Ephrin-A2 reverse signaling negatively regulates neural progenitor proliferation and neurogenesis

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The number of cells in an organ is regulated by mitogens and trophic factors that impinge on intrinsic determinants of proliferation and apoptosis. We here report the identification of an additional mechanism to control cell number in the brain: EphA7 induces ephrin-A2 reverse signaling, which negatively regulates neural progenitor cell proliferation. Cells in the neural stem cell niche in the adult brain proliferate more and have a shorter cell cycle in mice lacking ephrin-A2. The increased progenitor proliferation is accompanied by a higher number of cells in the olfactory bulb. Disrupting the interaction between ephrin-A2 and EphA7 in the adult brain of wild-type mice disinhibits proliferation and results in increased neurogenesis. The identification of ephrin-A2 and EphA7 as negative regulators of progenitor cell proliferation reveals a novel mechanism to control cell numbers in the brain.

[*Keywords*: SVZ; adult; neuronal progenitors; proliferation; ephrins; reverse signaling]

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Most neurons are generated before birth and neurogenesis in the adult brain is limited to a few regions. Cells with stem cell properties can be propagated in vitro from many regions of the adult brain and spinal cord, but the majority of these cells appear to be largely quiescent or only give rise to glial cells (Weiss et al. 1996; Johansson et al. 1999; Palmer et al. 1999; Horner et al. 2000; Yamamoto et al. 2001; Martens et al. 2002). High proliferative activity is limited to the dentate gyrus of the hippocampus and the lateral walls of the lateral ventricles. Stem cells in these niches give rise to neurons that migrate locally within the dentate and from the lateral ventricle wall to the olfactory bulb to integrate into the synaptic circuitry in the target area (Carlén et al. 2002; Gage 2002; van Praag et al. 2002; Carleton et al. 2003).

The number of new neurons that integrate in the brain is regulated both at the level of proliferation of stem/progenitor cells as well as the survival of newborn neurons. Although several different growth factors and cytokines can increase neural stem/progenitor cell proliferation when administered exogenously (Lie et al. 2004), little is known about the endogenous molecules regulating proliferation and neurogenesis within the stem cell niche.

The expression of ephrins and their Eph tyrosine kinase receptors in neural, intestinal, epidermal, and hematopoietic stem/progenitor cell populations in the adult (Conover et al. 2000; Batlle et al. 2002; Ivanova et al. 2002; Ramalho-Santos et al. 2002; Tumbar et al. 2004) suggests that they may modulate tissue homeostasis. Ephrins and Ephs are pivotal regulators of developmental processes such as axon guidance, cell migration, synapse formation, and vascular development (Wilkinson 2001; Holmberg and Frisén 2002; Palmer and Klein 2003), but much less is known about potential roles in the adult organism. Ephrins fall into two classes based on their mode of membrane attachment, where A-class ephrins are linked to the cell membrane by a GPI anchor and B-class ephrins have a transmembrane and a cytoplasmatic domain. Eph receptors are also divided in two classes, A and B, based on structural homology. Ephrin-As were thought to exclusively interact with A-class Ephs and B ephrins mainly with B-class Ephs (Gale et al. 1996), but it is now clear that there is a higher degree of promiscuity between the classes than previously anticipated (Himanen et al. 2004). Ephrins and Ephs mediate bidirectional signaling, where receptor signaling is referred to as forward signaling and ephrin signaling as reverse signaling (Cowan and Henkemeyer 2002). Whereas the mechanisms for ephrin-B signaling are be-

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ginning to be elucidated (Cowan and Henkemeyer 2002), mechanisms for ephrin-A reverse signaling, for which there is convincing biochemical and genetic evidence (Davy and Robbins 2000; Huai and Drescher 2001; Knoll et al. 2001; Cutforth et al. 2003), are largely unknown. Identification of signaling pathways used by ephrins and Ephs is often difficult due to a lack of simple models for the morphogenic events these molecules participate in, and the discovery of other effects of ephrin or Eph signaling would aid in the characterization of signaling pathways.

We have studied the role of A-class ephrins and Eph receptors in a neural stem cell niche in the adult brain. We report that ephrin-A2 and EphA7 negatively regulate neural progenitor proliferation, and that this effect is mediated by ephrin-A2 reverse signaling. Inhibition of the ephrin-A2/EphA7 interaction by infusion of soluble proteins or in mutant mice results in increased progenitor cell proliferation and neurogenesis in the adult brain. We propose that these molecules provide a feedback system between cells of different maturational states in the stem cell niche to modulate the number of cells that are generated in the adult brain.

Results

Complementary expression of ephrin-A2 and EphA7 in a neural stem cell niche

Analysis of the expression of all A-type ephrins and their EphA receptors in the mouse brain revealed prominent expression of ephrin-A2 and EphA7 in cells of the lateral ventricle wall (Fig. 1; Supplementary Fig. 1; data not shown). In addition, low levels of *EphA4* mRNA were detected by RT–PCR and in situ hybridization and very low levels of protein were seen in the lateral ventricle wall by immunohistochemistry with an antibody against EphA4 (data not shown). Neural stem cells reside in proximity to the lumen of the ventricular system both during embryogenesis and in the adult brain. EphA7 is expressed in the ventricular zone already at embryonic day 12.5, but expression of A ephrins in this region cannot be detected until later in embryonic development

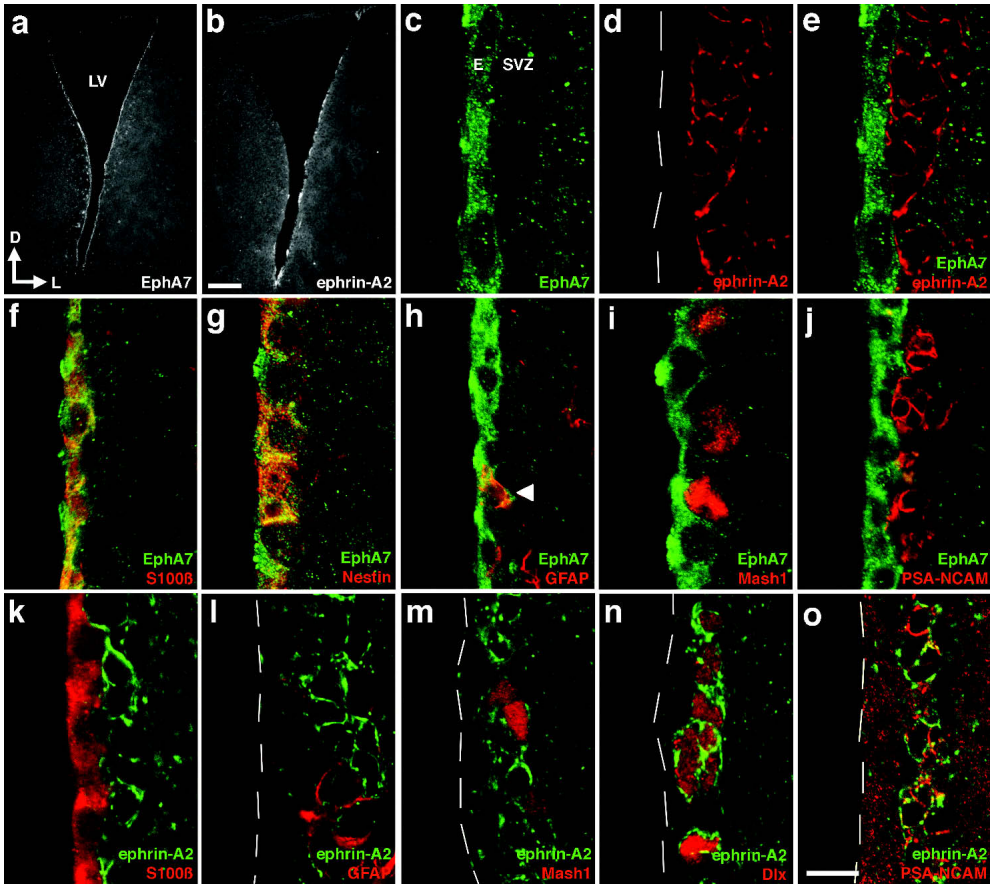

Figure 1. Ephrin-A2 and EphA7 in a neural stem cell niche in the adult brain. (*a*,*b*) Immunohistochemical localization of EphA7 and ephrin-A2 in the walls of the lateral ventricles (LV) in the adult mouse brain. (*c*–*e*) EphA7 and ephrin-A2 are expressed in complementary and mutually exclusive patterns. (*f*–*j*) EphA7 is seen in S100β-immunoreactive ependymal (E) cells and in a subset of GFAP-positive subventricular zone (SVZ) cells (arrowhead in *h*) but not in transit amplifying progenitor cells (Mash1$^+$) or migrating neuroblasts (PSA-NCAM$^+$). (*k*–*o*) In contrast, ephrin-A2-immunoreactivity is absent in ependymal cells and astrocytes and restricted to Mash1$^+$/Dlx$^+$ progenitor cells and PSA-NCAM$^+$ neuroblasts. Bars: *a*,*b*, 100 µm; *c*–*o*, 10 µm. D (dorsal) and L (lateral) indicate the orientation of all images.

(Zhang et al. 1996; Rogers et al. 1999). This expression pattern appears to be evolutionarily conserved, with expression of EphA receptors in the ventricular zone preceding ephrin-A2 expression, which starts late in embryogenesis in macaque monkeys (Donoghue and Rakic 1999).

EphA7 and ephrin-A2 are expressed in a complementary and mutually exclusive pattern in the adult mouse brain lateral ventricle wall (Fig. 1), with cells displaying the respective protein residing in direct physical proximity (Fig. 1c–e). EphA7-immunoreactive cells express nestin (Fig. 1g), a marker associated with neural stem cells (Lendahl et al. 1990). EphA7 is ubiquitously expressed by ependymal cells as well as by a subset of astrocytes in the subventricular zone (Fig. 1f–h), both being candidate neural stem cells (Doetsch et al. 1999; Johansson et al. 1999). EphA7 is not detectable in neural progenitor cells or PSA-NCAM-immunoreactive migratory neuroblasts in the lateral ventricle wall or the rostral migratory stream (Fig. 1i,j). In contrast, ephrin-A2 is expressed by Mash1- and Dlx-immunoreactive neural progenitor cells and PSA-NCAM-immunoreactive neuroblasts, but not ependymal cells or astrocytes (Fig. 1k–o).

Soluble ephrin and Eph proteins promote proliferation independent of clustering

Ephrins need to be clustered in the cell membrane, or artificially with antibodies, to activate Eph receptors (Davis et al. 1994). Unclustered soluble ephrins bind receptors but fail to activate them and therefore function as antagonists of Eph signaling (Davis et al. 1994). Infusion of antibody clustered ephrin-B2-Fc or EphB2-Fc into the lateral ventricle was shown to increase proliferation in the neural stem cell niche (Conover et al. 2000), leading to the suggestion that B-class ephrins and Eph receptors promote proliferation in this context (Alvarez-Buylla and Lim 2004). We delivered unclustered and clustered ephrin-A2-Fc and EphA7-Fc into the lateral ventricle of adult wild-type mice over a 3-d period via osmotic pumps (Fig. 2a,b). Surprisingly, infusion of unclustered and clustered ephrin-A2-Fc and Eph-A7-Fc all resulted in an increase in bromo-deoxyuridine (BrdU) incorporation in the lateral ventricle wall. The similar effect of clustered and unclustered proteins prompted us to infuse unclustered ephrin-B2-Fc, which also proved to increase proliferation to a similar extent as reported with clustered ephrin-B2 (Conover et al. 2000). Although these data indicate that both A- and B-class ephrins and Eph receptors can modulate proliferation, it is ambiguous whether both clustered and unclustered proteins act as agonists or antagonists, and thus whether ephrins and Eph receptors act as positive or negative regulators of proliferation. Moreover, it is unclear from the infusion of recombinant proteins whether ephrins have a physiological function in regulating cell proliferation.

Ephrin-A2 and EphA7 are negative regulators of proliferation

This prompted us to analyze *ephrin-A2*$^{-/-}$ and *EphA7*$^{-/-}$ mice (Feldheim et al. 2000; Holmberg et al. 2000). BrdU labeling of dividing cells in *ephrin-A2*$^{-/-}$ and *EphA7*$^{-/-}$ mice revealed an increase in the number of labeled cells in the lateral ventricle wall compared to wild-type littermates (Fig. 2c), suggesting that they negatively regulate cell proliferation. However, an increase in the number of BrdU-labeled cells in the mutants could alternatively be a consequence of a decrease in apoptosis of newborn cells in the lateral ventricle wall or an accumulation of newborn cells due to a migration defect. Quantification of the number of apoptotic cells in the lateral ventricle wall of *ephrin-A2* and *EphA7* null mice by TUNEL labeling did not reveal a significant difference compared to wild-type littermates (1.18 ± 0.55 cells/section in wild-type, 1.17 ± 0.58 in *ephrin-A2*$^{-/-}$, and 1.27 ± 0.62 in *EphA7*$^{-/-}$ mice, mean ± SEM). Analysis of the pattern of PSA-NCAM-immunoreactive neuroblasts in the lateral ventricle wall as well as labeling of cells in the ventricle wall by a DiI injection, which labels migratory cells (Johansson et al. 1999), did not reveal any migration defect (Fig. 3). Moreover, analysis of markers for cells at different maturational stages in the neural lineage as well as electron microscopic analysis of the lateral ventricle wall did not reveal an expansion of the subventricular zone, ectopic pattern, or accumulation of any cell type (Supplementary Fig. 2). Most importantly, the increased number of BrdU-labeled cells in the ventricle wall was paralleled by a similar increase in new-

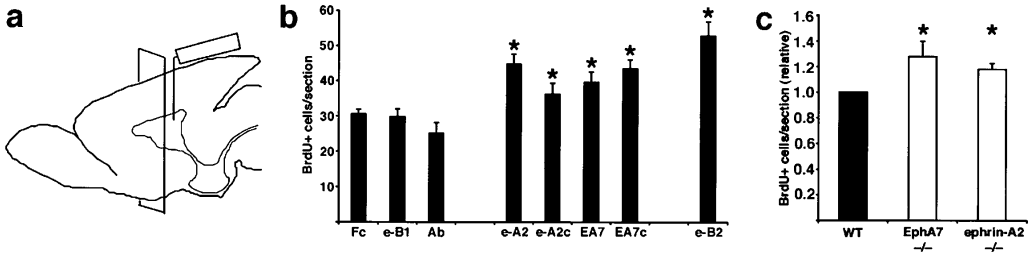

Figure 2. Ephrin-A2 and EphA7 negatively regulate proliferation. (*a*,*b*) Recombinant unclustered or antibody (ab), clustered (c), ephrin (e), or Eph-Fc (E) fusion proteins were infused into the lateral ventricle and BrdU incorporation was analyzed after 3 d. (*c*) The number of BrdU-incorporating cells in the lateral ventricle wall is increased in the lateral ventricle wall of the mutant mice. Mutant animals are compared to their respective control littermates and the value is given as relative to the wild type. Bars represent mean of three to eight independent experiments ± SEM. ANOVA was used for statistical analysis in *b* and Student's *t*-test was used in *c*. (*) $p < 0.05$.

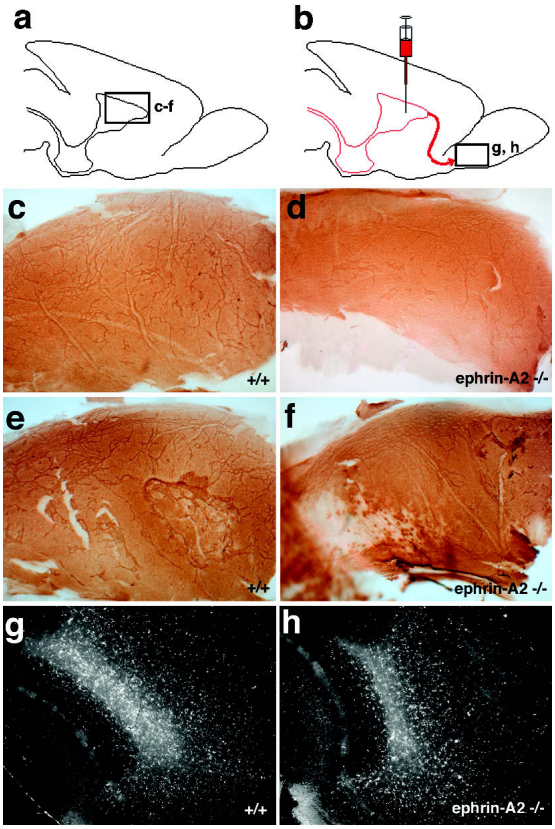

Figure 3. Loss of ephrin-A2 does not impede migration to the olfactory bulb. (*a*,*b*) Schematic drawings of the analyzed regions. (*c*–*f*) Normal pattern of chains of migrating PSA-NCAM-immunoreactive neuroblasts in the lateral ventricle wall visualized in whole-mount preparations. (*g*,*h*) Migrating neuroblasts labeled by an intraventricular DiI injection reach the olfactory bulb in a normal pattern in ephrin-A2 null mice.

born neurons in the olfactory bulb of *ephrin-A2* null mice (see below), establishing that migration to the olfactory bulb is not impeded.

To directly test whether the increase in BrdU labeling in the mutant mice was due to increased cell proliferation, we assessed the cell cycle length of dividing cells in the lateral ventricle wall. We quantified the proportion of PCNA-expressing cells in wild-type and mutant mice that were labeled after three pulses of BrdU 2, 4, and 6 h prior to analysis (Fig. 4a; Supplementary Fig. 3). Since PCNA is expressed throughout the cell cycle of mitotic cells, whereas BrdU is incorporated only in nuclei of cells in S phase, a shortening of the cell cycle will result in an increase in the proportion of PCNA-expressing cells that incorporate BrdU (Schmal 1983). We found a higher BrdU/PCNA labeling index in ephrin-A2 null mice compared to wild-type littermates (Fig. 4a), demonstrating a significant reduction in cell cycle length. A similar increase of the BrdU/PCNA index was observed in mice intracerebroventricularly infused with ephrin-A2-Fc (Fig. 4b).

Both ephrin-A2 and EphA7 are expressed in cultured primary neural stem/progenitor cells (neurospheres) from wild-type animals (Supplementary Fig. 4). Measurement of 3H-thymidine incorporation revealed a significant increase in cell proliferation in cultures from *ephrin-A2*$^{-/-}$ and *EphA7*$^{-/-}$ mice compared to wild-type littermates (Fig. 4c), without any difference in cell death or differentiation (Supplementary Fig. 4). After a neurosphere is formed from a single cell, an increasing heterogeneity will ensue as some cells within the clone will gain commitment to certain fates, making it difficult to conclude that the increase in proliferation is in the stem/progenitor population or in more restricted cells. To directly assay the number of neural progenitor cells that were generated in vitro, we quantified the number of primary cells that could form new secondary neurospheres. We found that the absolute number of secondary neurospheres was higher in *ephrin-A2* and *EphA7* null compared to wild-type cultures, but that this expansion was proportional to the increase in cell number (Fig. 4d). This demonstrates that ephrin-A2 and EphA7 suppress neural stem/progenitor cell proliferation in vitro without affecting the relative proportion of such cells to more committed cells.

Ephrin-A2 reverse signaling negatively regulates neural progenitor proliferation

The lateral ventricle wall harbors many cell types including all maturational stages from neural stem cells to neuroblasts. We next asked at what cellular stage in this lineage ephrin-A2 and EphA7 inhibit proliferation in

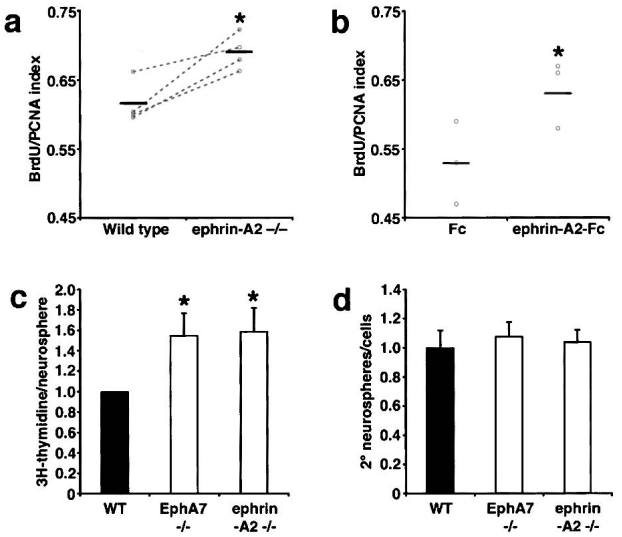

Figure 4. Ephrin-A2 and EphA7 are negative regulators of proliferation. (*a*,*b*) An increased BrdU/PCNA labeling index indicate a shorter cell cycle in cells of the lateral ventricle wall stem cell niche in *ephrin-A2* null mice compared to wild-type littermates and in ephrin-A2-Fc-infused mice. (*c*) Increased proliferation in *ephrin-A2* and *EphA7* null neurospheres. (*d*) The mutant neural stem/progenitor cells give rise to more cells, but with an unaltered frequency of cells capable of forming secondary neurospheres.

vivo. Since EphA7 is not the only EphA receptor expressed in the lateral ventricle wall and there may be some degree of redundancy, we focused the subsequent analyses on *ephrin-A2* null mice. The absolute majority of proliferating cells in the lateral ventricle wall are ephrin-A2-immunoreactive and represent Mash1- and/or Dlx-expressing transit amplifying progenitor cells (Figs. 1, 5a,b). Ependymal cells and astrocytes, which express EphA7, together only represent 2.2 ± 0.5% (mean ± SEM) of dividing cells in wild-type and 1.3 ± 0.5% in *ephrin-A2*$^{-/-}$ mice (Fig. 5b). Thus, the absence of ephrin-A2 does not increase the proliferation of EphA7-expressing cells. As in wild-type animals, the vast majority of BrdU incorporating cells in *ephrin-A2* null mice are progenitor cells (Fig. 5b), indicating that the increase in proliferation is in the progenitor cell compartment. Indeed, Dlx-immunoreactive cells, which constitute the largest subpopulation of progenitor cells, had a significantly shorter cell cycle in *ephrin-A2*$^{-/-}$ mice compared to wild-type littermates (Fig. 5c, Supplementary Fig. 3). Dlx is coexpressed with ephrin-A2 in wild-type animals (Fig. 1n), and the increased proliferation in the mutant mice is thus in cells normally expressing ephrin-A2 and not EphA7. This could indicate that ephrin-A2 cell autonomously regulates the proliferation of neural progenitor cells.

The negative regulation of proliferation by ephrin-A2 and EphA7 may thus be mediated by reverse signaling through ephrin-A2. The *EphA7* locus encodes several splice forms (Ciossek et al. 1995) and RT–PCR (data not shown), in situ hybridization, and immunohistochemistry revealed expression of full-length (EphA7-Fl) and a truncated receptor (EphA7-T1) in all ependymal cells in the lateral ventricle wall (Fig. 6a–d). These splice forms share identical extracellular domains, but EphA7-T1 lacks the kinase domain (Ciossek et al. 1995). EphA7-T1 is a dominant negative inhibitor of EphA7 signaling (Holmberg et al. 2000), raising the possibility that EphA7 may not primarily mediate forward signaling in the lateral ventricle wall but instead act as a ligand to induce ephrin-A2 reverse signaling. If forward signaling is mediating a certain effect, experimental expression of a truncated receptor would result in a loss of function phenotype by inhibition of full-length Eph receptor signaling (Wilkinson 2001; Cowan and Henkemeyer 2002). Conversely, if forward signaling is not required and the effect is mediated by ephrin reverse signaling, expression of a truncated receptor may increase reverse signaling and result in a gain of function. To directly test whether EphA7 forward signaling is required for the negative effect on neural progenitor proliferation, we engineered a retrovirus expressing EphA7-T1 and green fluorescent protein (GFP) and a control retrovirus expressing GFP only (Supplementary Fig. 5). Overexpression of EphA7-T1 in wild-type neurospheres resulted in a significant decrease in proliferation compared to cells infected with the GFP-expressing retrovirus (Fig. 6e). Thus, expression of EphA7-T1 has an effect opposite to the loss of the receptor (Fig. 4c) and represents a gain of function. Moreover, expression of EphA7-T1 significantly decreased proliferation in *EphA7*$^{-/-}$ neurospheres, establishing that forward signaling is redundant for the proliferation inhibiting effect of EphA7 (Fig. 6f). In contrast, expression of EphA7-T1 in *ephrin-A2*$^{-/-}$ neurospheres did not significantly affect proliferation, demonstrating the requirement of ephrin-A2 for EphA7-T1 to inhibit proliferation (Fig. 6g). The gain of function mediated by ectopic expression of EphA7-T1 in wild-type and *EphA7*$^{-/-}$ cells strongly indicates that the negative regulation of proliferation by EphA7 and ephrin-A2 is mediated by reverse signaling.

Ephrin-A2 negatively regulates adult neurogenesis

We next asked whether the increased proliferation of neural progenitor cells in *ephrin-A2* null mice was accompanied by an alteration in the number of mature cells that integrated in the target tissue. The majority of neurons born in the lateral ventricle wall migrate to the olfactory bulb. We found a 44% increase in the number of BrdU-labeled cells that integrate in the adult olfactory bulb of *ephrin-A2* null mice compared to wild-type littermates (Fig. 7a). A similar proportion of the adult-born cells in wild-type (63 ± 2.1%, mean ± SEM) and *ephrin-A2* null (71 ± 2.5%, $p > 0.05$) mice were NeuN-immuno-

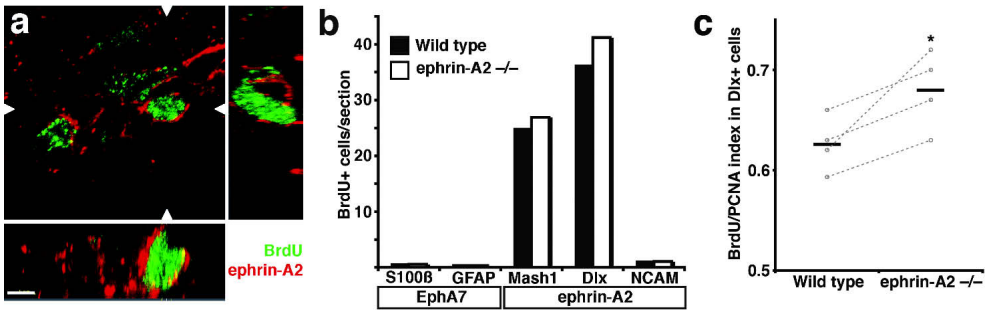

Figure 5. Ephrin-A2 regulates neural progenitor proliferation. (*a*) The majority of BrdU-incorporating cells are ephrin-A2-immunoreactive. (*b*) Analysis of BrdU incorporation in cells that express markers for cells at different stages in the neural lineage by confocal microscopy. The expression of ephrin-A2 or EphA7 in the different populations is indicated in the boxes *below*. (*c*) An increased BrdU/PCNA labeling index indicates a shortening of the cell cycle in Dlx$^+$ progenitor cells in *ephrin-A2*$^{-/-}$ mice. Bar, 5 µm. (*) $p < 0.05$.

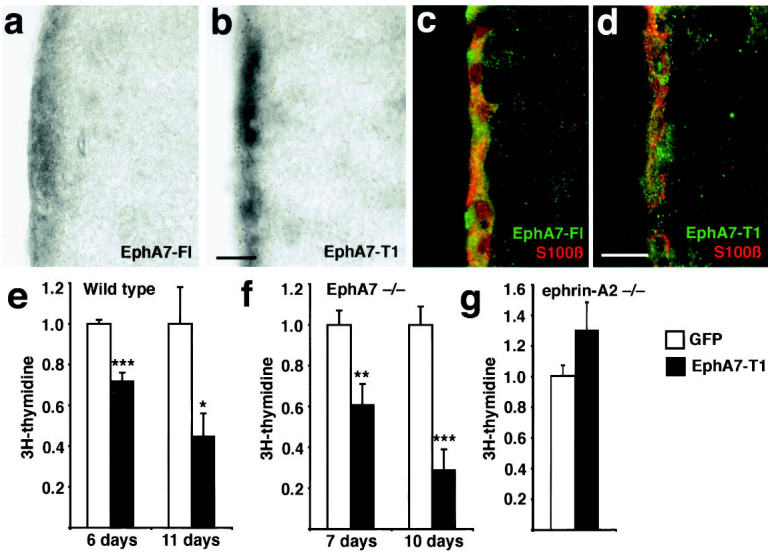

Figure 6. Reverse signaling negatively regulates progenitor proliferation. (*a*,*b*) Expression of mRNA encoding full-length *EphA7* (*EphA7-Fl*) and a kinase-deficient truncated splice form (*EphA7-T1*) in the lateral ventricle wall. (*c*,*d*) Immunoreactivity for both spliceforms is localized to S100β-positive ependymal cells. (*e*–*g*) Retroviral expression of EphA7-T1 in neurospheres suppresses proliferation in wild-type and *EphA7*$^{-/-}$ cells, but not in *ephrin-A2*$^{-/-}$ cells (shown as relative to cells transduced with GFP-expressing virus). Bars: *a*,*b*, 20 µm; *c*,*d*, 10 µm. (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.005$.

reactive neurons (Supplementary Fig. 6). This was paralleled by a 15% increase in cell density and 7% increase in cell number in the adult olfactory bulb (Fig. 7b,c; Supplementary Fig. 7). Analysis of nonneurogenic brain regions did not reveal any ectopic adult neurogenesis (data not shown) or increased cell density in the cortex or striatum (Supplementary Fig. 6) in *ephrin-A2* null mice, indicating that ephrin-A2 regulates the proliferation rate and neurogenesis but not its distribution. The number of new neurons that are born and integrate in the adult brain is regulated at multiple steps. The finding that the increased proliferation in the lateral ventricle wall is paralleled by a similar increase in the integration of neurons in the olfactory bulb suggests that the proliferative activity early in the lineage is an important determinant of the number of neurons that integrate in the target area.

Several studies have shown that infusion of mitogens can increase cell proliferation in the lateral ventricle wall and in some situations result in an increase in the number of adult-born neurons, suggesting that stimulation of neurogenesis from endogenous stem cells may be an attractive strategy for neuronal replacement (Lie et al. 2004). The identification of ephrin-A2 and EphA7 as negative regulators of neural progenitor cell proliferation raised the question of whether it may be possible to stimulate neurogenesis in the adult brain by blocking their interaction. We quantified the number of newborn cells in the adult olfactory bulb in animals that had received intracerebroventricular infusion of ephrin-A2-Fc for 1 wk and were allowed to survive for an additional 2 wk prior to analysis. Blocking the interaction between ephrin-A2 and EphA7 in this manner resulted in a 17% increase in the number of BrdU labeled cells in the olfactory bulb ($p < 0.05$, Fig. 7d), with a similar proportion displaying NeuN-immunoreactivity (69 ± 2.1% in mice receiving Fc and 77 ± 2.4% in animals given ephrin-A2-Fc, $p > 0.05$). The increase in neurogenesis in ephrin-A2-Fc-infused animals is similar in magnitude to that seen after FGF-2 infusion (15%) (Kuhn et al. 1997), but less than reported with VEGF (67%) (Schänzer et al. 2004). The increase in neurogenesis achieved by interfering with endogenous ephrin-A2 function with ephrin-A2-Fc and thereby disrupting the suppression of proliferation establishes inhibition of a negative regulator as a potential therapeutic strategy to expand a stem cell derived population in vivo.

Discussion

Infusion of several different growth factors and cytokines can modulate stem/progenitor cell mitosis in the adult brain (Lie et al. 2004), but much less is known about the endogenous molecules that regulate proliferation and neurogenesis. We show that ephrin-A2 and EphA7 are expressed in complementary and mutually exclusive patterns by cells at different maturational stages in the adult lateral ventricle wall neural stem cell niche. Inhibiting the interaction between ephrin-A2 and EphA7 by infusion of soluble proteins or in mutant mice results in increased progenitor cell proliferation. The ephrin–Eph interaction takes place at direct cell–cell contacts, suggesting that this pathway provides a feedback mechanism locally within the stem cell niche to control the number of cells produced.

The best described role for ephrins and Eph receptors is guiding growing axons and migrating cells. Visualization of progenitor cells and neuroblasts with molecular markers, by electron microscopy, and tracing in mutant animals did not reveal any disruption of the normal migratory pattern in the lateral ventricle wall or rostral migratory stream. Most importantly, quantification of the number of newly arrived (BrdU-positive) neurons in the olfactory bulb of *ephrin-A2* null mice demonstrated an increase parallel to the increase in proliferative cells in the ventricle wall, establishing that migration was not impeded. The increase in BrdU incorporation and shortening of the cell cycle in progenitor cells in the lateral

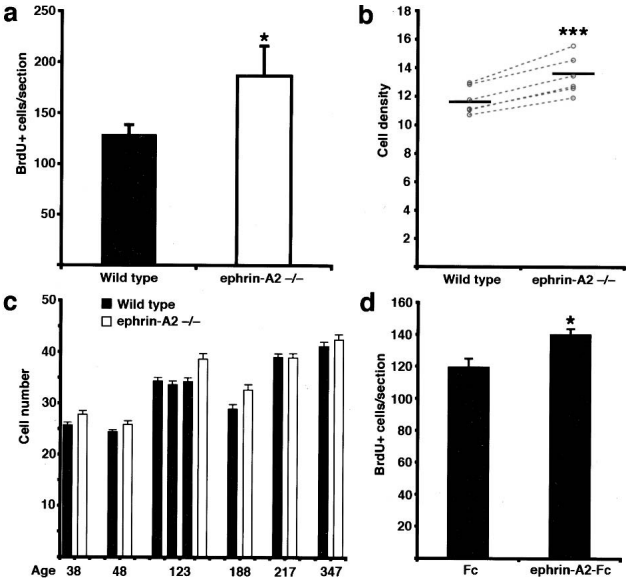

Figure 7. Negative regulation of neurogenesis. (*a*) The number of cells that are added to the olfactory bulb in adulthood is increased in the absence of ephrin-A2. (*b*,*c*) The cell density and number of cells in olfactory bulbs of wild-type and *ephrin-A2*$^{-/-}$ littermates were analyzed as described in Supplementary Figure 6. Litters of different age (days) are shown in *c*. (*d*) Infusion of ephrin-A2-Fc for 1 wk results in increased numbers of newborn cells 2 wk later. Error bars indicate SEM. (*) $p < 0.05$, (***) $p < 0.005$.

ventricle wall together with increased proliferation of neural progenitor cells in vitro established ephrin-A2 and EphA7 as negative regulators of cell proliferation.

The interaction between ephrins and Eph receptors can mediate bidirectional signaling (Cowan and Henkemeyer 2002). Several strategies can be used to dissociate the relative role of forward and reverse signaling in a certain situation. First, unclustered soluble ephrins and Ephs often inhibit signaling by their endogenous binding partner whereas clustered proteins often activate signaling (Davis et al. 1994), and analysis of the effect of clustered and unclustered ephrin and Eph proteins can provide insights into the role of activating and inhibiting ephrin and Eph signaling, respectively. We found that clustered and unclustered soluble ephrins/Ephs had indistinguishable effects on proliferation when infused into the lateral ventricle, not making this strategy informative for distinguishing between forward and reverse signaling in this context. The analysis of mutant mice allowed us to conclude that both clustered and unclustered A-class ephrins and Ephs act as antagonists in this situation. We do not know why clustered proteins failed to activate signaling, but it is possible that higher order clustering than that achieved with an antibody may be required. Second, truncated Eph receptors lacking the tyrosine kinase domain are incapable of forward signaling and act as dominant negatives, but still activate reverse signaling (Henkemeyer et al. 1996; Mellitzer et al. 1999; Xu et al. 1999). Expression of a truncated splice form of EphA7 in wild-type or EphA7 null primary neural progenitors resulted in a gain of function phenotype. This establishes that EphA7 forward signaling is not required for its negative regulation of proliferation, but that it mediates this function by activating ephrin-A2 reverse signaling. This is in line with the finding that the ephrin-A2/EphA7 interaction regulates the proliferation of ephrin-A2-expressing progenitor cells and not EphA7-expressing cells in vivo (Fig. 5). Moreover, the endogenous expression of the dominant negative truncated splice form EphA7-T1 in vivo (Fig. 6) raises the possibility that EphA7 may be incapable of forward signaling. There is evidence for ephrin-A reverse signaling regulating axon guidance and cell adhesion. The current study is, to our knowledge, the first indication of a role for ephrin-A reverse signaling in cell proliferation. Whereas several key components of the signal transduction pathways for ephrin-B reverse signaling have been identified (Cowan and Henkemeyer 2002), much less is known regarding ephrin-A reverse signaling. Signaling mechanisms for ephrins have been difficult to characterize, partly due to a lack of simple models for the morphogenic events in which these molecules participate. However, since cell proliferation is relatively easy to assay, the current data may suggest novel models that may aid in the identification of signaling pathways utilized by ephrins.

Infusion of antibody-clustered ephrin-B2-Fc or EphB2-Fc results in increased proliferation in the adult lateral ventricle wall (Conover et al. 2000), and it was suggested that B-class ephrins/Eph receptors act as positive regulators of proliferation (Alvarez-Buylla and Lim 2004). Do A- and B-class ephrins/Ephs have opposite functions in this context? Both A- and B-class ephrins/Eph-Fc proteins increase cell proliferation in the lateral ventricle wall, independent of clustering. That ephrin and Eph proteins give the same effect is difficult to reconcile with these proteins acting as positive regulators, since even though the ephrin or Eph protein could act as an activator, the endogenous binding partner would be antagonized. It thus appears likely that B-class ephrin/Ephs also act as negative regulators of proliferation in the neural stem cell niche. Considering the potential cross-talk between members of the A and B class (Himanen et al. 2004) it is even possible that they cooperate in the same process. The analysis of mice lacking ephrin-A2 or EphA7 made it possible to conclude that these proteins are negative regulators of proliferation, but in the case of the B class a genetic approach is more difficult since multiple members are expressed and several of the compound mutants do not reach adulthood.

The expression of ephrins and Ephs is altered in tumors of several different origins (Tang et al. 1999, 2000; Stephenson et al. 2001; Liu et al. 2002). Interestingly, overexpression of ephrins and Ephs in human neuroblastoma correlates with a favorable prognosis (Tang et al. 1999). The well-documented role of ephrins and Ephs in angiogenesis has led to the suggestion that the expression of ephrins/Ephs in tumors may influence vascular infiltration and thereby tumor growth (Palmer and Klein 2003). Tumors are often heterogeneous with respect to their cellular composition, and emerging evidence sug-

gests that a small subset of cells may act as stem cells for the transformed cell lineage (Pardal et al. 2003). Such tumor stem cells often share many properties with untransformed tissue stem cells (Pardal et al. 2003). The role of ephrin-A2 and EphA7 in modulating proliferation in the neural lineage raises the possibility that ephrins and Ephs may similarly negatively regulate proliferation of tumor stem cells in human brain tumors, which potentially could explain why overexpression correlates with a favorable prognosis. Ephrins/Ephs indeed negatively regulate the proliferation of human glioblastoma cells in vitro (our unpublished data), suggesting that similar pathways may control neurogenesis and tumor growth.

The identification of a pathway that negatively regulates progenitor cell proliferation reveals a novel control mechanism for neurogenesis in the brain. The expression of ephrins and Eph receptors in several stem/progenitor cell populations (Batlle et al. 2002; Ivanova et al. 2002; Ramalho-Santos et al. 2002; Tumbar et al. 2004) and the finding that they modulate cell cycle progression in *Caenorhabditis elegans* germ cells (Miller et al. 2003) suggest that this may be a general and evolutionarily conserved function for these genes.

Materials and methods

Mutant mice

EphA7 and *ephrin-A2* mutant mice (Feldheim et al. 2000; Holmberg et al. 2000) were genotyped by PCR. Both strains have a mixed 129/Sv and C57/bl6 genetic background and wild-type littermates were used as controls in all experiments.

Immunohistochemistry and in situ hybridization

Digoxygenin-labeled riboprobes complementary to the tyrosine kinase domain of EphA7-Fl or the 3′ untranslated region of EphA7-T1 were used for in situ hybridization. The following antibodies were used for immunohistochemistry: rabbit antiserum against PCNA (Oncogene), EphA7 (Santa Cruz Biotechnology), Dlx (raised against Distalless, but binds mouse Dlx family proteins; a gift from Grace Boekhoff-Falk, University of Wisconsin Medical School, Madison, WI), goat antiserum against ephrin-A2 (R&D Systems), and monoclonal antibodies against GFAP (clone G-A-5; Sigma), Mash1 (a gift from Jane Johnson, University of Texas, Southwestern Medical Center, Dallas, TX), Nestin (Rat401, DSHB), PSA-NCAM (5A5, DSHB, and 2-2B; Chemicon), and S100β (SH-B1; Sigma).

Infusion of recombinant proteins

Adult male C57 Bl/6 mice (B&K Universal) were anesthetized with 2,2,2-tribromethanol (400 mg/kg). Ephrin-A2-Fc, EphA7-Fc, ephrin-B1-Fc, ephrin-B2-Fc, Fc (200 µg/mL in PBS; R&D systems) or the clustering antibody (200 µg/mL goat anti-human IgG) were delivered with an osmotic pump (Alzet 1007D, delivering 0.5 µL/h) connected to a canula inserted 0.5 mm posterior and 0.7 mm lateral to Bregma, 2 mm below the dura mater in the right lateral ventricle. The proteins were in some experiments clustered by coincubation with anti-human IgG antibodies (Jackson Immunoresearch) at a 1:10 ratio for 1 h at room temperature.

Neurosphere cultures

Tissue dissociation and culture conditions were as described (Johansson et al. 1999). Differentiation of the neural stem cells was induced by plating on poly-L-ornithine coated slides in medium with 0.5% FCS but without EGF. The cells were fixed with 4% formaldehyde after 7 d of differentation and immunohistochemistry was performed as described (Johansson et al. 1999).

Cell proliferation analyses

BrdU (100 mg/kg; Sigma) was delivered by intraperitoneal injection, and was detected in tissue sections with mouse (Becton Dickson) or rat (clone BU1/75; Accurate) monoclonal antibodies. PCNA was visualized with mouse or rabbit antibodies (Oncogene). Three injections of BrdU were given 2, 4, and 6 h prior to sacrifice for analysis of proliferation in the lateral ventricle wall. For the analysis of addition of newborn cells to the olfactory bulb BrdU was injected once daily for 7 d, and the animals were sacrificed after an additional 14 d. Labeled cells were quantified as described in Supplementary Figure 3. 3H-thymidine (5 µCi/mL) was added to cultures of secondary neurospheres 4 h prior to analysis and incorporation was measured in a scintillation counter.

Analysis of cell migration

Mice were anesthetized with 2,2,2-tribromoethanol (400 mg/kg; Sigma), and 1 µL DiI (0.05% in DMSO; Molecular Probes) was stereotaxically injected into the left lateral ventricle. Coordinates for the injection site were 1 mm lateral to bregma and 1.2 mm ventral to dura. Four weeks after intracerebroventricular (ICV) injections, mice were transcardially perfused with PBS followed by fixative (4% paraformaldehyde, 0.05% glutaraldehyde in PBS), and brains were removed and post-fixed overnight at 4°C. Sagittal sections (40 µm) of the hemisphere contralateral to the ICV injection were cut with a vibratome. Sections were mounted on microscope slides, coverslipped, and analyzed with a Zeiss Axioplan fluorescence microscope.

Ectopic expression of EphA7-T1

A PCR product encoding EphA7-T1 was cloned into pCMMP-WPRE-IRES2:eGFP using PmeI and XhoI sites and the construct was verified by DNA sequencing. Vesicular stomatitis virus G-protein pseudotyped retroviral particles were produced using the packaging cell line 293 GPG. Primary neurosphere cultures were trypsinized, and after 5 h, infected with retroviral particles generated using the original vector or the one engineered to express EphA7-T1 at a multiplicity of infection of 2.5. After 3 d the infected secondary neurospheres were trypsinized and GFP-expressing cells were isolated using a FACSDiva flow cytometer. This resulted in pure cultures of infected cells. The sorted cells were cultured for 6 d, trypsinized, and plated in 24-well dishes, and proliferation was assessed by [3H]-thymidine uptake as described above.

Cell counts

See Supplementary Figures 3, 6, and 7 for a detailed description of cell counting procedures.

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