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EphrinB3 regulates cell proliferation and survival in adult neurogenesis

Jerome Ricard, Jessica Salinas, Lissette Garcia, and Daniel J. Liebl*

The Miami Project to Cure Paralysis and Department of Neurosurgery, University of Miami School of Medicine, 1095 NW 14th Terrace, R-48, Miami, FL 33136, USA

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Interactions between ephrins and their receptors have been implicated in many processes during central nervous system development. In the adult, ephrins and Eph receptors have been implicated in controlling cell proliferation and neuroblast migration, although there is no direct evidence for the role of ephrinB3 in these functions. In addition, activation of Eph receptors has been shown to regulate transduction pathways important in cell cycle control as well as cell death. We show that ephrinB3 contributes to the control of cell proliferation and survival in the adult subventricular zone (SVZ). EphrinB3$^{-/-}$ mice exhibit a significant increase in dividing cells along the lateral ventricle, and altered expression of proteins involved in cell cycle regulation. Gain-of-function approach by infusing soluble ephrinB3-Fc molecules in ephrinB3$^{-/-}$ can suppress cell proliferation to wild type levels. At the same time, ephrinB3 also regulates cell survival as greater numbers of cells die in the SVZ of ephrinB3$^{-/-}$ mice. Together, our results suggest that ephrinB3 negatively regulates cell cycle progression and cell apoptosis in the adult subventricular zone.

Keywords: Ephrin B3; Eph receptors; Stem cells; Subventricular zone; Apoptosis; Proliferation

Introduction

The subventricular zone (SVZ) is a principle region of neurogenesis in the adult rodent brain (Alvarez-Buylla and Garcia-Verdugo, 2002). In normal physiological conditions, neural stem/progenitor cells in the SVZ give rise to intermediate progenitors, also named transit-amplifying precursors, which in turn differentiate into neuroblasts that are destined for the olfactory bulb (OB). In the OB, these migrating neuroblasts terminally differentiate into interneurons (Luskin, 1993; Betarbet et al., 1996). The mechanisms that govern these events are tightly regulated during neurogenesis from stem cell to neuron. In particular, to ensure that there is an adequate pool of cells at each stage of differentiation, proliferation within these stages requires specific environmental and intracellular signaling mechanisms. In doing so, the stem/progenitor cells generate the intermediate progenitors while maintaining their own pool. The intermediate precursors may use repeated cell divisions to expand their numbers and thus greatly amplify the number of neuroblasts produced. Further divisions within the neuroblast population during migration contribute to the generation of a large amount of new neurons (Alvarez-Buylla et al., 2001).

Cell proliferation in the SVZ was shown to be increased by a variety of growth factors and hormones, including epidermal growth factor (EGF), fibroblastic growth factor (FGF), vascular endothelial growth factor (VEGF) and thyroid hormone (Doetsch et al., 2002a,b; Jin et al., 2002, 2003; Lemkine et al., 2005). Other molecules involved in the control of neural precursors proliferation include enhancers such as β1-integrins and retinoic acid (Leone et al., 2005; Wang et al., 2005) and repressors such as neurofibromin (Dasgupta and Gutmann, 2005). There is a tight link between cell cycle control and neurogenesis (Ohnuma and Harris, 2003), which is further supported by a reduction in cell proliferation and neuroblast generation in mice lacking expression of the E2F1 transcription factor (Cooper-Kuhn et al., 2002), and by an increase in the number of proliferating precursors in p107$^{-/-}$ mice (Vanderluit et al., 2004). An increase in proliferation in the SVZ is also found in p27$^{Kip1-/-}$ mice and is coupled to a decrease in neuroblast generation (Doetsch et al., 2002a,b). Similarly, in rats, a decrease in p27^{Kip1} expression in the SVZ following a stroke coincides with an increased cell proliferation (Zhang et al., 2004). In addition, cell death also controls cell numbers in the SVZ, probably through the release of yet non-identified diffusible factors (Agasse et al., 2004). Finally, physiological regulation may also be provided by sensory signals originating from the OB, where axotomy-induced deafferentation of the OB leads to an increase in proliferation in the SVZ (Mandairon et al., 2003). This increase in proliferation is coupled to an up-regulation of cell death. Conversely, others have reported decreased proliferation 3 months

* Corresponding author. Fax: +1 305 243 3914.
E-mail address: dliebl@miami.edu (D.J. Liebl).
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after bulbectomy (Kirschenbaum et al., 1999). It is still notable that OB inputs do not appear to be essential for neurogenesis to occur in the SVZ, suggesting that local cues and not diffusible OB factor may be required to regulate neurogenic events.

One group of molecules that has been recently implicated in proliferation of stem/progenitor cells in the SVZ is the ephrin family and their Eph receptors. Intraventricular infusions of ephrinB2-Fc or EphB2-Fc molecules lead to increases in the number of astrocytes (some of which may be the stem/progenitor cells) and transit-amplifying precursors, as well as a decrease in the number of neuroblasts after EphB2-Fc injections. RT-PCR experiments show that ephrinB2 and ephrinB3 ligands, as well as EphB1, EphB2, EphB3 and EphA4 receptors are expressed in the SVZ (Conover et al., 2000). Furthermore, EphB2 directly activates the proliferation of SVZ precursors in vitro (Katakowski et al., 2005). Recently, the A-class was implicated as well in the control of neurogenesis: ephrinA2 and EphA7 were identified as negative regulators of neural precursors proliferation (Holmberg et al., 2005). However, ephrinA1 does not affect telencephalon embryonic progenitors proliferation, but rather directs cells towards a neuronal fate (Aoki et al., 2004). Ephrins are membrane-bound ligands and tethered to the cell surface, by either a glycosylphosphatidylinositol (GPI)-anchor (A-class) or transmembrane domain (B-class). Eph receptors, which belong to the largest family of receptor tyrosine kinases, are also subdivided into two A- and B-classes, based on their preferential binding for one particular group of ligands. One exception is EphA4 as it binds ligands from both subclasses. Therefore, receptor activation by ligand binding is a direct result of cell–cell contact, and interactions between ephrins and Eph receptors can result in bidirectional signaling from both the ligand and receptor (Flanagan and Vanderhaeghen, 1998). Although the binding between ligands and receptors is considered very promiscuous, specific ligand–receptor interactions exist within each subclass (Blits-Huizinga et al., 2004).

Here, we examined a novel role for ephrinB3 in neurogenic proliferation of the SVZ. Examination of gene-targeted knockout mice for ephrinB3 revealed a significant increase in the number of proliferating stem/progenitor cells in the SVZ. We also observed significant increases in cell cycle activators and decreases in cell cycle inhibitors in the absence of ephrinB3. An increase in apoptosis in neurogenic regions is associated with the increase in proliferation in the absence of ephrinB3. We propose that ephrinB3 functions as a negative regulator of cell proliferation and cell apoptosis in the SVZ during adult neurogenesis.

Results

Expression of B-class ephrins in the adult SVZ and RMS regions

Neurogenesis continues to persist in the subventricular zone into adulthood, where stem/progenitor cells give rise to neuroblasts that migrate through the rostral migratory stream (RMS) to become interneurons in the olfactory bulb. Previous reports have implicated a role for B-class ephrins in the adult rodent SVZ, however, it is unclear which ephrin(s) may be involved (Conover et al., 2000). To further elucidate the role of ephrins in the SVZ, we initially examined the expression of this family of ligands in the subventricular zone. All three ephrinBs were observed in the SVZ or surrounding tissues, where ephrinB1 and ephrinB2 were expressed on cells residing in the SVZ while ephrinB3 was expressed in tissues surrounding the SVZ and RMS (Fig. 1). In particular, ephrinB1 was observed on cells that resided in the SVZ along the striatal, septal, and cortical sides (Fig. 1a). In addition, ephrinB1 was found to be co-expressed with GFAP (Figs. 1b–d), an astrocytic marker found also in stem/progenitor cells in the SVZ, but not PSA-NCAM (Figs. 1e–g). EphrinB2 was expressed in cells that resided in and around the SVZ and corpus callosum (CC) (Fig. 1h). Like ephrinB1, ephrinB2 was also co-expressed with GFAP and not PSA-NCAM; however, some of the GFAP-expressing cells have a ramified appearance that likely represents mature astrocytes while other GFAP-expressing cells appeared to be morphologically similar to stem/progenitor cells (Figs. 1i–n). In addition, we cannot rule out the possibility that ephrinB1 and ephrinB2 may also be expressed on ependymal cells, since cross-reactivity with anti-GFAP in such cells antibodies has been shown previously (Doetsch et al., 2002a,b). Co-expression of ephrinB1 and ephrinB2 with GFAP was confirmed using confocal microscopy (Figs. 1b–g insets, i–n insets). To examine ephrinB3 expression, we took advantage of a transgenic knock-in mouse where β-galactosidase (ephrinB3$^{\beta gal}$) replaces the cytoplasmic domain. X-gal staining was observed throughout the brain, including the cortex, striatum, septum, and CC; however, we did not observe staining within the SVZ or RMS (Figs. 1o, p). Figs. 1q–s represent an overlay image where PSA-NCAM-expressing neuroblasts where found to mainly reside along a border of ephrinB3 expression in the SVZ. To confirm whether ephrinB1 and ephrinB2 but not ephrinB3 are present in stem/progenitor cell populations, we examined mRNA expression in purified neurospheres. RT-PCR analysis using RNA from cultured neurospheres generated from adult SVZ cells showed expression of ephrinB1 and ephrinB2 but not ephrinB3, supporting our immunohistochemical observations (Fig. 1t). These findings also support the presence of ephrinB1 and ephrinB2 in the stem/progenitor cell population. We also found that EphB1, EphB3, and EphA4 receptors were all expressed in the neurospheres, which specifically contain stem/progenitor cell populations. Further analysis of carefully dissected SVZ tissue revealed that in addition to EphB1, EphB3, and EphA4 mRNA expression, EphB2, EphB4, and EphB6 mRNA transcripts were also localized to tissues in or closely associated to the SVZ (Fig. 1u). These studies demonstrate that multiple ephrins and Eph receptors are present in and/or around the SVZ, and may play important roles in regulating adult neurogenesis.

Deletion of ephrinB3 results in cell proliferation and not migration abnormalities in the subventricular zone

Previous reports have implicated ephrins in regulating cell migration and proliferation in the adult SVZ when ephrin-Fc or

Fig. 1. EphrinB ligands are expressed in the adult subventricular zone. EphrinB1 (red, a, c, f) is expressed in the walls of the ventricle, and co-localizes with GFAP (green, b, d) but not PSA-NCAM (green, e, g). EphrinB2 (green, h, j, m) is expressed along the ventricle and in the corpus callosum, and also co-localizes with GFAP (red, i, k) but not PSA-NCAM (red, l, n). Insets: high-magnification confocal images. Expression of ephrinB3 (identified using X-gal staining in ephrinB3$^{\beta gal}$ mice) is excluded from the RMS (o, sagittal section; inset: cross-section of the RMS) and SVZ (p, q). The area lining the ventricle devoid of ephrinB3 contains PSA-NCAM-stained neuroblasts (red, r, s), which seem to align along the ephrinB3 border (s). (t) Expression of ephrins and Eph receptors detected by RT-PCR in cultured neurospheres. (u) Expression of Eph receptors detected by RT-PCR in carefully dissected SVZ tissues.