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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 cell (NSPC) proliferation in early postnatal development in the SVZ. To examine NSPC 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 NSPC numbers in the developing SVZ.

1. 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 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 show that Gsh2-positive cells, located in the lateral SVZ in the adult and which are the major source of 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 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 (NSPCs) 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 a role for 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

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Table 1
Primers and annealing temperatures used for the RT-PCR.

Gene name	Primers	Annealing
ephrinB1	Sense: caccatcaagttccaagagt Reverse: cttctcttcctggttcacag	59 °C
ephrinB2	Sense: gtactgttggggacttttga Reverse: aatttcacatcttggtctgg	45 °C
ephrinB3	Sense: ctcccggggtgctgaagg Reverse: ccgccgtctccgccaacac	52 °C
EphB1	Sense: atccggaacccagctagtctcaag Reverse: ggtggtaaaggccgtgaagtctg	57 °C
EphB2	Sense: accaccggcccaagttcg Reverse: aagctggtgtagtccggtatcg	57 °C
EphB3	Sense: gtttatattgacccttttaccta Reverse: gtcgaccacggcacacttcc	55 °C
EphB4	Sense: gaatcccgctagcctcaaaa Reverse: tcagaactgctgggctggtc	57 °C
EphB6	Sense: gttccggctgcccccacctc Reverse: gagaagttgtcctcctggtagcac	57 °C
EphA4	Sense: aggaagtgagcattatggatga Reverse: tgctcctcgtgcccagcgtt	55 °C

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.

2. Materials and methods

2.1. 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. (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 100 mm 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 100 mm 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.

2.2. RT-PCR

RNA was extracted from NSPCs 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.

2.3. Proliferation and cell death assays

To assess proliferation *in vitro*, cells were plated in 96-well plates at 5000 cells/well and treated with 10 μM BrdU for 1 h 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 1 N HCl for 1 h 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 min. 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 h 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).

2.4. 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.

3. Results and discussion

We used NSPCs derived from P1 wild type mice grown in culture and their phenotype was assessed by immunostaining. All the cells were positive for nestin (Fig. 1b) and exhibited Sox-2 staining in the nucleus (Fig. 1e–g), confirming their neural progenitors' nature. A few nestin-positive cells stained for GFAP as well (Fig. 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 NSPCs. The NSPCs 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 (Fig. 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 NSPCs 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 (Fig. 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 (Fig. 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 NSPCs expressing EphB3.

Cultured NSPCs from wild type and EphB3 receptor knockout animals were incubated with 10 μM BrdU for 1 h and proliferating cells were counted (Fig. 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, NSPCs 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$^{-/-}$ animals. There was no statistically significant difference between wild type cells