Promotion of proliferation in the developing cerebral cortex by EphA4 forward signaling

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Eph receptors are widely expressed during cerebral cortical development, yet a role for Eph signaling in the generation of cells during corticogenesis has not been shown. Cortical progenitor cells selectively express one receptor, EphA4, and reducing EphA4 signaling in cultured progenitors suppressed proliferation, decreasing cell number. In vivo, EphA4−/− cortex had a reduced area, fewer cells and less cell division compared with control cortex. To understand the effects of EphA4 signaling in corticogenesis, EphA4-mediated signaling was selectively depressed or elevated in cortical progenitors in vivo. Compared with control cells, cells with reduced EphA4 signaling were rare and mitotically inactive. Conversely, overexpression of EphA4 maintained cells in their progenitor states at the expense of subsequent maturation, enlarging the progenitor pool. These results support a role for EphA4 in the autonomous promotion of cell proliferation during corticogenesis. Although most ephrins were undetectable in cortical progenitors, ephrin B1 was highly expressed. Our analyses demonstrate that EphA4 and ephrin B1 bind to each other, thereby initiating signaling. Furthermore, overexpression of ephrin B1 stimulated cell division of neighboring cells, supporting the hypothesis that ephrin B1-initiated forward signaling of EphA4 promotes cortical cell division.

KEY WORDS: Corticogenesis, Neurogenesis, Eph/ephrin, Intercellular communication, Ephrin B1, Mouse

INTRODUCTION

The intricate organization of the mature cerebral cortex is dependent on precisely tuned programs in the cells that seed this structure during development. To ensure proper formation, cortical cells are shaped by intrinsic genetic programs, signaling via soluble factors, interactions with substrates and differential cell adhesion as they undergo their stereotyped development during corticogenesis (McConnell, 1995). Here, we examine a contribution of intercellular communication via Eph family members in regulating progenitor proliferation in cortical germinal zones.

Eph receptors and ephrin ligands, surface bound signaling molecules that mediate communication between cells, are broadly expressed within the forming cerebrum (Mackarehtschian et al., 1999; Yun et al., 2003). Receptors and ligands must be tethered to the cell surface and clustered (Davis et al., 1994) in order to engage, generating autophosphorylation-dependent forward signaling in receptor-containing cells and/or reverse signaling in ligand-expressing cells (Davy et al., 1999; Holland et al., 1996; Holmberg et al., 2005; Kullander et al., 2001b). Initial studies designated A and B subclasses of Eph/ephrins and proposed that interactions occurred largely within each subgroup (Gale et al., 1996; Kullander et al., 2003). Activation of EphA4 influences diverse cellular processes in the nervous system: neuronal migration, differentiation and connectivity can all be guided by EphA4 activation (Conover et al., 2000; Dufour et al., 2003; Goldshmit et al., 2006; Helmbacher et al., 2000; Swartz et al., 2001; Walkenhorst et al., 2000). Furthermore, engagement of EphA4 can produce both positive and negative consequences, depending upon levels of stimulation and characteristics of participating cells (Castellani et al., 1998; Connor et al., 1998; Eberhart et al., 2004; Hansen et al., 2004). Moreover, EphA4 is broadly expressed, often coexists with ephrin ligands and can be involved in both cis- and trans-interactions (Homburger et al., 1999; Marquardt et al., 2005; Yin et al., 2004). Finally, a variety of downstream effectors of EphA4 have been identified that act to translate an extracellular signal into an intracellular response (Beg et al., 2007; Bourgin et al., 2007; Egea et al., 2005; Fawcett et al., 2007; Fu et al., 2007; Iwasato et al., 2007; Knoll and Drescher, 2004; Murai et al., 2003; Richter et al., 2007; Sahin et al., 2005; Shamah et al., 2001; Shi et al., 2007; Wegmeyer et al., 2007). Thus, EphA4 is a multifunctional receptor, and its effects are still being discerned.

In this study, we use in vitro and in vivo genetic and epigenetic approaches to characterize the role of EphA4 signaling in cortical proliferation during development. Our results support a role for EphA4-mediated forward signaling in the control of cell number in the forming cortex.

MATERIALS AND METHODS

Animals

All animal use and care was in accordance with institutional guidelines, the Yale IACUC protocol 2002-10908 and the Georgetown GUACUC protocol 06-022. CD-1 (Charles River Laboratories) or EphA4 mutant mice (P. Accepted 5 May 2009
Charney, INSERM, Paris, France), maintained on a C57Bl/6 strain (for 5-15 generations) and bred as heterozygotes (Helmbacher et al., 2000), were used. Wild-type, heterozygous and mutant alleles of EphA4 were generated in mendelian ratios. Body sizes of all genotypes were similar on the days of analyses. The day of the vaginal plug was embryonic day 0.5 (E0.5) and the day of birth, postnatal day 0 (P0).

In situ hybridization
Mouse embryos were collected, fixed, dehydrated, embedded in paraffin and sectioned sagittally (10-15 μm) for analysis of gene expression. In situ hybridizations were performed with 35S-labeled antisense probes to Eph family members, as previously described (Yun et al., 2003). Slides were dipped in autoradiographic emulsion, exposed for 2-6 weeks and developed. Our laboratory previously published an image of EphA4 expression at E12.5 (Yun et al., 2003). To obtain a profile of Eph family gene expression in cortical development, the density of silver grains was quantified in the proliferative zone (PZ), the intermediate zone (IZ) and the cortical plate (CP) of 3-5 sections of somatosensory cortex at E14.5 using ImageJ software. Probes with the same specific activity were used (see Figs S1 and S10 in the supplementary material). Silver grain density was converted to color intensity (Fig. 1A and Fig. 5A) or represented graphically (Fig. S1O and Fig. S1OK in the supplementary material).

Immunohistochemistry
Brains (E14.5-17.5) were fixed by immersion for 1-2 hours in 4% paraformaldehyde (PFA) at 4°C, washed in phosphate-buffered saline (PBS), cryoprotected in 30% sucrose overnight and frozen. Brains were stored at −80°C until 14 μm coronal cryosections were produced. Sections were mounted, air dried and stored desiccated at −20°C. Sections were incubated in block buffer plus (BB+; 2.5% goat serum, 2.5% donkey serum, 0.1% lysine, 0.4% Triton X-100 in PBS) for 30 minutes at room temperature (RT) and then incubated in primary antibody diluted with BB+ overnight at 4°C. After PBS washes, sections were incubated with Alexa-conjugated secondary antibodies (1:800), counterstained with fluorescent Nissl (1:500) or Bisbenzamide (1:1000), and mounted. The primary antibodies used, their source and dilutions are as follows: rabbit anti-GFP, Molecular Probes, 1:3000; mouse anti-Betatubulin (TuJ1, also known as Tubb3), Babco, 1:500-1000; mouse anti-R2C, Developmental Studies Hybridoma Bank (DSHB; under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242, USA), 1:5; mouse anti-nestin (rat-401), DSHB, 1:10; rabbit anti-EphA4, Zymed, 1:300; mouse anti-EphA4 Zymed, 1:500; rabbit anti-ephrin B1, Zymed, 1:250; rabbit anti-phospho-histone H3, Calbiochem, 1:200; goat anti-Sox2, R&D Systems, 1:20; mouse anti-BrdU, BD Biosciences, 1:100.

Expression vectors and expression analyses
Control vectors were either pCMV-eYFP-N1 (Clontech) or CAG-ΔsRed (Akiko Nishiyama, University of Connecticut, Storrs, CT, USA). The full-length (FL) EphA4 construct and shRNA for EphA4 (sA4) were provided by Mustafa Sahin (Harvard Medical School, Boston, MA, USA) and Nancy Ip (Hong Kong University of Science and Technology, Hong Kong, China), respectively, and were previously characterized (Fu et al., 2007). The dominant negative (DN) construct was produced using a DNA fragment generated by RT-PCR corresponding to the ectodomain and transmembrane region cloned in frame with YFP in pCMV-eYFP-N1, based upon previous studies (Hu et al., 2003). An additional shRNA for EphA4 targeting the 3′ UTR was generated using the pSUPER system and used in Fig. S9 in the supplementary material. Ephp bin coding sequence was produced via RT-PCR and cloned into an expression vector with the RSV LTR (Davy et al., 2004). COS7 cells were transfected with each construct and expression was evaluated by autofluorescence, immunohistochemistry, western blotting, live and fixed ligand binding, and receptor activation assays (Cheng and Flanagan, 1994; Gale et al., 1996). RT-PCR using primers corresponding to the ectodomain of each Eph receptor in either HEK293T or mouse [E14.5 cortex and neurospheres (Nphs)] was used to assess expression of endogenous genes. All amplified products were approximately 500 bp in length.

Binding studies
To examine ligand and receptor binding, COS7 cells were transfected with vectors encoding either EphA4 or ephrin B1. Two days later, cells were washed in PBS, fixed for 5 minutes with 4% PFA and incubated with 3 μg/ml of ephrin B1, ephrin A5, EphA4 or EphB2 reagents (ectodomains fused in frame with a human Fc fragment), or a control chimeric protein (Gale et al., 1996) for 1 hour at RT. Coverslips were washed with PBS, fixed in 4% PFA for 1 hour, washed with PBS again, incubated with an Alexa-conjugated anti-human Fc antibody for 1 hour, washed in PBS and mounted immediately. The number of positive cells per 100× field in each condition was recorded in five randomly designated positions on each of four coverslips.

Activation studies
To examine receptor activation, parallel plates of HEK293T cells were transfected with (1) YFP, (2) EphA4 (10%) and pSκ (90%), or (3) EphA4 (10%) and DN (90%) expression vectors. Two days after transfection, plates were either harvested immediately (0 minutes) or incubated with 5 μg/ml of clustered ephrin B1 or ephrin A5 reagents for 2 or 10 minutes. Cells were scraped in PBS and homogenized in RIPA buffer with protease inhibitors, and cell extracts were produced. Protein samples (15 μg/lane) were resolved on 10% SDS-PAGE gels, transferred to a nitrocellulose membrane and incubated with primary antibody for phosphorylated EphA (provided by Mustafa Sahin and Michael Greenberg, Harvard Medical School, Boston, MA, USA) overnight at 4°C in Blocking Solution [BS; 2.5% skimmed milk, 2.5% normal goat serum (NGS) in Tris-buffered saline (TBS)]. Following TBS washes, detection was carried out using a species-specific HRP-conjugated secondary antibody and chemiluminescence. Blots were stripped and reprobed for actin to confirm equal loading. Experiments were performed in triplicate. Band intensity was quantified and relative stimulation was calculated.

Neurosphere cultures
Neurospheres (Nsphs) were derived from dorsal telencephalons free of membranes and dissected from either CD-1 or EphA4 mutant E15.5 mouse embryos into Hank’s buffered salt solution (HBSS). A single cell suspension was produced by trituration and plated into serum-free Neurobasal medium containing 25 ng/ml human recombiant FGF2, 2% B27 supplement, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were plated at a density of 50,000-75,000 cells/ml, maintained at 37°C and supplemented with fresh media every other day. Following tail PCR, cells of similar EphA4 genotypes were pooled. Every 5-7 days cells were passed, dissociated by trituration and replated (Reynolds and Weiss, 1996). Following the third passage, wild-type cultures were transfected with YFP, YFP and DN or YFP and ephrin B1, using Lipofectamine 2000 (Invitrogen). One day before harvest, BrdU was added to the media (10 μM final concentration). Cells were harvested, fixed in 4% PFA, processed for BrdU and YFP immunocytochemistry, and counterstained with Bisbenzamide.

In utero electroporation (IUE)
Timed pregnant CD-1 or EphA4+/E14.5 (E14.5) mice were anaesthetized with 100 mg/kg Ketamine and 10 mg/kg Xylazine. Following laparotomy, a solution containing plasmid DNA (2-4 μg/μl of YFP, DsRed, sA4, DN or FL EphA4, or ephrin B1 in Tris-buffered 0.02% Fast Green) was injected through the uterine wall into the lateral ventricle of each embryo. Current pulses were delivered across the embryonic head using Tweezertrodes (BTX). Embryos were kept hydrated, placed back into the abdomen of the mother and gestation was allowed to proceed for 1-3 days. The survival rate was ~84% and the rate of transfection in surviving embryos was ~71%. Only embryos showing effective transfection in the somatosensory cortex were analyzed.

Cell proliferation analysis
Pregnant mice received an intraperitoneal injection of BrdU (50 μg/g) 2 hours before euthanasia. Embryonic brains were dissected, fixed, frozen and sectioned as described in Immunohistochemistry above. Sections were postfixed in 4% PFA for 10 minutes, washed in PBS, treated with 0.1% trypsin for 10-30 seconds and incubated in 100% fetal calf serum. Sections
were rinsed in PBS, incubated in 2 N HCl for 30 minutes at 37°C, rinsed in 0.1 M sodium borate pH 8.14, and incubated overnight with mouse anti-BrdU and rabbit anti-GFP in BB⁺. Standard immunohistochemistry was then performed. Sections (3-5) in 3-5 individuals were analyzed.

**Comununoprecipitation**

HEK293T cells were transfected with DsRed (control), EphA4 or ephrin B1. One day later, cells were trypsinized. The DsRed control cells were replated at a high density (100,000 cells/cm²). The EphA4 and ephrin B1 cells were mixed together and plated at the same high density. Three hours later, cells were harvested in immunoprecipitation (IP) buffer [1% NP-40, 50 mM Tris-HCl pH 8.0, 150 mM NaCl, Complete Protease Inhibitors (Roche)], incubated for 10 minutes at 4°C, and spun. The supernatant was then incubated with 20 μl protein A sepharose (GE Healthcare) and 5 μg of either anti-EphA4 or anti-IgG overnight at 4°C. Following washes with IP buffer, proteins bound to protein A were eluted by boiling and subjected to western blot analysis using either EphA4 or ephrin B1 antiserum.

Comununoprecipitation from brain tissue was performed as in Buchert et al. (Buchert et al., 1999). Briefly, E14.5 cortex was homogenized in 1 ml neurolysis (NL) buffer (20 mM Hepes pH 8.0, 1% NP-40, 0.2 M NaCl, 2 mM EGTA, and Complete Protease Inhibitors) using a dounce homogenizer, shaken vigorously at 4°C for 15 minutes, spun at 100,000 g for 1 hour and supernatant was collected. Brain lysate (1 mg) was precleared with protein A sepharose beads for 30 minutes at RT. In parallel, 20 μl sepharose was incubated for 30 minutes at RT with 10 μg of either anti-EphA4 or anti-IgG to allow antibody binding to beads. Precleared lysate was then mixed with prebound antibody/sepharose complexes, incubated overnight at 4°C and beads were washed in NL buffer. Proteins bound to protein A were eluted by boiling and subjected to western blot analysis using either EphA4 or ephrin B1 antiserum.

**Data analysis**

In the in vitro studies, 20-50 Nsphs per condition were imaged in each of at least three experiments. Both the number of transfected cells and BrdU⁺ cells were quantified. To compare between experiments, values were converted to a percentile of the maximal level in each experiment and then averaged between experiments. To analyze EphA4 mutant cortex, images of several similar mediolateral positions were obtained of Nissl-stained wild-type and EphA4⁺/⁻ cortex and processed in parallel (n=4 and 5, respectively). ImageJ was used to measure anteroposterior length, thickness of the cerebral wall and cortical area. Following IUE, 2-8 embryos per group (control, sa4, DN, Fl or ephrin B1) were analyzed at particular times after transfection or BrdU introduction. Only animals with transfected somatosensory cortex were included in the analyses. Images of three to five well-paced sections per animal were assessed using Adobe Illustrator. A 0.1 mm-wide rectangle that spanned the cerebral wall was placed over transfected cortex. Embryonic zones (PZ, IZ and CP) were identified using TuJ1 immunoreactivity and fluorescein Nissl staining, and were demarcated within this rectangle. The area of each zone was measured and the numbers of transfected or BrdU⁺ cells within each zone were recorded. Data were normalized to total cell number per mm², averaged for each embryo and data from several individuals were pooled. Data were tested for significance by one-way analysis of variance (ANOVA; P<0.05) and expressed as mean±s.e.m.

**RESULTS**

**Expression of EphA4 in cortical proliferative zones**

Cells of the developing cerebral cortex are generated in a precise temporal pattern that is translated into the spatial layout of the embryonic cerebral wall: immature, dividing cells populate the proliferative zone (PZ; comprises the ventricular zone (VZ) and subventricular zone (SVZ) in this study) close to the ventricle, migrating postmitotic cells exist in the intermediate zone (IZ) and more mature neurons reside in the cortical plate (CP) beneath the pia. The expression of Eph receptors in the embryonic cerebral mirrors this developmental progression, with particular molecules marking specific locations, thus producing a complex signaling landscape (Fig. 1A; see Fig. S1 in the supplementary material) (Mackaretschian et al., 1999; Yun et al., 2003) and supporting the concept that certain Eph signaling combinations uniquely impact discrete populations of cells within the forebrain. Despite widespread expression of several Eph receptors in the forebrain, cells of the cortical PZ selectively express only one receptor, EphA4 (Fig. 1A; see Fig. S1 in the supplementary material) (Kullander et al., 2001b; Yun et al., 2003). Analyses of RNA and protein distribution reveal that EphA4 is especially abundant within the proliferative zones of the cerebral cortex, with lower levels in the IZ and CP (Fig. 1A,B,C,F; see Fig. S1E,O in the supplementary material). Indeed, overlapping expression of EphA4 with Sox2 or phospho-histone H3 (pH3) reveals localization with markers of proliferating cells (Fig. 1C-H). The strong expression of EphA4 in the cortex is in contrast to the weaker and more diffuse embryonic expression of EphA7 (see Fig. S2 in the supplementary material), a receptor capable of affecting cell survival during corticogenesis (Depaepe et al., 2005). EphA4 is expressed by cells of the PZ throughout cortical development and is coexpressed with markers of cortical progenitor cells, such as RC2 (Ifaprc2 – Mouse Genome Informatics) and nestin (see Fig. S3A,C,G-I in the supplementary material), but not with mature neuronal markers (see Fig. S3D-F in the supplementary material). Thus, based upon its level of expression and localization, EphA4 is a candidate for affecting neural proliferation during corticogenesis.

**In vitro analyses of EphA4 signaling in neurosphere cultures**

To study EphA4 function, neurosphere (Nsph) cultures (Tropepe et al., 1999), Fgf2-responsive cortical progenitors, were used as an in vitro model, as Eph receptor expression patterns within E14.5 cortex were largely mimicked in these cultures (see Fig. S4A,B in the supplementary material). Nsph cultures derived from control and EphA4⁻/⁻ cortex that had been exposed to the nucleotide analog BrdU during their last day in culture (Fig. 2A,B) demonstrated that...
DNA synthesis was at lower levels in Nsphs derived from EphA4−/− (70±6%) than from control cortex (Fig. 2C). Parallel experiments of Nsph cultures transfected with YFP (control) or a function-reducing (FL) receptor was kinase inactive (KI; Fig. 3H, light gray bar). Together, these results support a role for EphA4 in cortical proliferation.

Despite Nsph cultures being a popular in vitro model, analyses of a role for Eph function in proliferation may be complicated in this experimental paradigm by interactions between Eph receptors and Fgf2 receptors (Arvanitis and Davy, 2008; Yokote et al., 2005). We evaluated at E17.5. Consistent with changes in BrdU incorporation, there were fewer transfected cells in the PZ of EphA4+/− cortex than WT cortex 3 days after IUE (Fig. 3H, compare dark gray and black bars). To confirm that this phenotype was dependent upon EphA4, full-length EphA4 (FL) was reintroduced into E14.5 EphA4−/− cortex by IUE and the number of transfected cells was similarly assessed. Expression of EphA4 significantly increased the number of transfected cells in the PZ of EphA4+/− cortex, demonstrating that EphA4 expression helps to overcome the EphA4−/− phenotype (Fig. 3H, white bar).

To determine a cause for the small EphA4−/− cortex, cell division was examined during corticogenesis. The number of mitotic cells was assessed in control and EphA4−/− embryos that had been exposed to BrdU two hours prior to sacrifice (Fig. 3E,F). These analyses revealed that fewer cells incorporated BrdU in EphA4−/− cortex than control mice (526±38 μm for WT versus 419±20 μm for EphA4−/−, Fig. 3C), resulting in smaller cortical area in EphA4−/− mice (Fig. 3D). No changes in cell death were observed in EphA4−/− cortex (see Fig. S6C,D in the supplementary material). To investigate the cellular and molecular basis of this difference, cortical cells in WT and EphA4−/− were labeled with YFP via in utero electroporation (IUE) (Tabata and Nakajima, 2001). Embryonic cortices were transfected in vivo at E14.5 and transfected cell numbers were evaluated at E17.5. Consistent with changes in BrdU incorporation, there were fewer transfected cells in the PZ of EphA4−/− cortex than WT cortex 3 days after IUE (Fig. 3H, compare dark gray and black bars). To confirm that this phenotype was dependent upon EphA4, full-length EphA4 (FL) was reintroduced into E14.5 EphA4−/− cortex by IUE and the number of transfected cells was similarly assessed. Expression of EphA4 significantly increased the number of transfected cells in the PZ of EphA4+/− cortex, demonstrating that EphA4 expression helps to overcome the EphA4−/− phenotype (Fig. 3H, white bar).

It is important to note that the fully functional receptor increased cell number significantly, wild-type numbers were not achieved. This might be due to developmental changes in EphA4−/− cortex that cannot be

**Analysis of EphA4−/− cortex**

To examine how EphA4 affects corticogenesis in vivo, the cortices of EphA4−/− mice were examined. Animals were similarly sized at birth, the gross appearance of EphA4−/− brains, including the cerebral cortex, was normal (Fig. 3A,B) (Helmbacher et al., 2000), and the anteroposterior (AP) length of the cortex was similar to that of control (WT) mice (see Fig. S6A in the supplementary material).
EphA4 promotes cortical proliferation

overcome with transient gene expression or unregulated FL promoting cell division but also affecting death and differentiation (see below), thus limiting overall numbers.

Differences in cerebral wall thickness, cortical area, BrdU incorporation and transfected cell number between WT and EphA4–/– cortex were significant yet not overwhelming. It is possible that molecular compensation by other Eph receptors in the absence of EphA4 is responsible for these modest effects and complicates the rescue by FL. Indeed, we find evidence of upregulation of related Eph genes in EphA4–/– cortex. For example, EphA5 is normally expressed within the IZ during corticogenesis and is downregulated by P0 (see Fig. S6E,F in the supplementary material) (Mackarehtschian et al., 1999; Yun et al., 2003). Although EphA5 expression was properly initiated within the EphA4–/– cortex (see Fig. S6G in the supplementary material), embryonic levels were low and the age-related decrease in expression in the cortex was not observed. Instead, elevated levels of EphA5 were present postnatally in EphA4–/– cortex (see Fig. S6H in the supplementary material). These data suggest that Eph gene expression in the forming cerebral cortex is coordinated and that expression patterns of other Eph receptors are perturbed in EphA4–/– cortex.

**In vivo analysis of EphA4 function**

To avoid genetic compensation, IUE of wild-type animals was used to alter Eph signaling transiently. Exogenous DNA was introduced into cells of the ventricular zone of mouse somatosensory cortex, a large and easily identifiable cortical region. When IUE of a control YFP vector was performed at the peak of corticogenesis (E14.5), transfected cells were visible within 24 hours, predominantly within the PZ (see Fig. S7A,C in the supplementary material). Three days after transfection, YFP-expressing cells spanned the cerebral wall, including a proportion of cells that resided in the CP 3 days after IUE (see Fig. S7B,C in the supplementary material), confirming that YFP-transfected cells undergo stereotyped maturation. Consistent with transfected cells undergoing division, total numbers of YFP+ cells increased over time (compare levels in Fig. S7D in the supplementary material and in Fig. 4E).

To investigate the role of EphA4 in corticogenesis, forward signaling was reduced via transfection of an shRNA specific for EphA4 (sA4) (Fu et al., 2007) or a DN EphA4 (DN) (see Fig. S4D in the supplementary material) (Xu et al., 1995). Initial transfection rates were similar (see Fig. S7D in the supplementary material), but DN+ and sA4+ cells were less abundant than YFP+ cells (Fig. 4A-C,E), and there were fewer dividing cells in those cerebral walls (Fig. 4F) 3 days after transfection. TUNEL assay excluded cell death as an explanation for the diminished transfected cell count; cell death increased in the DN-transfected brains, but only in neighboring untransfected cells (see Fig. S8 in the supplementary material). These data demonstrate that a reduction in EphA4 signaling suppresses normal cell proliferation. Levels of EphA4 signaling were also artificially elevated by transfection of a full-length (FL) construct (Fig. 4D). There were modest increases in transfected cell number (Fig. 4E) and cell division (Fig. 4F) when FL was expressed. Interestingly, at the later timepoint, whereas YFP+ and sA4+ cells were similarly distributed, DN+ and FL+ cells were abnormally dispersed within the cerebral wall. In mice transfected with YFP or sA4, the majority of transfected cells (~60%) resided within the PZ, with smaller proportions of transfected cells occupying the IZ and CP (~30% and ~10%, respectively; Fig. 4G). When DN was transfected, proportionally fewer DN+ cells resided within the PZ and relatively more populated the IZ: 49% of DN+ cells were housed within the PZ, with 42% located in the IZ (Fig. 4G). Conversely, overexpression of FL led to an increase in the proportion of transfected cells in the PZ and a decrease in the proportion within the CP (Fig. 4G). To confirm that FL could overcome the effects of sA4 or DN, rescue experiments were performed (see Fig. S9 in the supplementary material). Together, these data suggest that EphA4 promotes cortical progenitor cell proliferation and reduces the proportion of differentiated cells.

To determine whether this effect of EphA4 was cell autonomous, through the receptor itself, or non-cell autonomous, through a potential ligand, we asked whether transfected cells incorporated less BrdU when their EphA4 signaling was reduced. Indeed, sA4- and DN-containing cells incorporated significantly less BrdU than YFP-containing cells (Fig. 4H), supporting a model in which EphA4 forward signaling promotes cell division in receptor-containing cells.

**Ligands for EphA4 in cortical proliferative zones**

Expression levels of ephrins are low in the forming cortex, particularly within regions with dividing cells: ephrin A2 is diffusely expressed throughout the cerebral wall and ephrins A5 and B2 are...
Fig. 5. Ligand expression in cortical proliferative zones. (A) Levels of ephrin ligand expression, quantified from in situ hybridizations (see Fig. 5A–J in the supplementary material), of ephrin A ligands (green) and ephrin B ligands (yellow) in the proliferative zones (PZ), intermediate zone (IZ) and cortical plate (CP) of the E14.5 cerebral wall. Color intensity corresponds to density of silver grains from in situ hybridization samples. (B) In situ hybridization of ephrin B1 in the E14.5 cerebral cortex. (C–H) Immunohistochemical analyses of the cortical proliferative zone reveal expression of ephrin B1 (C,F; green) with EphA4 (D) or Sox2 (G; red), with merged images in E and H, respectively.

The most concentrated within the CP (Fig. 5A; see Fig. S10 in the supplementary material) (Kullander et al., 2001b; Mackarehtschian et al., 1999). By contrast, ephrin B1 is abundantly expressed in the PZ (Fig. 5A,B,C,F; see Fig. S10H in the supplementary material), colocalized with EphA4 in the PZ throughout cortical development (Fig. 5C–E) and coexpressed with markers of cortical progenitor cells, such as Sox2, nestin and RC2, but not neuronal markers (Fig. 5F–H; see Fig. S11 in the supplementary material). Thus, based upon both abundance and localization, ephrin B1 is a candidate for engaging EphA4 in promoting corticogenesis.

Previous studies demonstrated that EphA4 binds ligands permissively and is capable of engaging both ephrin A and B ligands (Gale et al., 1996; Kullander et al., 2003). Indeed, recognition of binding partners for EphA4 has progressed: ephrin A5 and ephrin B2 were shown to engage EphA4 in vitro (Gale et al., 1996), but binding of ephrin B3 to EphA4 was only detected later, in analyses of mutant mice in vivo (Kullander et al., 2003). To investigate whether an interaction between EphA4 and EphB4 exists, COS7 cells were transfected with YFP in combination with either an inert plasmid (control, C) or expression vectors encoding EphA4 or ephrin B1, and postfixation staining with ephrin and Eph reagents was performed. Compared with control-transfected cells, in which the low density of endogenous receptors and ligands was not detectable in this assay of fixed cells (Fig. 6A,C), EphA4-transfected cells bound ephrin B1, and EphB1-transfected cells bound EphA4 (Fig. 6B,D).

To assess the relative strength of EphA4/ephrin B1 binding, COS7 cells were again transfected, but this time serial dilutions of binding reagent were used for detection. EphA4-transfected cells bound ephrin A5, the cognate ligand, and ephrin B1, the novel ligand, and in all cases the number of positive cells increased according to the amount of binding reagent present (Fig. 6E). Still, there were more positive cells at each concentration of ephrin A5 than of ephrin B1, suggesting a weaker interaction with the latter ligand (Fig. 6E). Cells transfected with ephrin B1 bound EphB2, the previously characterized receptor, and EphA4, the novel receptor, in a dose-dependent manner (Fig. 6F), but in this case no obvious interaction preference was apparent.

To confirm a molecular interaction between EphA4 and ephrin B1, protein extracts were produced from either control-transfected cells or cells transfected with EphA4 or ephrin B1 that were subsequently mixed and grown together. EphA4 was concentrated following pulldown with the EphA4-specific antibody (Fig. 6G, top) and ephrin B1 copurified with EphA4 (Fig. 6G, bottom). EphA4/ephrin B1 engagement in this system appeared to be exclusively due to trans binding; cells cotransfected with both EphA4 and ephrin B1 and grown at low density to allow for cis- and prohibit trans-interactions did not result in coimmunoprecipitation of EphA4 and ephrin B1 (data not shown). To determine whether EphA4/ephrin B1 engagement occurs in vivo, similar analyses were performed using embryonic cortex. EphA4 was detectable in the cortical lysate and was enriched following precipitation with EphA4 antiserum (Fig. 6H, top). Importantly, ephrin B1 copurified with EphA4 (Fig. 6H, bottom), confirming an interaction in embryonic cortex. Thus, EphA4 and ephrin B1 interact both in vitro and in vivo.

To assess the functional consequences of an EphA4/ephrin B1 interaction, HEK293T cultures, cells that express few Eph receptors (see Fig. S4C in the supplementary material), were treated with clustered ephrin B1 and analyzed biochemically for EphA receptor activation. Parallel to previous ephrin A5 stimulation results, owing to low levels of endogenous receptors (see Fig. S4D in the supplementary material) cells transfected with YFP and treated with ephrin B1 displayed little receptor activation (Fig. 6I, left). Also similar to previous experiments (see Fig. S4D in the supplementary material), owing to high receptor density following EphA4 transfection, receptor activation was apparent in the absence of ligand (Fig. 6I, middle panel, left lane), but was elevated in the presence of ephrin B1 (Fig. 6I, middle panel, right lanes) (Davis et al., 1994; Gale et al., 1996). Importantly, coexpression of EphA4 and DN blocked ephrin B1-stimulated EphA4 activation (Fig. 6I, right panel).

To examine the relative levels of activation, EphA phosphorylation was quantified following stimulation with ephrin B1 (Fig. 6D, left) or ephrin A5 (Fig. 6D, right) and compared with baseline levels of phosphorylation. EphA4 activation in response to ephrin B1 was observed (150% at 5 minutes) and prolonged (sustained phosphorylation at 10 minutes; Fig. 6J, top left), whereas stimulation in response to ephrin A5 was stronger (200% at 5 minutes) but quicker (receptor became less phosphorylated at 10 minutes; Fig. 6J, top right). In both cases, EphA4 activation was eliminated in the presence of DN (Fig. 6J, bottom). Together, these data support an EphA4/ephrin B1 interaction based upon results from (1) cell-based binding, (2) biochemical interactions in transfected cells in culture, (3) biochemical interactions between native proteins in the developing cortex and (4) specific EphA4 activation following exposure to ephrin B1. Although structural analyses or additional protein chemistry could be performed to more fully understand this interaction, we conclude that EphA4 and ephrin B1 bind to one another and that the DN blocks ephrin B1-induced receptor activation.

Analysis of ephrin B1 function in vitro and in vivo

Ephrin B1 has previously been shown to play a role in promoting progenitor cell fate and inhibiting cortical neuronal differentiation (Qiu et al., 2008). To understand the role of ephrin B1 in regulating cortical proliferation, Nsph cultures transfected with YFP (Fig. 7A) or ephrin B1 (Fig. 7B) and then exposed to BrdU prior to harvest
EphA4 promotes cortical proliferation.

**DISCUSSION**

As the nervous system forms, Eph signaling affects several cellular processes including segregation (Mellitzer et al., 1999; Xu et al., 1999), migration (Conover et al., 2000; Karam et al., 2000) and parcellation (Miller et al., 2006; Vanderhaeghen et al., 2000) in early neurons, and axonal pathfinding (Castellani and Bolz, 1997; Dufour et al., 2003; Frisen et al., 1998; Helm bacher et al., 2000; Torii and Levitt, 2005) and synaptic transmission (Dalva et al., 2000; Kayser et al., 2006; Kayser et al., 2008; Torres et al., 1998) in more mature neurons. Eph signaling impacts neurogenesis in the adult olfactory system and hippocampus (Chumley et al., 2007; Conover et al., 2000; Depaepe et al., 2005; Holmberg et al., 2005), but the consequences of this mode of intercellular communication on the initial generation of cells in the cerebral cortex have not been clearly elucidated. Our genetic, epigenetic, histological and biochemical studies, both in vitro and in vivo, support a novel role for EphA4-mediated forward signaling, initiated by ephrin B1 binding, in the promotion of progenitor cell proliferation during corticogenesis.

Within the PZ, EphA4 and ephrin B1 are highly expressed and colocalize with radial glial markers. Although questions regarding heterogeneity of progenitor cells in the VZ (Gal et al., 2006; Malatesta et al., 2003) and the roles of intermediate progenitor cells within the SVZ (Mizutani et al., 2007; Noctor et al., 2007) exist, our results simply demonstrate that EphA4 signaling influences cortical proliferation. For example, when EphA4-mediated signaling is reduced, affected cells divide less, and when signaling is elevated, cells are more mitotically active. These results parallel findings that Eph signaling promotes cell division in the adult olfactory system (Conover et al., 2000) and the hippocampus (Chumley et al., 2007), as well as in non-neural stem cells (Fukai et al., 2008; Holmberg et al., 2006).

We demonstrate that ephrin B1 is coordinately expressed with, binds to and initiates forward signaling through EphA4. Our in vitro and in vivo results are consistent with an enhancement of cortical cell division by ephrin B1 via forward, but not reverse, signaling. Promotion of a cellular process by an EphA4/ephrin B1 interaction clarifies previous results that demonstrated that ephrin B1 interacts repulsively with EphB receptors but not with EphA4 (Mellitzer et al., 1999). Furthermore, the broad coexpression of EphA4 with ephrins in development and maturity (Mackarehtschian et al., 1999; Yun et al., 2003) argues that EphA4 is not always a repulsive receptor. Our results support a system in which ephrin B1 initiates forward signaling via EphA4 to stimulate cortical cell division. This role is consistent with the findings that overexpressed EphA4 (Fig. 4E) or ectopic ephrin B1 (Qiu et al., 2008) antagonize neuronal differentiation.

Engagement of Eph receptors and ephrin ligands can result in bidirectional events, with signaling cascades initiated in both receptor- and ligand-expressing cells (Davy et al., 2004; Holland et al., 1996; Kullander et al., 2001b), and the consequences of forward
and reverse signaling can be functionally dissected (Davy et al., 2004; Holmberg et al., 2005; Kullander et al., 2001b). This was the case in our in vitro and in vivo studies: EphA4 forward signaling promoted cell division during corticogenesis in gain- and loss-of-function paradigms, whereas reverse signaling via ephrin B1 had a minor effect on cell proliferation (Fig. 8). Consistent with this model, ephrin B1 conditional mutants had fewer mitotic cells within their cerebral cortex, although it was not determined whether the effect was autonomous or not (Qiu et al., 2008). Differences in the results of overexpression studies are likely to be related to the cell types manipulated; our elevation of ephrin B1 was in proliferating cells, whereas Qiu et al. overexpressed ephrin B1 exclusively in differentiating neurons. Our results also support the idea that excessive and unbalanced reverse signaling can impact cell survival, consistent with other overexpression studies (Depaepe et al., 2005). From these results, we conclude that the major biological role of EphA4 signaling in corticogenesis is to regulate proliferation (Fig. 8).

Stereotyped placement of cortical cells of particular maturational states in specific locations is a hallmark in the construction of a functional cerebral cortex: dividing cells reside near the ventricle, more mature cells occupy the most superficial locations, and migrating cells are sandwiched in between. Unique combinations of Eph receptors are expressed by cells within each developmental niche (Yun et al., 2003), such that a spatial, stage-specific sequence of Eph signaling is apparent (Fig. 8). This developmental patterning supports a continuous role for Eph signaling, using different signaling partners at different times, in the maturation of cortical cells. Indeed, ectopic expression of EphA4 resulted in the maintenance of the proliferative state (Fig. 4), a result phenocopied when ephrin B1 was ectopically expressed in postmitotic neurons (Qiu et al., 2008). Our finding that DN+ but not sA4+ cells are mislocalized within the cerebral wall suggests that a related receptor, silenced by the DN but not the sA4, or the excessive reverse signaling initiated by the DN but not the sA4, might mediate migration. In parallel, the build-up of FL+ cells in the PZ, at the expense of differentiated neurons in the CP, demonstrates that these states are linked. In these ways, our results reveal the coordination that exists between division and differentiation, and highlight possible roles for discrete Eph signals in maintaining this balance.

In summary, our study demonstrates that EphA4/ephrin B1 signaling plays a role in the modulation of cortical progenitor cell proliferation. Characterizing the balance that exists between the generation of cells and their subsequent differentiation is essential for understanding the organization and function of the cerebral cortex, as well as for providing insights into instances of malfunction and their potential therapies.

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References


