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Neural progenitors proliferation is inhibited by EphB3 in the developing subventricular zone

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Abstract

The subventricular zone (SVZ) of the mammalian forebrain is a major source of multipotent stem cells during development, and contributes to neurogenesis throughout the lifespan of the organism. Several studies described molecules regulating adult neurogenesis, however, few of them have examined neurogenesis in the early postnatal period. Adult neurogenesis is regulated in part by ephrinB3 and its receptors, so we examined the role of EphB3 on neural stem/progenitor cells (NSPCs) proliferation in early postnatal development in the SVZ. To examine NSPCs proliferation, we used BrdU incorporation in both cultured NSPCs and neonatal gene-targeted knockout mice, as well as Ki67 immunostaining in EphB3^{-/-} mice. We observed a significant increase in proliferation in cultured NSPCs derived from EphB3^{-/-} mice and in the SVZ of EphB3^{-/-} mice. These studies support an anti-proliferative role for EphB3 in regulating NSPCs numbers in the developing SVZ.

Keywords

EphB3; neural progenitor cell; neonate

INTRODUCTION

The subventricular zone (SVZ) of the mammalian forebrain (reviewed extensively in Levison, 2006) is a region central to both neurogenesis and gliogenesis in the developing brain. During embryonic development, the germinal zone lining the ventricles (called ventricular zone (VZ)) generates the vast majority of cells which constitute the telencephalon. It harbors radial glial cells that are multipotent progenitors generating first the neurons populating the multiple cortical layers, then glial cells as well. The SVZ, a secondary proliferative zone that originates as a continuum of the VZ (Tramontin et al., 2003), starts expanding during the last third of prenatal development and peaks in size

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during the early post-natal period (around one week after birth). The radial glia disappears around 7–10 days post-natally (Alves et al., 2002) but some of it persists in the adult SVZ as stem cells sharing astrocyte characteristics (Doetsch et al., 1999). Numerous studies in rodents have demonstrated that stem cells capable of both self-renewal and differentiation into various cell types reside within the SVZ.

Fate-mapping studies to elucidate the origin of the proliferating cells of the adult SVZ have been performed (Young et al., 2007) and they show for instance that Gsh2-positive cells, located in the lateral SVZ in the adult and which are the major source of the neurons migrating into the olfactory bulb (OB), originate from the ganglionic eminences in the developing embryo. The early post-natal SVZ is still a source of glial cells that can populate the neighboring striatum, the dorsal cortical gray matter (mostly around post-natal day 2 in rodents) or the dorsal white matter (mostly around post-natal day 14 in rodents) (Levison and Goldman, 1993; Spadafora et al., 2010; Suzuki and Goldman, 2003).

Unlike the early post-natal SVZ that can still generate glial cells that can take dorsal and lateral migration routes, the adult SVZ remains an area of active neurogenesis under normal physiological conditions where glial differentiation is inhibited (Lim et al., 2000). The SVZ contributes cells to the olfactory bulb, which starts during the neonatal period (Luskin, 1993) and continues into adult life. Studies in adult mice have demonstrated that a population of SVZ neural stem cells/progenitors (NSPC) begins to differentiate into neuroblasts that migrate rostrally out of the SVZ along a specific pathway called the rostral migratory stream to the OB where they become functional interneurons. Previous studies have demonstrated the role of ephrins/Eph receptors in this process, as well as in the regulation of NSPC proliferation and survival in the adult SVZ (Conover et al., 2000; Holmberg et al., 2005; Ricard et al., 2006).

Eph receptors are a family of growth and guidance molecules expressed widely in the brain and in other tissues. These proteins have many important functions during brain development, including axonal guidance/fasciculation, dendritic spine morphogenesis and remodeling, midline development, and synaptic regulation (Pasquale, 2008). They constitute the largest known subgroup of transmembrane receptor tyrosine kinases with 14 known members in warm blooded vertebrates. There are two subfamilies of receptors: the EphA receptors bind to A-class ephrin ligands which are anchored to the membrane by a glycosylphosphatidylinositol (GPI) linkage, and the EphB ones bind B-class ephrins that are transmembrane spanning. There is a broad overlap of binding specificities within the subclasses, suggesting shared activities and possible redundancy among family members.

We have examined the role of the EphB3 receptor on the proliferation in the SVZ of postnatal day 1 (P1) mice. Using gene-targeted knockout mice, we demonstrate that EphB3 is a negative regulator of NSPC proliferation both *in vitro* and *in vivo* in the early postnatal SVZ.

MATERIALS AND METHODS

Cell culture

NSPCs were isolated from the SVZ of post-natal day 1 (P1) wild type or EphB3^{-/-} CD1 mice and grown as adherent cells according to Scheffler *et al.* (Scheffler et al., 2005). Briefly, the tissue of the lateral ventricle wall on the striatal side was dissected out and was dissociated in Hanks' Balanced Salt Solution containing 1.33 mg/ml trypsin, 0.7 mg/ml hyaluronic acid and 0.2 mg/ml kynurenic acid. The cells were plated in 100mm dishes in N5 medium (DMEM:F12 containing 5% FBS, 1% N2 supplement, 35 µg/ml bovine pituitary extract, 20 ng/ml EGF, 20 ng/ml FGF and antibiotics). The next day, unattached cells were

collected and replated onto a 100mm dish and grown to confluence in N5 medium. Cells were passed no more than ten times and plated at 500,000 cells per dish in N5 medium.

RT_PCR

RNA was extracted from NSPC using the Trizol reagent (Invitrogen) and cDNA was synthesized from 1 µg RNA using the ImProm-II kit (Promega). The PCR reaction was performed for 30 cycles with the primers presented in table 1.

Proliferation and cell death assays

To assess proliferation *in vitro*, cells were plated in 96-well plates at 5,000 cells/well and treated with 10 µM BrdU for 1 hour at 37°C. Cells were fixed with 4% paraformaldehyde, washed with PBS and permeabilized for 10 min in 0.4% Triton-X100. The DNA was denatured by incubating in 1N HCl for 1 hour at 37°C and the pH was neutralized in 0.1 M borate pH 8.5. The plate was then blocked in 5% BSA/0.2% Triton-X100 for 30 minutes. The anti-BrdU antibody (Roche, 1/100) was applied overnight at 4°C. Alexa Fluor 488-conjugated secondary antibody (Invitrogen) was used for 30 min at room temperature. Cell nuclei were counterstained with Hoechst and the ratio of BrdU-positive cells to total nuclei was determined by the Target Activation Bioapplication assay with plates scanned in the ArrayScan Reader (Thermo Scientific Cellomics).

The generation of the EphB3^{-/-} mice has been described previously (Orioli et al., 1996). Genotyping was performed by PCR analysis. P1 mice were injected intraperitoneally with 50 µg BrdU/g of body weight and perfused intracardially with 4% paraformaldehyde 1 hour later. Brains were removed and post-fixed overnight in the same solution, cryopreserved in 30% sucrose, and frozen in OCT (Tissue Tek). Coronal cryostat brain sections were taken at 30 µm intervals through the ventricles and sections were stained for BrdU as detailed above. TUNEL staining was performed on PFA-fixed tissue using the ApopTag Plus Fluorescein In Situ detection kit (Chemicon) according to the manufacturer's instructions. Cell count analysis was conducted using the StereoInvestigator software (MicroBrightField).

Statistical analysis

The data were analyzed using the SigmaStat software. The Student's *t*-test was used to compare two groups. One-way ANOVA analysis followed by Tukey test was performed for multiple pairwise comparisons.

RESULTS AND DISCUSSION

We used NSPC derived from P1 wild type mice grown in culture and their phenotype was assessed by immunostaining. All the cells were positive for nestin (Figure 1b) and exhibited Sox-2 staining in the nucleus (Figure 1e–g), confirming their neural progenitors' nature. A few nestin-positive cells stained for GFAP as well (Figure 1c) as described previously (Scheffler et al., 2005). Furthermore, we observed their ability to differentiate into neurons and glia upon mitogens withdrawal (not shown). The cells were characterized in terms of B-class ephrin/Eph receptor expression. The expression of EphB3 and EphA4 in neonatal SVZ via radiolabeled probes has been shown previously (Liebl et al., 2003). More sensitive RT-PCR analysis showed that ephrinB3 and its receptors EphB3 and EphA4 were expressed in cultured NSPC. The NSPC were also found to express the other B-class ligands, ephrinB1 and ephrinB2, as well as several other B-class receptors, namely EphB1, EphB2, EphB4 and EphB6 (Figure 2a). It could be argued that our culture was a mixed one because of the presence of a subpopulation of GFAP⁺/nestin⁺ cells. However, the pattern of expression was very similar to the one observed in adult SVZ progenitors (Ricard et al., 2006) with ephrinB3 being one notable exception. Indeed, ephrinB3 is not expressed in adult NSPC

whereas a transcript was detected in the P1 cells. Upon further examination using cells derived from ephrinB3-lacZ animals (in which a fusion protein formed of the extracellular domain of ephrinB3 and the beta-galactosidase is expressed), we did not observe any protein expression using X-gal staining (data not shown). The band observed on the gel may denote the presence of an untranslated transcript as we ruled out contamination with DNA in our samples (Figure 2, lane 18 “RT(–) control”). *In vivo*, the expression of ephrinB3 at P1 (assessed using X-gal staining on tissues harvested from ephrinB3-lacZ mice) was found to be similar to the one observed in the adult: it was observed in the septum, the corpus callosum (CC) and the striatum (Figure 2b). However, contrary to the adult, the levels of expression in the CC and the striatum were very faint. The expression was never seen on cells lining the ventricle, as is the case in the adult (Ricard et al., 2006). It is conceivable that ephrinB3 expressed in the striatum bordering the germinal zone could signal on NSPC expressing EphB3.

Cultured NSPC from wild type and EphB3 receptor knockout animals were incubated with 10 μ M BrdU for one hour and proliferating cells were counted (Figure 3). Data are shown as percentage of proliferating cells to total cells, as determined by Hoechst nuclear staining. Cells derived from mice lacking EphB3 expression demonstrated a significant increase in proliferation as compared with wild type ones. However, NSPC derived from EphA4^{−/−} animals did not exhibit any change in their proliferation rate. Cells lacking the expression of both receptors showed also a greater rate of proliferation, as seen in cells derived from single EphB3 knock-out animals. There was no statistically significant difference between wild type cells and EphA4^{−/−} ones ($p=0.745$), nor was there any between cells lacking the expression of EphB3 or both EphB3 and EphA4 receptors ($p=0.965$), arguing in favor of a role for EphB3 in the control of proliferation but not for EphA4 (Figure 3). In order to assert that the increased rate of proliferation observed in cultured EphB3^{−/−} cells was cell-autonomous, we transfected them with a plasmid encoding for EphB3 and measured their proliferation by assessing BrdU incorporation. Our results showed that expression of EphB3 (in at least around 30% of the cells according to our observations) did not induce any change in the proliferation rate of the NSPC as compared to cells transfected with a mock vector (not shown). However, we are convinced that the faster proliferation exhibited by the EphB3^{−/−} results directly from the absence of the receptor. Our cultures are very homogeneous despite the presence of a few GFAP-positive cells inherent to the system used and they all express nestin and Sox2.

To determine if EphB3 deficiency also leads to increased proliferation *in vivo*, BrdU incorporation was determined by injecting P1 animals and counting BrdU-stained cells on frozen brain sections. As observed previously *in vitro*, a significant increase in proliferation was observed in EphB3^{−/−} mice. Furthermore, Ki67-staining of brain sections also showed increased cell cycle activity in EphB3^{−/−} animals as compared to wild type mice (Figure 4). As an increase in proliferation can be at times accompanied by an increase in cell death, we sought to determine whether there is a change in the rate of apoptosis between wild type and knockout animals. Comparison between wild type and EphB3^{−/−} apoptosis in the SVZ via TUNEL staining showed no discernable *in vitro* or *in vivo* differences (wild type: 1554 ± 339 vs EphB3^{−/−}: 2313 ± 725 ; $p=0.293$). This is different from what is observed in the adult animals as less cell death was reported from the EphB3^{−/−} adult mice (Theus et al., 2010), probably because of a “dependence receptor” effect as seen with EphA4 (Furne et al., 2009). The germinal niche of the neonatal mouse is likely to be different from the adult one and more studies are needed to identify all the regulatory factors affecting proliferation and cell death in the SVZ. The development of the bulb is also very different between these two stages and inputs to the SVZ from the bulb could affect proliferation as well.

There is a growing body of knowledge regarding the role of ephrins in regulating NSPC proliferation in the adult brain. Initial studies revealed that infusion of ephrinB2 or EphB2 into the lateral ventricle increases cell proliferation (Conover et al., 2000), thus suggesting a role for ephrins in controlling proliferation. Subsequent studies using knockout animals further implicated ephrins as negative regulators. Adult mice deficient in ephrinA2 or its receptor EphA7, demonstrate higher numbers of proliferating cells in the lateral ventricle. This effect can also be produced by infusing either ligand or receptor into the ventricle of wild type mice (Holmberg et al., 2005). Previously reported studies in our laboratory have examined a similar role for ephrinB3, the primary ligand for EphB3. Using BrdU incorporation and Ki67 staining, we found that proliferation in the SVZ is increased in ephrinB3^{-/-} mice with an associated change in cell cycle proteins. Infusion of ephrinB3 soluble ligand into the ventricles of these knockout mice suppresses proliferation to wild type levels, thus strongly implicating ephrinB3 as a negative regulator of NSPC proliferation in the adult SVZ (Ricard et al., 2006). It is noteworthy that the paper by Conover *et al.* described an increase in proliferation after infusion of ephrinB2 or EphB2, whereas all the subsequently published papers described ephrins as inhibitory. Holmberg *et al.* also reported that infusing ephrins had the same effect on proliferation as the one observed in the knockout animals. It is likely that the infusions disrupt the interaction between endogenous ligands and receptors, resulting in the lifting of the pro-inhibitory signal triggered by ephrins. Areas outside of the SVZ have also been studied, and ephrinA2 and ephrinA3 have been found to inhibit progenitor proliferation in the cerebral cortex via EphA7 signaling (Jiao et al., 2008). In the hippocampus, absence of EphB1 leads to an increase in proliferation and altered positioning of the progenitors (Chumley et al., 2007). EphB receptors are also involved in the regulation of the stem cell niche in the intestine, although in an opposite manner (Holmberg et al., 2006). Mice lacking expression of both EphB2 and EphB3 exhibit a decrease in proliferation in the colon crypts and these two receptors promote cell cycle reentry in the intestine.

Since the EphB3^{-/-} neonates exhibit a higher rate of proliferation and a similar level of cell death, one could anticipate a greater number of cells in the adult and a larger brain. Moreover, a decrease in cell death in the adult mice (Theus et al.) could exacerbate the increase in cell numbers. We actually never observed any gross abnormalities between wild type and EphB3^{-/-} adult animals. The regulation of the neurogenesis process by ephrins and Eph receptors could occur at many levels. As an example, we have observed that ephrinB3^{-/-} mice exhibit more proliferation in their SVZ, a defective migration to the OB and a lower level of cell death within the OB, which ultimately could result in roughly the same number of newly born neurons being integrated into the circuitry (Ricard et al., 2006).

We sought to examine the role of ephrinB3 receptors in regulating proliferation in the neonatal SVZ, a structure that differs from its adult counterpart in both size and function. Similar to its role in the adult, the SVZ generates neurons for the developing olfactory bulb during late fetal and early postnatal life. However, the neonatal SVZ also gives rise to cortical and subcortical interneurons (Inta et al., 2008), and its major role during this time is to give rise to glial cells of the neocortex and developing white matter. It is in fact the major source of myelinating oligodendrocytes in the forebrain (Levison and Goldman, 1993) and, in rodents, the SVZ reaches its peak of proliferation during the first week of postnatal life (Levison, 2006; Lewis and Lai, 1974). Proliferation in the SVZ diminishes substantially over the first weeks of postnatal life but little is known about the factors affecting this decline. We proposed that ephrinB3, via interactions with EphB3 and/or EphA4, may be one factor affecting NSPC proliferation during this developmentally distinct period of time, and we were interested in examining whether ephrinB3/receptor interactions would affect NSPC proliferation in a similar or opposite manner than that observed in the adult. Here we report that neonatal NSPC express EphB3 and EphA4, similarly to the adult NSPC. We looked at

the effect of knocking out both EphB3 and EphA4 individually, as well as knocking them out together, and found that only EphB3^{-/-} cells demonstrate increased proliferation both *in vitro* and *in vivo*. The marked effect of eliminating EphB3 on neonatal NSPC suggests that it plays a significant role in regulating progenitor cell numbers during this time of life. Since ephrinB3 has also an anti-proliferative role on NSPC (Ricard et al., 2006), it is likely to signal onto EphB3 to regulate proliferation. In summary, our results clearly implicate EphB3 in the regulation of proliferation in the neonatal SVZ and suggest that some mechanisms regulating NSPC proliferation at the adult stage are already in place in the neonatal brain.

RESEARCH HIGHLIGHTS

EphB3 regulates proliferation in the neonatal subventricular zone.

EphB3^{-/-} neural progenitors proliferate more in culture.

Cell death is unchanged in the neonatal EphB3^{-/-} SVZ, unlike the adult.

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REFERENCES

- Alves JA, Barone P, Engelender S, Froes MM, Menezes JR. Initial stages of radial glia astrocytic transformation in the early postnatal anterior subventricular zone. *J Neurobiol* 2002;52:251–265. [PubMed: 12210108]
- Chumley MJ, Catchpole T, Silvany RE, Kernie SG, Henkemeyer M. EphB receptors regulate stem/progenitor cell proliferation, migration, and polarity during hippocampal neurogenesis. *J Neurosci* 2007;27:13481–13490. [PubMed: 18057206]
- Conover JC, Doetsch F, Garcia-Verdugo JM, Gale NW, Yancopoulos GD, Alvarez-Buylla A. Disruption of Eph/ephrin signaling affects migration and proliferation in the adult subventricular zone. *Nat Neurosci* 2000;3:1091–1097. [PubMed: 11036265]
- Doetsch F, Caille I, Lim DA, Garcia-Verdugo JM, Alvarez-Buylla A. Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. *Cell* 1999;97:703–716. [PubMed: 10380923]
- Furne C, Ricard J, Cabrera JR, Pays L, Bethea JR, Mehlen P, Liebl DJ. EphrinB3 is an anti-apoptotic ligand that inhibits the dependence receptor functions of EphA4 receptors during adult neurogenesis. *Biochim Biophys Acta* 2009;1793:231–238. [PubMed: 18948148]
- Holmberg J, Armulik A, Senti KA, Edoff K, Spalding K, Momba S, Cassidy R, Flanagan JG, Frisen J. Ephrin-A2 reverse signaling negatively regulates neural progenitor proliferation and neurogenesis. *Genes Dev* 2005;19:462–471. [PubMed: 15713841]
- Holmberg J, Genander M, Halford MM, Anneren C, Sondell M, Chumley MJ, Silvany RE, Henkemeyer M, Frisen J. EphB receptors coordinate migration and proliferation in the intestinal stem cell niche. *Cell* 2006;125:1151–1163. [PubMed: 16777604]
- Inta D, Alfonso J, von Engelhardt J, Kreuzberg MM, Meyer AH, van Hooft JA, Monyer H. Neurogenesis and widespread forebrain migration of distinct GABAergic neurons from the postnatal subventricular zone. *Proc Natl Acad Sci U S A* 2008;105:20994–20999. [PubMed: 19095802]
- Jiao JW, Feldheim DA, Chen DF. Ephrins as negative regulators of adult neurogenesis in diverse regions of the central nervous system. *Proc Natl Acad Sci U S A* 2008;105:8778–8783. [PubMed: 18562299]
- Levison, SW. Mammalian subventricular zones: their roles in brain development, cell replacement, and disease. Springer; 2006.

- Levison SW, Goldman JE. Both oligodendrocytes and astrocytes develop from progenitors in the subventricular zone of postnatal rat forebrain. *Neuron* 1993;10:201–212. [PubMed: 8439409]
- Lewis PD, Lai M. Cell generation in the subependymal layer of the rat brain during the early postnatal period. *Brain Res* 1974;76:520–525. [PubMed: 4850922]
- Liebl DJ, Morris CJ, Henkemeyer M, Parada LF. mRNA expression of ephrins and Eph receptor tyrosine kinases in the neonatal and adult mouse central nervous system. *J Neurosci Res* 2003;71:7–22. [PubMed: 12478610]
- Lim DA, Tramontin AD, Trevejo JM, Herrera DG, Garcia-Verdugo JM, Alvarez-Buylla A. Noggin antagonizes BMP signaling to create a niche for adult neurogenesis. *Neuron* 2000;28:713–726. [PubMed: 11163261]
- Luskin MB. Restricted proliferation and migration of postnatally generated neurons derived from the forebrain subventricular zone. *Neuron* 1993;11:173–189. [PubMed: 8338665]
- Orioli D, Henkemeyer M, Lemke G, Klein R, Pawson T. Sek4 and Nuk receptors cooperate in guidance of commissural axons and in palate formation. *Embo J* 1996;15:6035–6049. [PubMed: 8947026]
- Pasquale EB. Eph-ephrin bidirectional signaling in physiology and disease. *Cell* 2008;133:38–52. [PubMed: 18394988]
- Ricard J, Salinas J, Garcia L, Liebl DJ. EphrinB3 regulates cell proliferation and survival in adult neurogenesis. *Mol Cell Neurosci* 2006;31:713–722. [PubMed: 16483793]
- Scheffler B, Walton NM, Lin DD, Goetz AK, Enikolopov G, Roper SN, Steindler DA. Phenotypic and functional characterization of adult brain neurogenesis. *Proc Natl Acad Sci U S A* 2005;102:9353–9358. [PubMed: 15961540]
- Spadafora R, Gonzalez FF, Derugin N, Wendland M, Ferriero D, McQuillen P. Altered fate of subventricular zone progenitor cells and reduced neurogenesis following neonatal stroke. *Dev Neurosci* 2010;32:101–113. [PubMed: 20453463]
- Suzuki SO, Goldman JE. Multiple cell populations in the early postnatal subventricular zone take distinct migratory pathways: a dynamic study of glial and neuronal progenitor migration. *J Neurosci* 2003;23:4240–4250. [PubMed: 12764112]
- Theus MH, Ricard J, Bethea JR, Liebl DJ. EphB3 limits the expansion of neural progenitor cells in the subventricular zone by regulating p53 during homeostasis and following traumatic brain injury. *Stem Cells* 28:1231–1242. [PubMed: 20496368]
- Tramontin AD, Garcia-Verdugo JM, Lim DA, Alvarez-Buylla A. Postnatal development of radial glia and the ventricular zone (VZ): a continuum of the neural stem cell compartment. *Cereb Cortex* 2003;13:580–587. [PubMed: 12764031]
- Young KM, Fogarty M, Kessaris N, Richardson WD. Subventricular zone stem cells are heterogeneous with respect to their embryonic origins and neurogenic fates in the adult olfactory bulb. *J Neurosci* 2007;27:8286–8296. [PubMed: 17670975]

Figure 1. Culture of neural progenitors *in vitro*. All the cells express nestin (**b**, green) and a subset is also positive for GFAP (**c**, red). **d**) merged image including the Hoechst nuclear staining (**a**). The transcription factor Sox-2 is found in all the cells (**f**, green) and the localization was always nuclear (**g**, merged image with Hoechst nuclear staining in **e**). Scale bars: 50 μm.

Figure 2.

a) Multiple ephrins and Eph receptors are expressed in wild type P1 NSPC as shown by RT-PCR. cDNA was generated from RNA extracted from cultured NSPC and amplified by PCR using the primers described in table 1. **b)** EphrinB3 (blue color, X-gal staining) is expressed outside the germinal zone: it is expressed strongly in the septum and faintly in the corpus callosum, as well as in the striatum bordering the few layers of cells constituting the germinal zone of the SVZ (unstained, brown appearance). It is likely that ephrinB3 bordering the SVZ signals onto NSPCs of the germinal zone to control proliferation.

Figure 3.

EphB3-deficient NSPCs proliferate more than wild type NSPCs *in vitro*. BrdU incorporation is greater in EphB3^{-/-} and EphB3^{-/-}/EphA4^{-/-} than in wild type or EphA4^{-/-} cells. BrdU was added to cultured NSPCs for 1 hour (10 μ M). After fixation, the incorporated BrdU was detected by immunostaining and the ratio of positive cells was determined (against total population labeled with Hoechst). *: $p=0.002$ (wild type vs EphB3^{-/-}); #: $p=0.001$ (wild type vs EphB3^{-/-}/EphA4^{-/-}).

Figure 4.

Cell proliferation in the SVZ of EphB3^{-/-} mice is greater than in wild type mice. Postnatal day 1 mice were injected with 50 µg/g body weight BrdU intraperitoneally and perfused transcardially with 4% PFA after 1 hour. BrdU was detected by immunostaining on 30 µm cryosections and the positive cells along the ventricles were counted on every 10th section using the StereoInvestigator software. **a)** Photograph of wild type P1 SVZ stained for BrdU incorporation. **b)** Photograph of EphB3^{-/-} P1 SVZ showing BrdU incorporation greater than wild type. Scale bar: 250 µm. **c)** The number of proliferating cells is greater in EphB3^{-/-} SVZ as seen by both BrdU and Ki67 staining. *: p=0.043 and **: p=0.002 (wild type vs EphB3^{-/-}).

Table 1

Primers and annealing temperatures used for the RT-PCR

Gene name		Primers	Annealing
ephrinB1	sense:	caccatcaagttccaagagt	59 ° C
	reverse:	cttctctcctggttcacag	
ephrinB2	sense:	gtactgttggggacttttga	45 ° C
	reverse:	aatttcacatcttggtctgg	
ephrinB3	sense:	ctcccgggggtgctgaagg	52 ° C
	reverse:	ccgccgtctccgccaacac	
EphB1	sense:	atccggaaccagctagtctcaag	57 ° C
	reverse:	ggtggtaaaggccgtgaagtctg	
EphB2	sense:	accaccggccaagtctg	57 ° C
	reverse:	aagctggtgtagtccggtatcg	
EphB3	sense:	gtttatattgaccttttaccta	55 ° C
	reverse:	gtcgaccacggcacacttc	
EphB4	sense:	gaatcccgtagcctcaaaa	57 ° C
	reverse:	tcagaactgctgggctggtc	
EphB6	sense:	gttcggctgccccacctc	57 ° C
	reverse:	gagaagtgtcctcctggtagcac	
EphA4	sense:	aggaagtgagcattatggatga	55 ° C
	reverse:	tgtctctcgtgccagcgtt	