid-free BSA, and 0.02% pluronic acid F-127 (Invitrogen) in Krebs' solution (132 mM NaCl, 4 mM KCl, 1.4 mM $MgCl_2$, 1 mM $CaCl_2$, 6 mM glucose, 10 mM HEPES, pH 7.4), in an incubator with 5% CO_2 and 95% atmospheric air at 37°C. After a 10 min postloading period at room temperature in the same medium without fura-2 and pluronic acid, to obtain a complete hydrolysis of the probe, the glass coverslip was mounted on an RC-20 chamber in a PH3 platform (Warner Instruments) on the stage of an inverted fluorescence microscope (Axiovert 200; Carl Zeiss). Cells were continuously perfused with Krebs' solution and stimulated by applying high-potassium Krebs' solution (containing 50 mM KCl, isosmotic substitution with NaCl) or 100 μM histamine. Solutions were added to the cells by a fast-pressurized (95% air, 5% CO_2 atmosphere) system (AutoMate Scientific). The variations of $[Ca^{2+}]_i$ were evaluated by quantifying the ratio of the fluorescence emitted at 510 nm after alternate excitation (750 ms) at 340 and 380 nm, using a Lambda DG4 apparatus (Sutter Instrument) and a 510 nm bandpass filter (Carl Zeiss) before fluorescence acquisition with a 40× objective and a CoolSNAP HQ digital camera (Roper Scientific). Acquired values were processed using the MetaFluor software (Molecular Devices). Histamine/KCl values for fura-2 fluorescence ratio were calculated to determine the extent of neuronal maturation in cultures.

To investigate the involvement of the phosphoinositide-3-kinase (PI3K)/AKT signaling pathway in response to Ang-1, SVZ neurospheres were treated with 500 ng/ml human recombinant Ang-1 and/or 20 nM rapamycin (Tocris Bioscience) for 7 d. On the second day, the treatments were renewed. Rapamycin inhibits mammalian target of rapamycin kinase (mTOR), a serine-threonine kinase of the downstream signal molecules of PI3K/AKT kinases that, in neurons, is involved in the control

size of the soma as well as the directional axonal growth and dendritic tree development (Swiech et al., 2008).

Immunostainings. After fixation in 4% PFA for 30 min at room temperature, nontreated SVZ cells (48 h or 7 d after depositing on poly-D-lysine) or SVZ spheres, obtained by centrifugation in a Cellspin I (Tharmac), were permeabilized, and nonspecific binding sites were blocked with 1% Triton X-100 and 3% BSA dissolved in PBS for 30 min at room temperature or with 0.5% Triton X-100 and 6% BSA dissolved in PBS for 1 h and 30 min at room temperature. SVZ cells were subsequently incubated overnight at 4°C with the following primary antibodies, all of which had been prepared in PBS containing 0.1% Triton X-100 and 0.3% BSA: rabbit monoclonal anti-DCX (1:200; no. 4604; Cell Signaling Technology), goat polyclonal anti-DCX (1:200; sc-8066; Santa Cruz Biotechnology), mouse monoclonal anti-Tau (1:800; no. 4019; Cell Signaling Technology), mouse monoclonal anti-GFAP (1:500; no. 3670; Cell Signaling Technology), rabbit polyclonal anti-NG2 (1:100; AB5320; Millipore Bioscience Research Reagents), rabbit polyclonal anti-Nestin (1:250; ab5968; Abcam), mouse monoclonal anti-Nestin (1:200; MAB353; Millipore Bioscience Research Reagents), rabbit monoclonal anti-Ang-1 (1:100; ab8451; Abcam), goat polyclonal anti-Tie-2 (1:10; AF313; R&D Systems; 1:50; sc-31266; Santa Cruz Biotechnology), and monoclonal rabbit anti-neuronal class III β-tubulin (1:750; MRB-435P; Covance). Thereafter, the coverslips were rinsed in PBS and incubated for 1 h at room temperature with the appropriate secondary antibodies: Alexa Fluor 594 goat anti-mouse, Alexa Fluor 594 rabbit anti-goat, Alexa Fluor 568 donkey anti-goat, Alexa Fluor 488 rabbit anti-mouse, and Alexa Fluor 488 donkey anti-rabbit all from Invitrogen. After an additional rinse in PBS, SVZ cell nuclei were stained with Hoechst 33342 (2 μg/ml in PBS) for 5 min at room temperature. Finally, the preparations were mounted using Dako fluorescent medium.

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To investigate the involvement of the stress-activated protein kinase (SAPK)/c-Jun N-terminal kinase (JNK) signaling pathway in response to Ang-1 stimulation, SVZ neurospheres were treated with 500 ng/ml human recombinant Ang-1 and or 20 μM anthra[1,9-cd]pyrazol-6(2*H*)-one (SP600125) (Sigma-Aldrich), an inhibitor of SAPK/JNK, for 6 h at 37°C. At the end of each incubation protocol, immunocytochemistry against phosphorylated (activated) forms of the SAPK/JNK kinase, namely phospho-stress-activated protein kinase (P-SAPK)/JNK, was performed, using as a primary antibody rabbit polyclonal anti-phospho-(Thr183/Tyr185)-SAPK/JNK (1:100; 9251S; Cell Signaling Technology). To ascertain that P-SAPK/JNK localization was associated with axons, double-labeling immunocytochemistry was performed to visualize both P-SAPK/JNK and Tau. As secondary antibodies, Alexa Fluor 594 goat anti-mouse and Alexa Fluor 488 donkey anti-rabbit were used (Invitrogen). Each experiment included a series of control cultures not subjected to any drugs.

In *in vivo* experiments, 6- to 8-week-old mice were daily injected with 50 mg/kg BrdU for 5 d and after this time anesthetized and perfused with 0.9% NaCl and subsequently with 4% PFA. Brains were collected in 4% PFA and left at 4°C overnight, until transference to a 30% sucrose solution. Twenty micrometer coronal and sagittal brain slices were obtained by using a Leica CM3050 S Cryostat (Leica Microsystems). Brain slices were stained for BrdU (rat anti-BrdU; 1:50; OBT0030; AbD Serotec; mouse anti-BrdU; 1:100; Invitrogen), Tie-2 (goat anti-Tie-2; 1:10; R&D Systems), DCX (rabbit anti-DCX; 1:200; Cell Signaling Technology), epidermal growth factor (EGF) receptor [mouse anti-EGF receptor (EGFR); 04-290; 1:200; Millipore Bioscience Research Reagents], CD31 (rat anti-CD31; 1:100; 550274; BD Biosciences Pharmingen), Ang-1 (rabbit anti-Ang-1; 1:200; Abcam), and tyrosine hydroxylase (TH) (rabbit anti-TH; 1:500; ab112; Abcam). Secondary antibodies used were Alexa Fluor donkey anti-goat 633, donkey anti-rabbit 488, goat anti-mouse 488, goat anti-rat 488, and goat anti-rat 594 (Invitrogen). Fluorescent images were recorded using a confocal microscope (LSM 510 Meta; Carl Zeiss) or a fluorescent microscope (Axioskop 2 Plus; Carl Zeiss).

Isolation of total RNA from SVZ cells. Total RNA was isolated from SVZ spheres and total murine placenta, using TRI reagent (Sigma-Aldrich) according to the instructions of the manufacturer. Cells were gently homogenized in guanidium thiocyanate and phenol and allowed to stand at room temperature to secure the complete dissociation of nucleoprotein complexes. Chloroform was added, allowing a clear isolation of RNA in the resultant aqueous phase. Then, the RNA was precipitated with isopropanol, and the pellet was washed with 75% (v/v) ethanol, dried at room temperature, redissolved in diethylpyrocarbonate-treated water, and stored at −80°C until use. The total amount of RNA was quantified by optical density (OD) measurements at 260 nm, and the purity was evaluated by measuring the ratio of OD at 260 and 280 nm (RNA/DNA calculator GeneQuant II; GE Healthcare). In addition, RNA quality was assessed by gel electrophoresis.

Reverse transcription-PCR analysis. Ang-1 and Tie-2 mRNA expression was determined by reverse transcription (RT)-PCR. First, cDNA was obtained from the transcription of 2 μg of RNA using M-MuLV Reverse Transcriptase RNase H^- and oligo-dT_{15} primers (Bioron). PCR was performed in a 50 μl reaction system (Bioron) containing 5 μl of template cDNA, 1 μl (0.2 μM) of deoxynucleotide mix, 5 μl of 10× PCR buffer [160 mM $(NH_4)_2SO_4$, 670 mM Tris-HCl, pH 8.8, 0.1% Tween 20, 25 mM $MgCl_2$], 0.2 μl (0.2 μM) of upstream primer, 0.2 μl (0.2 μM) of downstream primer, a variable volume of water, and 0.25 μl (5000 U/ml) of DNA-free sensitive TaqDNA polymerase (Bioron). Primers used in PCRs were as follows: Ang-1, forward primer, 5′-TGCATTCTTCG-CTGCCATTCT-3′, and reverse primer, 5′-ATTGCCCATGTTGAATC-CGGT-3′; Tie-2, forward primer, 5′-ATGTGGAAGTCGAGAGGCGAT-3′, and reverse primer, 5′-CCCTGAACCTTATACCGGATGA-3′ (Sigma-Aldrich); and β-actin, forward primer, 5′-GACTACCTCATGAAGA-TCCT-3′, and reverse primer, 5′-ATCTTGATCATGGTGCTG-3′ (MWG Biotech). PCR products of each sample were subjected to electrophoresis in a 1.5% agarose gel and stained with ethidium bromide. Negative controls were performed without RNA sample, which was replaced by water. Positive controls were total murine placenta mRNA samples. Photographs were taken in a Versa-Doc Imaging System (model 3000; Bio-Rad).

Western blot analysis. For the evaluation of neuronal and glial differentiation in control versus Ang-1-treated condition, 6- to 8-d-old neurospheres were plated onto six-well plates previously coated with poly-D-lysine, allowed to adhere for 24 h in the presence of SFM, and treated in the absence (control) or in the presence of 500 ng/ml Ang-1. The medium was renewed after 48 h. Seven days after the first treatment, the cells were harvested by scraping in 0.1% Triton X-100 PBS. Six- to 8-d-old free floating neurosphere were used for detection of Ang-1 and Tie-2. Mouse placenta was used as a positive control. SVZ cells or placental tissue were homogenized in a homogenizer system in 50 mM Tris-HCl, 0.5% Triton X-100, supplemented with 1 mM PMSF, 1 mM dithiothreitol (DTT), 1 μg/ml chymostatin, 1 μg/ml leupeptin, 1 μg/ml antipain, 5 μg/ml pepstatin A (all from Sigma-Aldrich), pH 7.4, at 4°C. The supernatant was collected after centrifugation at 14,000 rpm for 10 min, at 4°C. Protein concentration was measured by the BCA method and samples were treated with SDS-PAGE sample buffer [6× concentrated: 350 mM Tris, 10% (w/v) SDS, 30% (v/v) glycerol, 0.6 M DTT, 0.06% (w/v) bromophenol blue], boiled 5 min at 95°C, and stored at −80°C until use for Western blotting analysis. Proteins (60 and 80 μg of total protein for Ang-1 and Tie-2 immunoblots, respectively; 5 μg for GFAP and βIII tubulin immunoblots) were separated by SDS-PAGE on acrylamide/bisacrylamide gels and transferred onto PVDF (polyvinylidine difluoride) membranes using a gel transfer apparatus. After blockade of the nonspecific binding sites with 5% nonfat milk in PBS containing 0.5% Tween (Sigma-Aldrich) (PBST), for 1 h at room temperature, the membranes were probed with 1:3000 rabbit anti-Ang-1 (Abcam), 1:250 goat anti-Tie-2 (R&D Systems), 1:20,000 rabbit anti-GFAP (G9269; Sigma-Aldrich), and 1:1000 mouse anti-βIII tubulin

(Covance) at 4°C overnight. Membranes were washed with PBST, incubated for 1 h at room temperature with alkaline phosphatase-conjugated secondary antibodies (anti-rabbit, 1:20,000; anti-goat, 1:5000; both from GE Healthcare), and visualized using ECF reagent (GE Healthcare) on the Storm 860 Gel and Blot Imaging System (GE Healthcare). Immunoblots were reprobed with β-actin (mouse anti-β-actin; 1:2000; Sigma-Aldrich; anti-mouse; 1:10,000; GE Healthcare) antibody to ensure equal sample loading and densitometric analyses were performed using the ImageQuant software.

Statistical analysis. In all experiments, except for immunocytochemistry of cytospin preparations, measurements were performed in the border of SVZ neurospheres where migrating cells form a cell pseudo-monolayer (Gage, 2000). For SCCI experiments, the percentage of neuronal-like responding cells (with a histamine/KCl ratio <0.8) was calculated on the basis of one microscopic field per coverslip, containing ~100 cells (magnification, 40×). Except where otherwise specified, the experiments were at least replicated in three independent culture preparations. Within each experiment, three coverslips for each condition were analyzed. Percentages of NeuN-, BrdU-, or TUNEL-immunoreactive cells in SVZ cell cultures were calculated from cell counts in five independent microscopic fields in each coverslip with a 40× objective (~200 cells per field). Quantification of the number of neuritic ramifications positive for P-SAPK/JNK per neurosphere, as well as the total neuritic length per neurosphere (at 6 h), was performed in two independent cultures (two coverslips for each condition) in ~20 nonoverlapping fields per coverslip using digital images (magnification, 20×). Software used was Axiovision, release 4.6 (Carl Zeiss). Data are expressed as means ± SEM. Statistical significance was determined by using the unpaired two-tailed Student *t* test, with $p \leq 0.05$ considered to represent statistical significance.

Results

SVZ cells express both Ang-1 and its receptor Tie-2

The expression of both Ang-1 and the Tie-2 receptor was first investigated in SVZ neurospheres in proliferative conditions. Briefly, primary neurospheres were grown from single SVZ dissociated cells in SFM containing 10 ng/ml EGF and 5 ng/ml FGF-2. After 4–5 d, neurospheres were collected and processed for RT-PCR, Western blot, and immunocytochemistry. Transcripts of mRNA for Ang-1 and Tie-2 were detected by RT-PCR in SVZ neurospheres (Fig. 1*A*). Additionally, by Western blotting, expression of Ang-1 and Tie-2 protein was demonstrated in SVZ cells (Fig. 1*B*). Moreover, Tie-2 and Ang-1 were detected by immunocytochemistry in nestin-positive SVZ neurospheres (Fig. 1*C*,*Cc1*,*Cc2* or *D*,*Dd1*,*Dd2*, respectively) (for the independent channels, see supplemental data S1, available at www.jneurosci.org as supplemental material). These results suggest that SVZ neurospheres secrete Ang-1 that may signal in an autocrine/paracrine manner to modulate the SVZ cell dynamic.

To disclose whether Tie-2 expression is maintained on differentiation, SVZ neurospheres were seeded onto poly-D-lysine and allowed to differentiate in SFM devoid of growth factors for 48 h to 7 d. During this period of time, cells migrate out of the neurospheres and form a pseudo-monolayer so-called "carpet," constituted of neurons, oligodendrocytes, and astrocytes in different stages of maturation. Expression of the Tie-2 receptor is maintained along the neuronal lineage as immature neurons expressing DCX (Fig. 1*E*), as well as Tau-positive mature neurons (Fig. 1*F*), are immunoreactive for Tie-2 (for the independent channels, see supplemental data S1, available at www.jneurosci.org as supplemental material). Nevertheless, expression of Tie-2 is not restricted to the neuronal lineage as both GFAP-positive astrocytes and NG2-positive oligodendrocyte precursors are found immunopositive for Tie-2 (Fig. 1*G*,*H*, respectively) (for the independent channels, see supplemental data S1, available at www.jneurosci.org as supplemental material). These results were confirmed with another anti-Tie-2 primary antibody (supplemental data S2*a–f*, available at www.jneurosci.org as supplemental material). Negative controls were performed to confirm the specificity of the antibodies used for the detection of the Tie-2 receptor and Ang-1 (data not shown). A brain slice double-labeled for CD31 and Tie-2 was used as a positive control as vessels expressed Tie-2 (supplemental data S3, available at

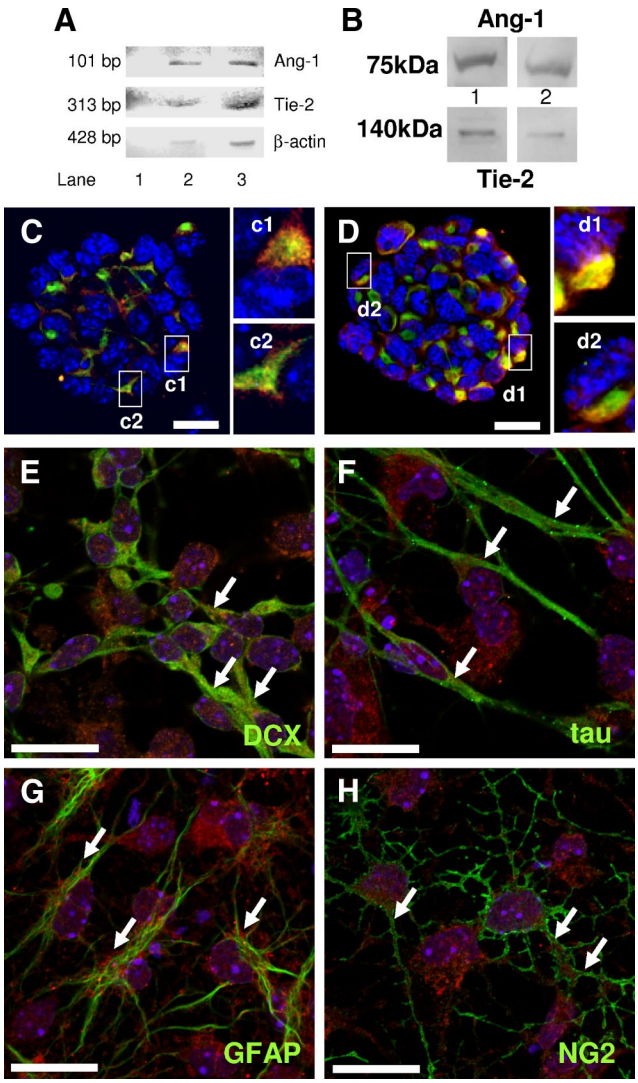

Figure 1. SVZ cells express both Ang-1 and Tie-2. ***A***, RT-PCR detection of Ang-1 and Tie-2 mRNAs in SVZ neurospheres. Lanes 1 and 2 correspond to the negative control (nontemplate negative control) and positive control (total mRNAs from mouse placental tissue), respectively. Lane 3 corresponds to SVZ neurospheres. Ang-1, ~101 bp; Tie-2, ~313 bp. ***B***, Detection of Tie-2 and Ang-1 proteins by Western blotting in SVZ neurospheres. Lane 1 corresponds to the positive control (total proteins form mouse placenta), and lane 2 corresponds to SVZ proliferating cells. ***C***, ***D***, Representative fluorescent confocal digital images depicting Tie-2 and Ang-1 immunoreactivity in SVZ neurospheres (red staining for Tie-2 and Ang-1; green staining for nestin, as a marker of immature cells) ***c1*** and ***c2***, and ***d1*** and ***d2*** are magnifications of squares in ***C*** and ***D***, respectively. ***E***, The Tie-2 receptor is maintained in SVZ cells migrating out of a neurosphere 2 d after plating, and it is expressed in neuroblasts (red staining for Tie-2; green staining for DCX, a marker of immature neurons). ***F***, The Tie-2 receptor is also maintained in SVZ cell-derived neurons after 7 d of differentiation (red staining for Tie-2; green staining for the Tau protein, an axonal marker). ***G***, ***H***, Tie-2 expression is retained in astrocytes (red staining for Tie-2; green staining for GFAP) and oligodendrocyte progenitor cells (red staining for Tie-2; green staining for NG2 chondroitin sulfate proteoglycan, a marker of oligodendrocyte progenitors), respectively. The arrows indicate regions of Tie-2 labeling. Hoechst 33342 staining (blue) was used to visualize cell nuclei. Scale bars, 20 μm.

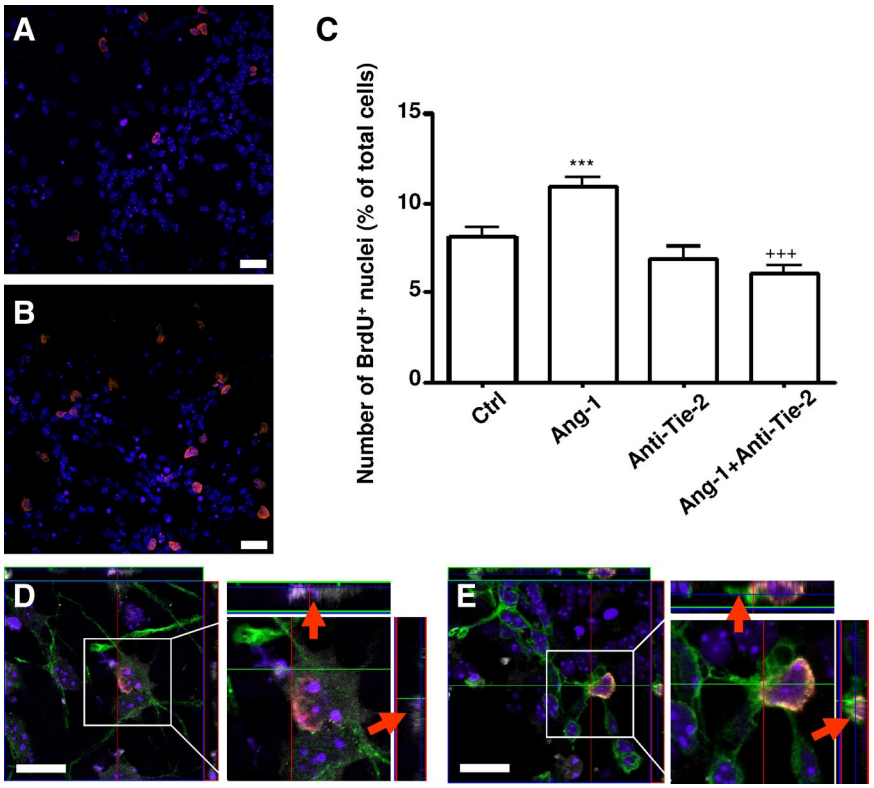

Figure 2. Ang-1 modulates cell proliferation in mouse SVZ cell cultures, an effect mediated by the Tie-2 receptor. ***A***, ***B***, Representative fluorescent confocal digital images of BrdU (red nuclei) and Hoechst 33342 staining (blue nuclei) in a control culture and in a culture treated with 500 ng/ml Ang-1, respectively. ***C***, Bar graph depicts the number of BrdU-positive cells, expressed as percentages of the total number of counted nuclei, in control cultures and in cultures exposed to 500 ng/ml Ang-1 and/or 5 μg/ml anti-Tie-2 neutralizing antibody for 48 h. Data are expressed as mean ± SEM. ***$p < 0.001$, using the unpaired Student *t* test for comparison with SVZ control cultures. $^{+++}p < 0.001$, using the unpaired Student *t* test for comparison with Ang-1-treated SVZ cultures. ***D***, ***E***, Representative *z*-stack confocal digital images of control SVZ cultures showing BrdU-positive cells (in red) positive for Tie-2 (white staining) and DCX (green staining) (***D***) or nestin (green staining) (***E***). The arrows indicate areas of triple labeling. Hoechst 33342 staining (blue) was used to visualize cell nuclei. Scale bars, 20 μm.

www.jneurosci.org as supplemental material). The wide expression of the Tie-2 receptor suggests that Ang-1 may modulate proliferation and differentiation in the SVZ neurogenic niche.

Ang-1 stimulates cell proliferation and exerts no effect on cell death

We first determined whether Ang-1 modulates cell proliferation. For that purpose, we applied increasing concentrations of Ang-1 (10, 100, and 500 ng/ml) for 48 h on SVZ cells in differentiation conditions. BrdU, an analog of the thymidine nucleotide, was added during the last 4 h of the culture session and cells in the S-phase integrated BrdU in their DNA. After fixation, BrdU was immunorevealed and positive nuclei were counted. Representative immunostainings for BrdU in control and 500 ng/ml Ang-1-treated conditions are shown in Figure 2, *A* and *B*, respectively. A significant increase in the number of BrdU-immunopositive nuclei was obtained in cultures incubated with 100 and 500 ng/ml but not with 10 ng/ml Ang-1 compared with control (control: 8.13 ± 0.52%, $n = 15$ coverslips, 12,912 cells counted; 10 ng/ml Ang-1: 7.99 ± 0.67%, $n = 10$ coverslips, 7816 cells counted; 100 ng/ml Ang-1: 10.07 ± 0.53%, $n = 7$ coverslips, 7567 cells counted, $p < 0.05$; 500 ng/ml Ang-1: 11.00 ± 0.43%, $n = 15$ coverslips, 14,935 cells counted, $p < 0.001$). As the most marked effect was obtained with 500 ng/ml Ang-1, we used this concentration for the following studies, as depicted in Figure 2*C*.

To investigate whether the Tie-2 receptor mediates the pro-proliferative effects of Ang-1, SVZ cell cultures were coexposed to 500 ng/ml Ang-1 and 5 μg/ml anti-Tie-2 neutralizing antibody for 48 h followed by BrdU immunoassays. As depicted on Figure 2*C*, the proliferative effect of 500 ng/ml Ang-1 was prevented in the presence of the anti-Tie-2 antibody (5 μg/ml anti-Tie-2 plus 500 ng/ml Ang-1: 6.08 ± 0.44%, $n = 6$ coverslips; 5885 cells counted; $p < 0.001$ vs Ang-1 alone). Hence, Ang-1 promotes proliferation of SVZ cells via Tie-2 binding.

To further disclose the progenitor phenotypes of the cells induced to proliferate on Tie-2 binding, triple immunocytodetections were performed. Some of the BrdU-positive cells were simultaneously positive for Tie-2 and DCX, expressed by neuroblasts (Fig. 2*D*), and for Tie-2 and Nestin, a marker of immature cells (Fig. 2*E*) (see independent channels in supplemental Fig. S4, available at www.jneurosci.org as supplemental material). These data demonstrate that Ang-1 via Tie-2 binding induces proliferation at least in neuroblasts and immature cells.

In addition, we performed TUNEL staining to examine the effects of Ang-1/Tie-2 on apoptosis. Exposure of SVZ cells to 500 ng/ml Ang-1 and/or 5 μg/ml anti-Tie-2 for 48 h did not affect the number of TUNEL-positive nuclei (control: 18.57 ± 1.53%, $n = 10$ coverslips, 4542 cells counted; 500 ng/ml Ang-1: 19.56 ± 2.88%, $n = 4$ coverslips, 3876 cells counted; 5 μg/ml anti-Tie-2 plus 500 ng/ml Ang-1: 23.02 ± 5.37%, $n = 6$ coverslips, 4468 cells counted).

Ang-1 modulates cell proliferation through the ERK/MAPK kinase pathway

Proliferation in SVZ cultures has been reported mainly to depend on the activation of the ERK/MAPK kinase pathway (Learish et al., 2000; Agasse et al., 2008b; Bernardino et al., 2008; Nicoleau et al., 2009). To test whether Ang-1 binding to Tie-2 results in the downstream activation of this pathway, BrdU incorporation assays were performed in the presence of both 500 ng/ml Ang-1 and 20 μM U0126, a highly selective inhibitor of both MEK1 and MEK2, activators of ERK1/2.

As expected, incubation with the MAPK kinase inhibitor alone decreased significantly the normal proliferative activity inherent to SVZ cultures (control: 6.32 ± 0.49%, $n = 6$ coverslips, 5468 cells counted; control plus 20 μM U0126: 3.36 ± 0.31%, $n = 6$ coverslips, 4898 cells counted; $p < 0.001$) (Fig. 3), demonstrating the specificity of U0126 to inhibit proliferation associated to ERK activation. We verified that DMSO, the solvent used to resuspend U0126, was not toxic, by performing TUNEL. In fact, this solvent, diluted 10,000 times from our 20 mM stock solution, did not increase cell death (control: 18.57 ± 1.53%, $n = 10$ coverslips, 7966 cells counted; DMSO, 1/10,000: 17.04 ± 0.34%, $n = 2$ coverslips, 2185 cells counted). Increase of proliferation obtained with 500 ng/ml Ang-1 is abolished in the presence of U1026 (500 ng/ml Ang-1 plus 20 μM U0126: 4.29 ± 0.50% positive nuclei,

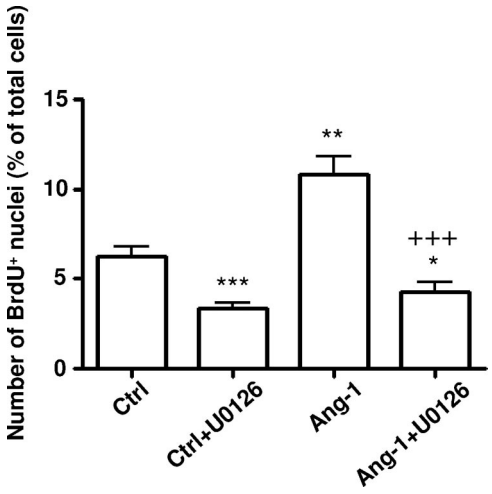

Figure 3. Ang-1 modulates cell proliferation in mouse SVZ cell cultures, an effect mediated by the MAPK/ERK kinase pathway. Bar graph depicts the number of BrdU-positive cells, expressed as percentages of the total number of nuclei per culture, in control cultures and in cultures exposed to 500 ng/ml Ang-1 and/or 20 μM U0126, a highly selective inhibitor of MEK1 and -2, for 48 h. Data are expressed as a mean ± SEM. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, using the unpaired Student *t* test for comparison with SVZ control cultures. $^{+++}p < 0.001$, using the unpaired Student *t* test for comparison with Ang-1-treated SVZ cultures.

$n = 6$ coverslips, 4577 cells counted, $p < 0.05$ compared with control, $p < 0.001$ compared with Ang-1-treated condition) (Fig. 3). Therefore, the pro-proliferative effect of Ang-1 is associated with ERK1/2 MAPK kinase pathway activation.

Ang-1 induces neuronal differentiation via Tie-2 and mTOR activation

To unravel whether Ang-1 promotes neuronal differentiation, cells were treated with 500 ng/ml Ang-1 for 7 d. After that, neuronal differentiation was evaluated after the immunorevelation of the neuronal-specific nuclear protein NeuN (Fig. 4*A*,*B*). Ang-1 induced a significant increase in the number of NeuN-immunoreactive cells compared with the control condition (control: 11.64 ± 1.25%, $n = 12$ coverslips, 9566 cells counted; 500 ng/ml Ang-1: 18.62 ± 1.22%, $n = 9$ coverslips, 8619 cells counted; $p < 0.001$) (Fig. 4*C*). Interestingly, addition of 5 μg/ml anti-Tie-2 neutralizing antibody to the culture together with 500 ng/ml Ang-1 prevented the proneurogenic effect of Ang-1 (5 μg/ml anti-Tie-2 plus 500 ng/ml Ang-1: 13.86 ± 1.42%, $n = 9$ coverslips, 7221 cells counted; $p < 0.05$ vs Ang-1 alone) (Fig. 4*C*). Incubation of the cells with the anti-Tie-2 antibody had no effect on the number of NeuN-positive cells (5 μg/ml anti-Tie-2: 12.49 ± 1.93%, $n = 4$ coverslips, 2883 cells counted) (Fig. 4*C*). These results suggest that Ang-1 increases morphological neuronal differentiation via Tie-2 binding.

Neurogenesis may occur detrimentally to glial differentiation. To disclose this point, SVZ neurospheres were seeded onto poly-D-lysine-coated culture dishes and incubated during 7 d in the absence or the presence of 500 ng/ml Ang-1. Relative amount of astrocytes and neurons were quantified by Western blotting. Figure 4, *D* and *E*, depicts the immunoblots for βIII tubulin and GFAP. Quantification revealed that the amount of GFAP was similar in control and Ang-1-treated condition (control: 100.00 ± 16.80%; 500 ng/ml Ang-1: 81.94 ± 4.86%; $n = 3$ independent experiments). However, Ang-1 increased the βIII tubulin levels, compared with control (control: 100.00 ± 11.39%; 500 ng/ml Ang-1: 137.20 ± 14.90%; $n = 3$ independent experiments). Consistently, immunoreactivity to βIII tubulin was increased in SVZ cultures treated in the presence of Ang-1 (Fig. 4*F*,*G*). This suggests that Ang-1 induces neuronal differentiation but does not decrease the number of glial cells generated.

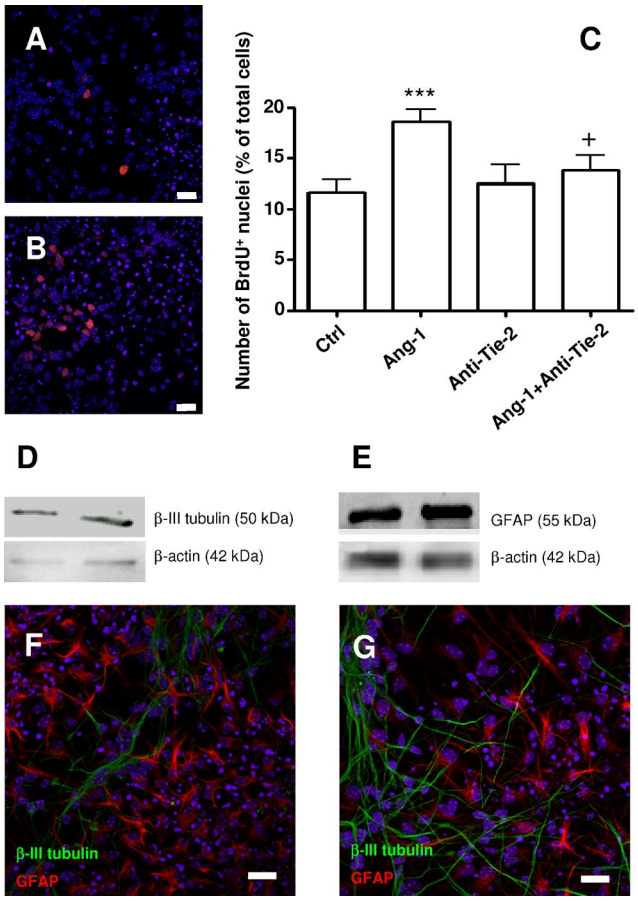

Figure 4. Ang-1 induces neuronal differentiation in mouse SVZ cell cultures via Tie-2 activation. ***A***, ***B***, Representative fluorescent photomicrographs of NeuN-positive neurons (red nuclei) and Hoechst 33342 staining (blue nuclei) in control SVZ cultures (***A***) and in cultures treated with Ang-1 (***B***). Scale bar, 20 μm. ***C***, Bar graph depicts the number of NeuN-positive cells, expressed as percentages of the total number of cells per culture, in control cultures and in cultures treated with 500 ng/ml Ang-1 and/or 5 μg/ml anti-Tie-2 neutralizing antibody for 7 d. Data are expressed as mean ± SEM. ***$p < 0.001$, using the unpaired Student *t* test for comparison with SVZ control cultures. $^{+}p < 0.05$, using the unpaired Student *t* test for comparison with SVZ cultures treated with Ang-1. ***D***, ***E***, Representative Western blots for βIII tubulin (***D***) and GFAP (***E***)—the β-actin blots are provided as loading controls— of total protein extract from SVZ cells treated for 7 d in the absence (control) or the presence of 500 ng/ml Ang-1. ***F***, ***G***, Representative confocal digital images of SVZ cell cultures treated for 7 d in the absence (control) (***F***) or the presence of 500 ng/ml Ang-1 (***G***) and stained for βIII tubulin (green staining) and GFAP (red staining). Hoechst 33342 staining (blue) was used to visualize cell nuclei. Scale bars, 50 μm.

We then evaluated the functional neuronal differentiation in SVZ cultures using a method settled at our laboratory, based on the monitoring of the variations of $[Ca^{2+}]_i$ in single cells in response to 50 mM KCl and 100 μM histamine stimulations (Agasse et al., 2008a,b). Membrane depolarization of neuronal cells after exposure to high KCl concentrations leads to the opening of voltage-sensitive calcium channels and massive influx of calcium into the cytoplasm (Ambrósio et al., 2000), whereas stimulation with histamine specifically triggers an increase in $[Ca^{2+}]_i$ in immature SVZ cells (Tran et al., 2004). Taking this into consideration, we demonstrated that a low histamine/KCl ratio of response (<0.8) is characteristic of SVZ-derived neurons (Agasse et al., 2008a). So SVZ neurospheres were treated during 7 d as

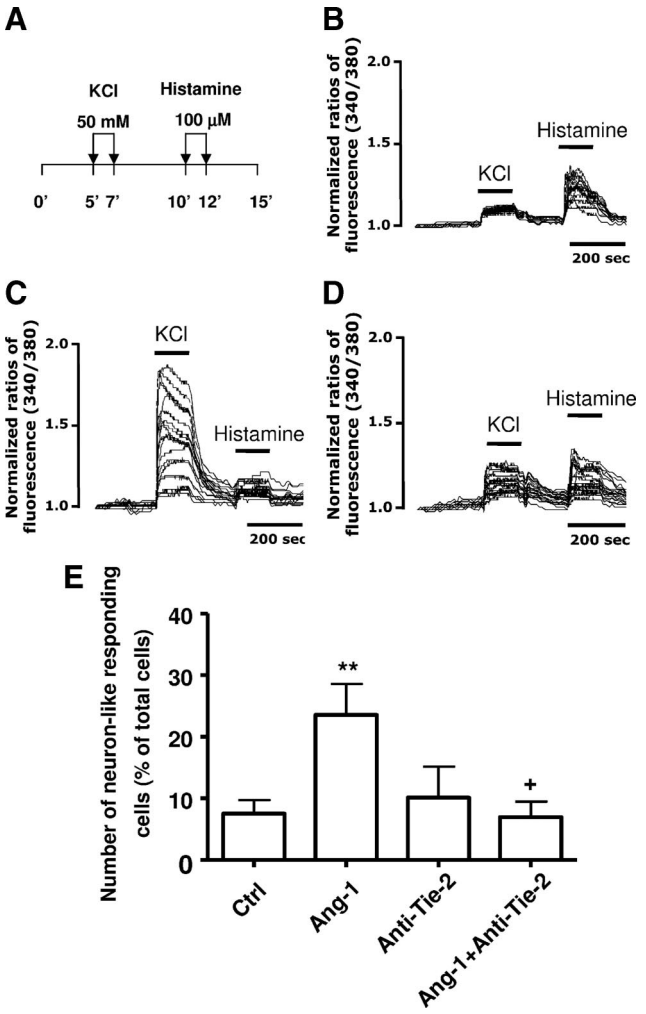

Figure 5. Ang-1 increases the generation of neuronal-like responding cells in mouse SVZ cell cultures via Tie-2 activation. ***A***, SVZ cultures were perfused continuously with Krebs' solution for 15 min and stimulated for 2 min (from minute 5 to minute 7) with 50 mM KCl and for 2 min (from minute 10 to minute 12) with 100 μM histamine. ***B–D***, Shown are representative single-cell calcium imaging profiles of response of 20 cells in a control culture (***B***), in a 500 ng/ml Ang-1-exposed culture (***C***), and in a culture treated with both 500 ng/ml Ang-1 and 5 μg/ml anti-Tie-2 neutralizing antibody (***D***). ***E***, Bar graph depicts the percentages of neuronal-like responding cells in SVZ control cultures and in cultures exposed to Ang-1 and/or anti-Tie-2 for 7 d. Data are expressed as mean ± SEM. $^{**}p < 0.01$, using the unpaired Student *t* test for comparison with SVZ control cultures. $^{+}p < 0.05$, using the unpaired Student *t* test for comparison with SVZ cultures treated with Ang-1.

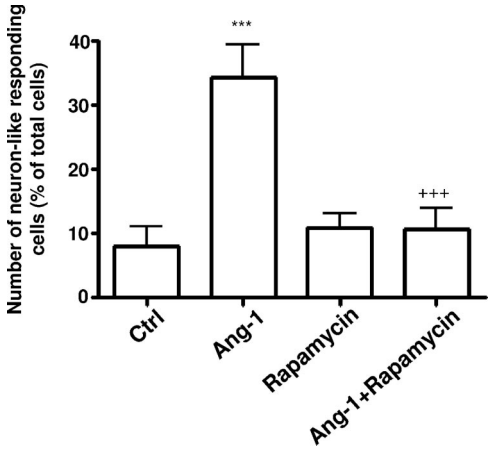

Figure 6. Ang-1 increases the generation of neuronal-like responding cells in mouse SVZ cell cultures via mTOR. SVZ cultures were perfused continuously with Krebs' solution for 15 min and stimulated for 2 min (from minute 5 to minute 7) with 50 mM KCl and for 2 min (from minute 10 to minute 12) with 100 μM histamine. The bar graph depicts the percentages of neuronal-like responding cells in SVZ control cultures and in cultures exposed to 500 ng/ml Ang-1 and/or 20 nM rapamycin for 7 d. Data are expressed as mean ± SEM. $^{***}p < 0.001$, using the unpaired Student *t* test for comparison with SVZ control cultures. $^{+++}p < 0.001$, using the unpaired Student *t* test for comparison with SVZ cultures treated with Ang-1.

aforementioned and then loaded with the fura-2 AM calcium probe, perfused continuously for 15 min with Krebs' solution, and briefly (2 min) stimulated with 50 mM KCl and 100 μM histamine (Fig. 5*A*). Figure 5, *B–D*, depicts representative profiles of responses displayed by control cultures (Fig. 5*B*), Ang-1-treated alone (Fig. 5*C*), or together with the anti-Tie-2 antibody (Fig. 5*D*). Quantification of the number of cells presenting a neuronal-like profile of response is represented in Figure 5*E*. Control cultures showed a predominant immature-like profile, characterized by cells mainly increasing $[Ca^{2+}]_i$ in response to histamine (Fig. 5*B*,*E*). In contrast, Ang-1-treated SVZ cells displayed an increase in the number of cells increasing $[Ca^{2+}]_i$ in response to KCl but not to histamine stimulation comparing with control cells, consistent with a neuronal-like profile (Fig. 5*C*,*E*) (control: 7.50 ± 2.20%, 1927 cells analyzed, $n = 14$ coverslips; 500 ng/ml Ang-1: 23.50 ± 5.06%, 1419 cells analyzed, $n = 11$ coverslips; $p < 0.01$). In the presence of both Ang-1 and anti-Tie-2 antibody, cells responded mainly as observed in the control condition. Indeed, the proneurogenic effect of Ang-1 was prevented by incubation with the anti-Tie-2 antibody (5 μg/ml anti-Tie-2 plus 500 ng/ml Ang-1: 6.95 ± 2.52%, 1080 cells analyzed, $n = 9$ coverslips; $p < 0.05$ vs 500 ng/ml Ang-1 alone) (Fig. 5*D*,*E*). In addition, we verified that incubation of cells with the anti-Tie-2 antibody alone did not affect neuronal differentiation (5 μg/ml anti-Tie-2: 10.09 ± 5.02%, 836 cells analyzed, $n = 5$ coverslips) (Fig. 5*E*). Together, these results show that exogenous addition of 500 ng/ml Ang-1 induces neuronal differentiation in SVZ cells, an effect mediated through Tie-2 activation.

In another set of experiments, SVZ cells were coincubated for 7 d with 20 nM rapamycin and 500 ng/ml Ang-1. Rapamycin inhibits mTOR, a serine-threonine kinase of the downstream signaling pathway of the PI3K/AKT kinases. In cultures treated with both rapamycin and Ang-1, fewer functional neurons were obtained compared with cultures treated with Ang-1 alone (control: 8.05 ± 2.93%, 2189 cells analyzed, $n = 19$ coverslips; 500 ng/ml Ang-1: 34.43 ± 4.99%, 2003 cells analyzed, $n = 14$ coverslips, $p < 0.0001$; 20 ng/ml rapamycin plus 500 ng/ml Ang-1: 10.57 ± 3.42%, 1435 cells analyzed, $n = 12$ coverslips, $p < 0.001$ vs 500 ng/ml Ang-1 alone; 20 ng/ml rapamycin: 10.83 ± 2.46%, 1163 cells analyzed, $n = 10$ coverslips; DMSO, 1/100,000: 6.65 ± 1.67%, 967 cells analyzed, $n = 8$ coverslips) (Fig. 6), demonstrating that mTOR mediates neuronal differentiation induced by Ang-1 stimulation. This shows that Ang-1-induced neurogenesis is dependent on the activation of mTOR. Previously, we verified that DMSO, diluted 10,000 times from our 20 mM stock solution, was not toxic by performing TUNEL (as indicated above). Because DMSO used for resuspend rapamycin was even more diluted (1/100,000) we did not repeat the TUNEL assays.

Ang-1 promotes neuronal maturation via the activation of the SAPK/JNK pathway

We showed that Ang-1 increased the number of morphologically (NeuN expression) and functionally (increase of intracellular calcium after KCl depolarization) differentiated neurons in SVZ cultures. In fact, it was known that fully developed and functional

neurons extend a single axon. Recently, activation of the SAPK/JNK MAPK pathway has been shown to be related to axonogenesis (Oliva et al., 2006). Moreover, we verified that two proneurogenic factors, neuropeptide Y (NPY) and tumor necrosis factor-α (TNFα), increased the number and total length of ramifications immunoreactive for the phosphorylated form of JNK, P-JNK, in SVZ cultures. Additionally, P-JNK-positive ramifications colocalized with the immunoreactivity to the axon-specific protein Tau (Agasse et al., 2008b; Bernardino et al., 2008) but not with the MAP-2 (microtubule-associated protein 2) expressed in dendrites (see supplemental Fig. S7, available at www.jneurosci.org as supplemental material).

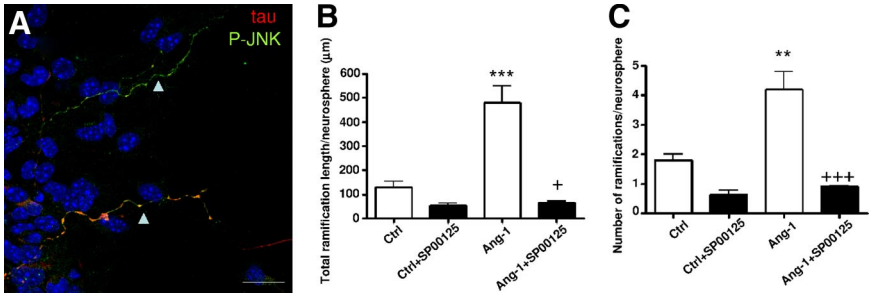

Figure 7. Ang-1 induces activation of SAPK/JNK pathway on growing axons. ***A***, Representative fluorescent confocal digital images depicts the P-SAPK/JNK (green), Tau protein (red), and Hoechst 33342 staining (blue nuclei) in cultures treated with 500 ng/ml Ang-1 for 6 h. Growing axons (double labeled for P-SAPK/JNK and Tau) are indicated by arrowheads. Scale bar, 20 μm. ***B***, ***C***, Bar graphs depict the total length (in micrometers) of P-JNK-positive ramifications and the number of ramifications per neurosphere. Data are expressed as mean ± SEM. Measurements were done in ~20 nonoverlapping fields in each coverslip from two independent culture preparations using digital images (magnification, 20×). $^{**}p < 0.01$; $^{***}p < 0.001$, using the unpaired Student *t* test for comparison with SVZ control cultures. $^{+}p < 0.05$, $^{+++}p < 0.001$ using the unpaired Student *t* test for comparison with SVZ cultures treated with Ang-1.

Therefore, we verified whether Ang-1 activates the SAPK/JNK signaling pathway. Exposure of SVZ cells to 500 ng/ml Ang-1 for 6 h increased P-JNK immunoreactivity in neurites and growth cone-like structures emerging from the neurospheres compared with control cultures. Double immunocytochemistry was performed to visualize whether P-JNK is associated with the axon-specific protein Tau (Fig. 7*A*). P-JNK is indeed localized predominantly in Tau-positive axons. Quantification of the total length of ramifications per neurosphere (Fig. 7*B*) as well as the number of ramifications per neurosphere (Fig. 7*C*) showed that 500 ng/ml Ang-1 increases significantly both parameters compared with control cultures (total length of ramification per neurosphere: control, 128.70 ± 24.21 μm; Ang-1, 479.50 ± 70.51 μm, $p < 0.001$; number of ramifications per neurosphere: control, 1.81 ± 0.19; Ang-1, 4.20 ± 0.61, $p < 0.01$).

The same experiments were performed in the presence of the 20 μM SP600125, an inhibitor of JNK activity. It has been previously demonstrated that SP600125 reduces the JNK phosphorylation (Oliva et al., 2006). Conversely, P-JNK immunoreactivity decreased in SP00125-treated cultures. In fact, both number (Fig. 7*C*) and length of P-JNK ramifications (Fig. 7*B*) per neurospheres decreased in cultures treated with SP600125 alone or together with 500 ng/ml Ang-1 (total length of ramification per neurosphere: control, 128.70 ± 24.21 μm; Ang-1 plus SP00125, 66.70 ± 5.60 μm; $p < 0.05$; number of ramifications per neurosphere: control, 1.81 ± 0.19; Ang-1 plus SP00125, 0.92 ± 0.02; $p < 0.001$) demonstrating the specificity of the P-JNK labeling. Decrease in P-JNK immunoreactivity is associated with a decrease in Tau expression, pointing to a crucial role of P-JNK activation in Ang-1-mediated axonogenesis. Together, these data show that Ang-1 promotes axonogenesis via activation of the SAPK/JNK pathway in SVZ cultures.

Tie-2 is expressed in neuronal progenitors and neurons along the SVZ–rostral migratory stream–olfactory bulb system *in vivo*

In vivo, neuroblasts and stem/progenitor cells proliferate in the SVZ (Coskun et al., 2001; Chojnacki et al., 2009). *In vitro*, some proliferating DCX-positive neuroblasts and immature Nestin-positive progenitor cells expressed Tie-2, suggesting that signaling via Tie-2 may induce these cells to proliferate. To determine whether Tie-2 receptors are present in proliferating neuroblasts and progenitors *in vivo*, brain slices from BrdU-injected mice were immunostained for Tie-2, EGFR as a marker of progenitor cells, and DCX. As depicted in Figure 8, *A* and *B*, some EGFR/BrdU-positive progenitors and DCX/BrdU-positive neuroblasts express the Tie-2 receptor (for independent channels, see supplemental data S5, available at www.jneurosci.org as supplemental material), suggesting that Tie-2 may be involved in proliferation *in vivo*. Moreover, using immunocytochemistry, Ang-1 was detected in microvessels crossing the SVZ and close to the ependymal layer (supplemental Fig. S6, available at www.jneurosci.org as supplemental material). Therefore, it is possible that locally available Ang-1 may favor proliferation via Tie-2 binding.

Ang-1 triggers neuronal differentiation in SVZ cultures and the expression of Tie-2 by DCX-positive neuroblasts as well as in Tau-positive neurons suggests that Tie-2 may be involved in neuronal maturation. Accordingly, Ang-1 promotes axonal development *in vitro*. To further support that Tie-2 signaling may be involved in neuronal maturation *in vivo*, the expression of Tie-2 by migrating neuroblasts and TH periglomerular cells was investigated in the rostral migratory stream (RMS) and olfactory bulb (OB), respectively. Tie-2 expression was found in some DCX-positive migrating neuroblasts in the RMS and TH-positive periglomerular interneurons in the OB, suggesting a role of Tie-2 signaling in SVZ-derived neuronal differentiation in the SVZ–OB system (Fig. 8*C*,*D*, respectively) (for independent channels, see supplemental Fig. S5, available at www.jneurosci.org as supplemental material).

Discussion

The present work intended to disclose the effects of the angiogenic factor Ang-1 on SVZ neurogenesis. We showed that Nestin-positive SVZ cells express Ang-1 and its receptor Tie-2, suggesting an autocrine/paracrine regulation of the SVZ cell dynamic via Ang-1.

Treatment of SVZ cells with 500 ng/ml Ang-1 did not affect SVZ cell death or survival. However, Ang-1 is known to promote cell survival in endothelial cells (Fujikawa et al., 1999; Papapetropoulos et al., 2000). In nonendothelial cells, antiapoptotic properties of Ang-1 have been described, but only on injury paradigm: Ang-1 protects embryonic cortical neurons and progenitor cells from apoptosis induced by serum deprivation and hypoxia (Valable et al., 2003; Bai et al., 2009b). Therefore, Ang-1 does not promote survival of SVZ cells in basal conditions but could protect them on injury.

Incubation of SVZ cells with 500 ng/ml Ang-1 induced proliferation, an effect mediated via Tie-2 and ERK1/2 kinase activation, the canonical pathway mediating proliferation in the SVZ

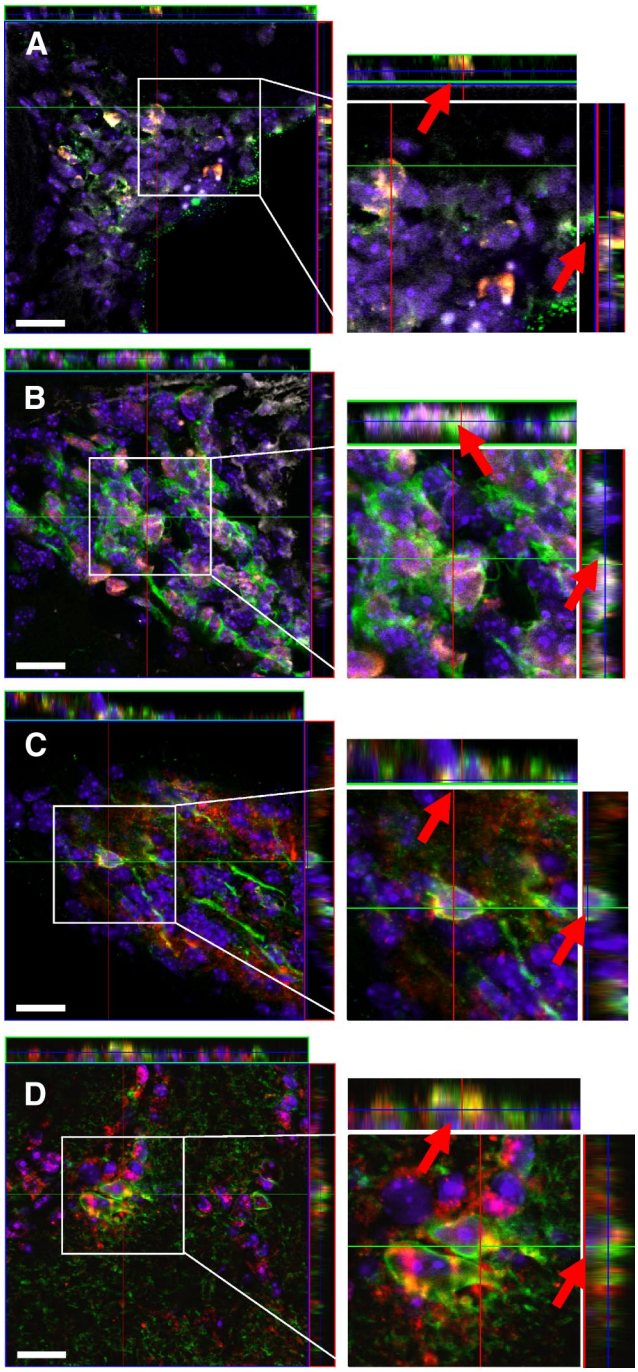

Figure 8. Tie-2 is expressed in neurons along the SVZ, the RMS, and the olfactory bulb. ***A***, ***B***, Representative *z*-stack confocal digital images of the SVZ showing BrdU-positive stem/progenitor cells (red nuclear staining for BrdU and green staining for EGFR) (***A***) and BrdU-positive neuroblasts (red nuclear staining for BrdU and green staining for DCX) (***B***), both cell types expressing Tie-2 (white staining). ***C***, Representative *z*-stack confocal digital image of the RMS showing a DCX neuroblast (green staining) expressing Tie-2 (red staining). ***D***, Representative *z*-stack confocal digital image of TH-expressing periglomerular cells (green staining) expressing Tie-2 (red staining). The arrows indicate regions of triple labeling. Hoechst 33342 staining (blue) was used to visualize cell nuclei. Scale bars, 20 μm.

(Learish et al., 2000). Although some studies failed to show a pro-proliferative effect of Ang-1 in endothelial cells (Huang et al., 1999; Takakura et al., 2000; Velazquez et al., 2002), others showed that Ang-1 triggers proliferation via ERK1/2 in murine brain endothelial cells and human umbilical vein endothelial cells (HUVECs) (Koblizek et al., 1998; Kanda et al., 2005; Abdel-Malak et al., 2008). Activation of the PI3K/AKT kinases also mediates the Ang-1-induced proliferation in endothelial cells (Kanda et al., 2005; Abdel-Malak et al., 2008). However, in SVZ cultures, ERK1/2 activation seems to prevail on PI3K/AKT pathway as total inhibition of Ang-1-induced proliferation was obtained in the presence of a MEK inhibitor.

Exogenous administration of Ang-1 increases the number of neurons through the activation of Tie-2. Ang-1 similarly elicits proneurogenic effects in embryonic mouse neural progenitors (Bai et al., 2009a), increasing the proportion of βIII tubulin neurons through Tie-2 binding and PI3 kinase activation. A growing body of evidence suggests the involvement of mTOR in neuronal differentiation. The serine-threonine kinase mTOR is one of the downstream signaling molecules of the PI3K/AKT pathway. Rapamycin-mediated inhibition of mTOR prevents neuronal differentiation induced by insulin in neuronal precursors from the rat embryonic telencephalon and decreases the number of neurons in neuroblastoma cell cultures (Han et al., 2008; Zeng and Zhou, 2008). Consistently, fewer neurons were obtained in SVZ cultures coincubated with rapamycin and Ang-1 compared with Ang-1 alone, demonstrating that mTOR mediates Ang-1-induced neuronal differentiation.

mTOR may be activated by SAPK/JNK kinases, which, in our study, promote neuronal maturation. Consistently, SP600125 (SAPK inhibitor II) was shown to decrease mTOR activation in H1299 lung cancer cells (Jin et al., 2009). Moreover, mTOR may be one convergence point between Notch and Tie-2 signaling cascades leading to proliferation and neuronal differentiation. Indeed, intracerebroventricular injection of Ang-2 and Tie-2 activation promote the expansion of the hairy and enhancer of split 3 (Hes3)-positive precursors pool in the rat SVZ (Androutsellis-Theotokis et al., 2009). The transcription factor Hes3 belongs to the Hes/Hey gene family that mediates transcriptional responses to Notch activation. Intracerebroventricular injection of the Notch ligand Delta-like 4 (Dll4) elicits similar responses to Ang-2, suggesting that Tie-2 and Notch receptors activate similar pathways promoting self-renewal and proliferation (Androutsellis-Theotokis et al., 2009). In stem-derived cell cultures from the embryonic mouse brain, transcription of the Hes3 gene after Dll4 binding to Notch is mediated by the consequent phosphorylation of AKT and mTOR (Androutsellis-Theotokis et al., 2006). Although we did not investigate the involvement of mTOR in Ang-1-induced proliferation, we showed that mTOR mediates the proneurogenic effects elicited by Tie-2 activation. Consistently, Androutsellis-Theotokis et al. (2006) showed that newly generated cells after Dll4 intracerebroventricular injection expressed DCX. In addition, Notch activation induces proliferation of neural progenitors after ischemia in the rat brain (X. Wang et al., 2009). Additional studies are needed to unravel the role of mTOR in mediating proliferation and neuronal differentiation after Tie-2 activation.

In the present study, the involvement of ERK/MAP kinases in neuronal differentiation was not assessed. However, the ERK pathway is involved in neuronal differentiation induced by bone morphogenetic protein 4, FGF-2, and nerve growth factor in neuronal precursor and mouse bone marrow stromal cells (Yang et al., 2008; Moon et al., 2009; Washio et al., 2009). Here, mTOR inhibition completely blocked Ang-1/Tie-2-mediated neuronal differentiation, suggesting that ERK/MAP kinases may not be critical. Consistently, ERK inhibition is required to induce differentiation in neural stem-derived cell cultures (B. Wang et al., 2009).

We further investigated the capacity of Ang-1 to promote neuronal maturation and neurite outgrowth in SVZ cultures.

Ang-1 increases neurite length in neural progenitors derived from the embryonic mouse brain (Bai et al., 2009a) and in dorsal root ganglion cell cultures (Kosacka et al., 2005, 2006). *In vivo*, Ward et al. (2005) showed that Ang-1 increases the dendritic arborization of motor cortex and hippocampal neurons. To assess whether Ang-1 triggers neurite outgrowth, we measured the number and length of neurites immunoreactive for P-SAPK/JNK in 6-h-treated cultures. Activation of the JNK pathway is involved in axonal sprouting and neurite outgrowth but not in dendritic growth (Waetzig et al., 2005; Oliva et al., 2006). Ang-1 treatment increased the number and length of P-JNK-positive axons. The SAPK/JNK pathway is generally activated on cellular stress such as stimulation by proinflammatory cytokines, leading mainly to apoptosis (Karin and Gallagher, 2005). In endothelial cells, serum deprivation evokes apoptosis and is accompanied by an increase of the P-JNK levels. Ang-1 attenuates serum deprivation-induced apoptosis via inhibition of the SAPK/JNK pathway (Harfouche et al., 2003). Regarding neurite outgrowth, Ang-1 triggers neurite outgrowth in PC12 cells without affecting levels of JNK phosphorylation (Chen et al., 2009). Despite the fact that the cellular model is different, discrepancies may arise from the method used to evaluate P-JNK levels. Indeed, Western blotting requires a considerable amount of protein. We detected P-JNK by immunocytochemistry, which is associated with thin neurites and therefore may not represent a sufficient amount to be detected in Western blotting.

In the present paper, Ang-1 promotes both proliferation and neuronal differentiation. As differentiation generally requires exit of the cell cycle, there may be an apparent contradiction. However, SVZ cell cultures consist of a heterogeneous population of postmitotic and cycling cells, as well as DCX-positive neuroblasts that retain the capacity to proliferate *in vitro* and *in vivo* (Coskun et al., 2001; Li et al., 2009). Additionally, factors able to promote proliferation and neuronal differentiation have been described previously. NPY, for instance, promotes proliferation and neuronal differentiation in the rodent dentate gyrus and SVZ (Howell et al., 2005; Agasse et al., 2008b; Decressac et al., 2009). TNFα and VEGF display pro-proliferative and proneurogenic capacities in SVZ cultures (Jin et al., 2002; Bernardino et al., 2008; Wittko et al., 2009). Considering the heterogeneity of SVZ cell types, pro-proliferative factors such as Ang-1 are susceptible of modulating proliferation of cycling cells. Factors promoting neuronal differentiation, Ang-1 here, may commit early postmitotic cells to the neuronal lineage. It has been recently demonstrated that Ang-2 increases the commitment of mouse SVZ cells to neurons, likely involving the binding of the transcription factor C/EBPβ (CCAAT-enhancer-binding protein β) to the promoter of βIII tubulin gene (Liu et al., 2009). Retinoic acid promotes proliferation of SVZ cells and commitment to a neuronal fate of P19 cells through epigenetic regulation of the ngn-1 gene expression (T. W. Wang et al., 2005; Wu et al., 2009). Hence, Ang-1 pro-proliferative and prodifferentiation effects may account for the diversity of the targeted cells.

Recently, Ang-2 was shown to increase neuronal differentiation in SVZ cultures via Tie-2 binding (Liu et al., 2009). Moreover, intracerebroventricular administration of Ang-2 increased proliferation in the rat SVZ (Androutsellis-Theotokis et al., 2009). The similarities of Ang-2 and Ang-1 effects on SVZ are quite puzzling as Ang-2 is a competitive antagonist of Tie-2 in endothelial cells and cancels the antiapoptotic and promigratory effects of Ang-1. However, Ang-2 may also stimulate Tie-2. Indeed, in the absence of Ang-1, Ang-2 binds to Tie-2 in HUVECs, promotes Tie-2 and PI3K-AKT activation, and acts similarly to Ang-1 as a prosurvival factor in a serum deprivation paradigm (Yacyshyn et al., 2009; Yuan et al., 2009). Nevertheless, in the presence of Ang-1, Ang-2 antagonizes the activity of Ang-1 (Yuan et al., 2009). Ang-2 is a less potent activator of Tie-2 compared with Ang-1 (Yuan et al., 2009). Hence, it is conceivable that Ang-1 and Ang-2 activate the SVZ-expressed Tie-2 receptor.

The system Ang-1/Tie-2 may play a role in the SVZ *in vivo*, as Ang-1 labeling is found in ependymal cells and in microvessels crossing the SVZ. This is in accordance with previous reports showing that sources of Ang-1 include perivascular astrocytes, endothelial cells, ependymal cells, and the choroid plexus (Acker et al., 2001; Nourhaghighi et al., 2003; Ward et al., 2005; Ohab et al., 2006; Tonchev et al., 2007; Fukuhara et al., 2008; Horton et al., 2010). Moreover, Ang-1 mRNA was detected in SVZ from adult mice (Liu et al., 2009), and we showed that proliferating DCX-positive neuroblasts and EGFR-positive progenitors express Tie-2. Together, these observations suggest that the basal neurogenic activity in the SVZ *in vivo* may partly account for the local secretion of Ang-1 and identify Ang-1 as a component of the neurogenic niche.

In conclusion, the proneurogenic effect of Ang-1 opens new perspectives for brain repair. A better understanding of the neurovascular niche and of endothelial cell-derived soluble factors may be of extreme relevance to allow the development of new strategies to enhance neuronal replacement using SVZ stem cells.

References

Abdel-Malak NA, Srikant CB, Kristof AS, Magder SA, Di Battista JA, Hussain SN (2008) Angiopoietin-1 promotes endothelial cell proliferation and migration through AP-1-dependent autocrine production of interleukin-8. Blood 111:4145–4154.

Acker T, Beck H, Plate KH (2001) Cell type specific expression of vascular endothelial growth factor and angiopoietin-1 and -2 suggests an important role of astrocytes in cerebellar vascularization. Mech Dev 108:45–57.

Agasse F, Bernardino L, Silva B, Ferreira R, Grade S, Malva JO (2008a) Response to histamine allows the functional identification of neuronal progenitors, neurons, astrocytes, and immature cells in subventricular zone cell cultures. Rejuvenation Res 11:187–200.

Agasse F, Bernardino L, Kristiansen H, Christiansen SH, Ferreira R, Silva B, Grade S, Woldbye DP, Malva JO (2008b) Neuropeptide Y promotes neurogenesis in murine subventricular zone. Stem Cells 26:1636–1645.

Ambrósio AF, Silva AP, Malva JO, Mesquita JF, Carvalho AP, Carvalho CM (2000) Role of desensitization of AMPA receptors on the neuronal viability and on the $[Ca^{2+}]_i$ changes in cultured rat hippocampal neurons. Eur J Neurosci 12:2021–2031.

Androutsellis-Theotokis A, Leker RR, Soldner F, Hoeppner DJ, Ravin R, Poser SW, Rueger MA, Bae SK, Kittappa R, McKay RD (2006) Notch signalling regulates stem cell numbers in vitro and in vivo. Nature 442:823–826.

Androutsellis-Theotokis A, Rueger MA, Park DM, Mkhikian H, Korb E, Poser SW, Walbridge S, Munasinghe J, Koretsky AP, Lonser RR, McKay RD (2009) Targeting neural precursors in the adult brain rescues injured dopamine neurons. Proc Natl Acad Sci U S A 106:13570–13575.

Bai Y, Cui M, Meng Z, Shen L, He Q, Zhang X, Chen F, Xiao J (2009a) Ectopic expression of angiopoietin-1 promotes neuronal differentiation in neural progenitor cells through the AKT pathway. Biochem Biophys Res Commun 378:296–301.

Bai Y, Meng Z, Cui M, Zhang X, Chen F, Xiao J, Shen L, Zhang Y (2009b) An Ang1-Tie-2-PI3K axis in neural progenitor cells initiates survival responses against oxygen and glucose deprivation. Neuroscience 160:371–381.

Bernardino L, Agasse F, Silva B, Ferreira R, Grade S, Malva JO (2008) Tumor necrosis factor-alpha modulates survival, proliferation, and neuronal differentiation in neonatal subventricular zone cell cultures. Stem Cells 26:2361–2371.

Chen X, Fu W, Tung CE, Ward NL (2009) Angiopoietin-1 induces neurite outgrowth of PC12 cells in a Tie-2-independent, beta1-integrin-dependent manner. Neurosci Res 64:348–354.

Chojnacki AK, Mak GK, Weiss S (2009) Identity crisis for adult periven-

tricular neural stem cells: subventricular zone astrocytes, ependymal cells or both? Nat Rev Neurosci 10:153–163.

Coskun V, Venkatraman G, Yang H, Rao MS, Luskin MB (2001) Retroviral manipulation of the expression of bone morphogenetic protein receptor Ia by SVZa progenitor cells leads to changes in their p19(INK4d) expression but not in their neuronal commitment. Int J Dev Neurosci 19:219–227.

Decressac M, Prestoz L, Veran J, Cantereau A, Jaber M, Gaillard A (2009) Neuropeptide Y stimulates proliferation, migration and differentiation of neural precursors from the subventricular zone in adult mice. Neurobiol Dis 34:441–449.

Fujikawa K, de Aos Scherpenseel I, Jain SK, Presman E, Christensen RA, Varticovski L (1999) Role of PI 3-kinase in angiopoietin-1-mediated migration and attachment-dependent survival of endothelial cells. Exp Cell Res 253:663–672.

Fukuhara S, Sako K, Minami T, Noda K, Kim HZ, Kodama T, Shibuya M, Takakura N, Koh GY, Mochizuki N (2008) Differential function of Tie-2 at cell–cell contacts and cell–substratum contacts regulated by angiopoietin-1. Nat Cell Biol 10:513–526.

Gage FH (2000) Mammalian neural stem cells. Science 287:1433–1438.

Han J, Wang B, Xiao Z, Gao Y, Zhao Y, Zhang J, Chen B, Wang X, Dai J (2008) Mammalian target of rapamycin (mTOR) is involved in the neuronal differentiation of neural progenitors induced by insulin. Mol Cell Neurosci 39:118–124.

Harfouche R, Gratton JP, Yancopoulos GD, Noseda M, Karsan A, Hussain SN (2003) Angiopoietin-1 activates both anti- and proapoptotic mitogen-activated protein kinases. FASEB J 17:1523–1525.

Hirao A, Arai F, Suda T (2004) Regulation of cell cycle in hematopoietic stem cells by the niche. Cell Cycle 3:1481–1483.

Horton BN, Solanki RB, Kulesza P, Ardelt AA (2010) Localization of angiopoietin-1 and Tie-2 immunoreactivity in rodent ependyma and adjacent blood vessels suggests functional relationships. J Histochem Cytochem 58:53–60.

Howell OW, Doyle K, Goodman JH, Scharfman HE, Herzog H, Pringle A, Beck-Sickinger AG, Gray WP (2005) Neuropeptide Y stimulates neuronal precursor proliferation in the post-natal and adult dentate gyrus. J Neurochem 93:560–570.

Huang XL, Takakura N, Suda T (1999) In vitro effects of angiopoietins and VEGF on hematopoietic and endothelial cells. Biochem Biophys Res Commun 264:133–138.

Jin HO, Seo SK, Woo SH, Kim ES, Lee HC, Yoo DH, Choe TB, Hong SI, Kim JI, Park IC (2009) SP600125 negatively regulates the mammalian target of rapamycin via ATF4-induced Redd1 expression. FEBS Lett 583:123–127.

Jin K, Zhu Y, Sun Y, Mao XO, Xie L, Greenberg DA (2002) Vascular endothelial growth factor (VEGF) stimulates neurogenesis in vitro and in vivo. Proc Natl Acad Sci U S A 99:11946–11950.

Kanda S, Miyata Y, Mochizuki Y, Matsuyama T, Kanetake H (2005) Angiopoietin 1 is mitogenic for cultured endothelial cells. Cancer Res 65:6820–6827.

Karin M, Gallagher E (2005) From JNK to pay dirt: jun kinases, their biochemistry, physiology and clinical importance. IUBMB Life 57:283–295.

Koblizek TI, Weiss C, Yancopoulos GD, Deutsch U, Risau W (1998) Angiopoietin-1 induces sprouting angiogenesis in vitro. Curr Biol 8:529–532.

Kosacka J, Figiel M, Engele J, Hilbig H, Majewski M, Spanel-Borowski K (2005) Angiopoietin-1 promotes neurite outgrowth from dorsal root ganglion cells positive for Tie-2 receptor. Cell Tissue Res 320:11–19.

Kosacka J, Nowicki M, Kacza J, Borlak J, Engele J, Spanel-Borowski K (2006) Adipocyte-derived angiopoietin-1 supports neurite outgrowth and synaptogenesis of sensory neurons. J Neurosci Res 83:1160–1169.

Kuroda H, Ohtsuru A, Futakuchi M, Kawashita Y, Nagayama Y, Fukuda E, Namba H, Shirai T, Kanematsu T, Yamashita S (2002) Distinctive gene expression of receptor-type tyrosine kinase families during rat hepatocarcinogenesis. Int J Mol Med 9:473–480.

Learish RD, Bruss MD, Haak-Frendscho M (2000) Inhibition of mitogen-activated protein kinase kinase blocks proliferation of neural progenitor cells. Brain Res Dev Brain Res 122:97–109.

Lee OH, Xu J, Fueyo J, Fuller GN, Aldape KD, Alonso MM, Piao Y, Liu TJ, Lang FF, Bekele BN, Gomez-Manzano C (2006) Expression of the receptor tyrosine kinase Tie-2 in neoplastic glial cells is associated with integrin beta1-dependent adhesion to the extracellular matrix. Mol Cancer Res 4:915–926.

Li X, Tang X, Jablonska B, Aguirre A, Gallo V, Luskin MB (2009) $p27^{KIP1}$ regulates neurogenesis in the rostral migratory stream and olfactory bulb of the postnatal mouse. J Neurosci 29:2902–2914.

Liu XS, Chopp M, Zhang RL, Hozeska-Solgot A, Gregg SC, Buller B, Lu M, Zhang ZG (2009) Angiopoietin 2 mediates the differentiation and migration of neural progenitor cells in the subventricular zone after stroke. J Biol Chem 284:22680–22689.

Lledo PM, Alonso M, Grubb MS (2006) Adult neurogenesis and functional plasticity in neuronal circuits. Nat Rev Neurosci 7:179–193.

Makinde T, Agrawal DK (2008) Intra and extravascular transmembrane signalling of angiopoietin-1-Tie-2 receptor in health and disease. J Cell Mol Med 12:810–828.

Moon BS, Yoon JY, Kim MY, Lee SH, Choi T, Choi KY (2009) Bone morphogenetic protein 4 stimulates neuronal differentiation of neuronal stem cells through the ERK pathway. Exp Mol Med 41:116–125.

Nicoleau C, Benzakour O, Agasse F, Thiriet N, Petit J, Prestoz L, Roger M, Jaber M, Coronas V (2009) Endogenous hepatocyte growth factor is a niche signal for subventricular zone neural stem cell amplification and self-renewal. Stem Cells 27:408–419.

Nourhaghighi N, Teichert-Kuliszewska K, Davis J, Stewart DJ, Nag S (2003) Altered expression of angiopoietins during blood-brain barrier breakdown and angiogenesis. Lab Invest 83:1211–1222.

Ohab JJ, Fleming S, Blesch A, Carmichael ST (2006) A neurovascular niche for neurogenesis after stroke. J Neurosci 26:13007–13016.

Oliva AA Jr, Atkins CM, Copenagle L, Banker GA (2006) Activated c-Jun N-terminal kinase is required for axon formation. J Neurosci 26:9462–9470.

Papapetropoulos A, Fulton D, Mahboubi K, Kalb RG, O'Connor DS, Li F, Altieri DC, Sessa WC (2000) Angiopoietin-1 inhibits endothelial cell apoptosis via the AKT/survivin pathway. J Biol Chem 275:9102–9105.

Parati EA, Bez A, Ponti D, de Grazia U, Corsini E, Cova L, Sala S, Colombo A, Alessandri G, Pagano SF (2002) Human neural stem cells express extraneural markers. Brain Res 925:213–221.

Patan S (2000) Vasculogenesis and angiogenesis as mechanisms of vascular network formation, growth and remodeling. J Neurooncol 50:1–15.

Poncet S, Gasc JM, Janzer RC, Meyer S, Juillerat-Jeanneret L (2003) Expression of Tie-2 in human peripheral and autonomic nervous system. Neuropathol Appl Neurobiol 29:361–369.

Schänzer A, Wachs FP, Wilhelm D, Acker T, Cooper-Kuhn C, Beck H, Winkler J, Aigner L, Plate KH, Kuhn HG (2004) Direct stimulation of adult neural stem cells in vitro and neurogenesis in vivo by vascular endothelial growth factor. Brain Pathol 14:237–248.

Segi-Nishida E, Warner-Schmidt JL, Duman RS (2008) Electroconvulsive seizure and VEGF increase the proliferation of neural stem-like cells in rat hippocampus. Proc Natl Acad Sci U S A 105:11352–11357.

Shin HY, Kim JH, Phi JH, Park CK, Kim JE, Kim JH, Paek SH, Wang KC, Kim DG (2008) Endogenous neurogenesis and neovascularization in the neocortex of the rat after focal cerebral ischemia. J Neurosci Res 86:356–367.

Sun Y, Jin K, Childs JT, Xie L, Mao XO, Greenberg DA (2006) Vascular endothelial growth factor-B (VEGFB) stimulates neurogenesis: evidence from knockout mice and growth factor administration. Dev Biol 289:329–335.

Suri C, Jones PF, Patan S, Bartunkova S, Maisonpierre PC, Davis S, Sato TN, Yancopoulos GD (1996) Requisite role of angiopoietin-1, a ligand for the TIE-2 receptor, during embryonic angiogenesis. Cell 87:1171–1180.

Swiech L, Perycz M, Malik A, Jaworski J (2008) Role of mTOR in physiology and pathology of the nervous system. Biochim Biophys Acta 1784:116–132.

Takakura N, Watanabe T, Suenobu S, Yamada Y, Noda T, Ito Y, Satake M, Suda T (2000) A role for hematopoietic stem cells in promoting angiogenesis. Cell 102:199–209.

Thurston G, Suri C, Smith K, McClain J, Sato TN, Yancopoulos GD, McDonald DM (1999) Leakage-resistant blood vessels in mice transgenically overexpressing angiopoietin-1. Science 286:2511–2514.

Tonchev AB, Yamashima T, Guo J, Chaldakov GN, Takakura N (2007) Expression of angiogenic and neurotrophic factors in the progenitor cell niche of adult monkey subventricular zone. Neuroscience 144:1425–1435.

Tran PB, Ren D, Veldhouse TJ, Miller RJ (2004) Chemokine receptors are

expressed widely by embryonic and adult neural progenitor cells. J Neurosci Res 76:20–34.

Valable S, Bellail A, Lesné S, Liot G, Mackenzie ET, Vivien D, Bernaudin M, Petit E (2003) Angiopoietin-1-induced PI3-kinase activation prevents neuronal apoptosis. FASEB J 17:443–445.

Velazquez OC, Snyder R, Liu ZJ, Fairman RM, Herlyn M (2002) Fibroblast-dependent differentiation of human microvascular endothelial cells into capillary-like 3-dimensional networks. FASEB J 16:1316–1318.

Waetzig V, Czeloth K, Hidding U, Mielke K, Kanzow M, Brecht S, Goetz M, Lucius R, Herdegen T, Hanisch UK (2005) c-Jun N-terminal kinases (JNKs) mediate pro-inflammatory actions of microglia. Glia 50:235–246.

Wang B, Gao Y, Xiao Z, Chen B, Han J, Zhang J, Wang X, Dai J (2009) Erk1/2 promotes proliferation and inhibits neuronal differentiation of neural stem cells. Neurosci Lett 461:252–257.

Wang TW, Zhang H, Parent JM (2005) Retinoic acid regulates postnatal neurogenesis in the murine subventricular zone-olfactory bulb pathway. Development 132:2721–2732.

Wang X, Mao X, Xie L, Greenberg DA, Jin K (2009) Involvement of Notch1 signaling in neurogenesis in the subventricular zone of normal and ischemic rat brain in vivo. J Cereb Blood Flow Metab 29:1644–1654.

Ward NL, Putoczki T, Mearow K, Ivanco TL, Dumont DJ (2005) Vascular-specific growth factor angiopoietin 1 is involved in the organization of neuronal processes. J Comp Neurol 482:244–256.

Washio A, Kitamura C, Jimi E, Terashita M, Nishihara T (2009) Mechanisms involved in suppression of NGF-induced neuronal differentiation of PC12 cells by hyaluronic acid. Exp Cell Res 315:3036–3043.

Wittko IM, Schänzer A, Kuzmichev A, Schneider FT, Shibuya M, Raab S, Plate KH (2009) VEGFR-1 regulates adult olfactory bulb neurogenesis and migration of neural progenitors in the rostral migratory stream *in vivo*. J Neurosci 29:8704–8714.

Wu M, Zhang Y, Wu NH, Shen YF (2009) Histone marks and chromatin remodelers on the regulation of neurogenin1 gene in RA induced neuronal differentiation of P19 cells. J Cell Biochem 107:264–271.

Yacyshyn OK, Lai PF, Forse K, Teichert-Kuliszewska K, Jurasz P, Stewart DJ (2009) Tyrosine phosphatase beta regulates angiopoietin-Tie-2 signaling in human endothelial cells. Angiogenesis 12:25–33.

Yamashita T, Ninomiya M, Hernández Acosta P, García-Verdugo JM, Sunabori T, Sakaguchi M, Adachi K, Kojima T, Hirota Y, Kawase T, Araki N, Abe K, Okano H, Sawamoto K (2006) Subventricular zone-derived neuroblasts migrate and differentiate into mature neurons in the post-stroke adult striatum. J Neurosci 26:6627–6636.

Yancopoulos GD, Davis S, Gale NW, Rudge JS, Wiegand SJ, Holash J (2000) Vascular-specific growth factors and blood vessel formation. Nature 407:242–248.

Yang H, Xia Y, Lu SQ, Soong TW, Feng ZW (2008) Basic fibroblast growth factor-induced neuronal differentiation of mouse bone marrow stromal cells requires FGFR-1, MAPK/ERK, and transcription factor AP-1. J Biol Chem 283:5287–5295.

Yuan HT, Khankin EV, Karumanchi SA, Parikh SM (2009) Angiopoietin 2 is a partial agonist/antagonist of Tie-2 signaling in the endothelium. Mol Cell Biol 29:2011–2022.

Yuasa H, Takakura N, Shimomura T, Suenobu S, Yamada T, Nagayama H, Oike Y, Suda T (2002) Analysis of human TIE-2 function on hematopoietic stem cells in umbilical cord blood. Biochem Biophys Res Commun 298:731–737.

Zeng M, Zhou JN (2008) Roles of autophagy and mTOR signaling in neuronal differentiation of mouse neuroblastoma cells. Cell Signal 20:659–665.

Zhao C, Deng W, Gage FH (2008) Mechanisms and functional implications of adult neurogenesis. Cell 132:645–660.