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Trk signaling regulates neural precursor cell proliferation and differentiation during cortical development

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Increasing evidence indicates that development of embryonic central nervous system precursors is tightly regulated by extrinsic cues located in the local environment. Here, we asked whether neurotrophin-mediated signaling through Trk tyrosine kinase receptors is important for embryonic cortical precursor cell development. These studies demonstrate that inhibition of TrkB (Ntrk2) and/or TrkC (Ntrk3) signaling using dominant-negative Trk receptors, or genetic knockdown of TrkB using shRNA, caused a decrease in embryonic precursor cell proliferation both in culture and in vivo. Inhibition of TrkB/C also caused a delay in the generation of neurons, but not astrocytes, and ultimately perturbed the postnatal localization of cortical neurons in vivo. Conversely, overexpression of BDNF in cortical precursors in vivo promoted proliferation and enhanced neurogenesis. Together, these results indicate that neurotrophin-mediated Trk signaling plays an essential, cell-autonomous role in regulating the proliferation and differentiation of embryonic cortical precursors and thus controls cortical development at earlier stages than previously thought.

KEY WORDS: TrkB, TrkC, BDNF, Neurogenesis, Gliogenesis, Self-renewal, Cortical precursors, Neural stem cells, In utero electroporation, Akt, Mouse

INTRODUCTION

Development of the cerebral cortex is achieved through a common pool of precursor cells that sequentially generate neurons and glial cells. Emerging evidence indicates that whereas intrinsic cues are important in cortical precursor development, differences in the availability of growth factors determine precursor survival, proliferation and the appropriate timed genesis of neurons versus astrocytes (Miller and Gauthier, 2007). The neurotrophins are growth factors that are best known for regulating the biology of central nervous system (CNS) neurons, but that also regulate development of at least some precursor cells (Huang and Reichardt, 2003). At least two members of the neurotrophin family, BDNF and NT3 (also known as Ntf3 – Mouse Genome Informatics), along with their preferred tyrosine kinase receptors, TrkB and TrkC (Ntrk2 and Ntrk3, respectively – Mouse Genome Informatics), are expressed in the cortical ventricular/subventricular zones (VZ/SVZ) during the period of cortical neurogenesis (Maisonpierre et al., 1990; Tessarollo et al., 1993; Behar et al., 1997; Fukumitsu et al., 1998; Fukumitsu et al., 2006). Moreover, culture work indicates that: (1) NT3 selectively regulates cell cycle exit and neuronal differentiation in cortical progenitors (Ghosh and Greenberg, 1995; Lukaszewicz et al., 2002); and (2) cortical precursors themselves synthesize and secrete the neurotrophins BDNF and NT3, which promote their survival and differentiation in an autocrine/paracrine fashion by activating TrkB/TrkC receptors (Barnabé-Heider and Miller, 2003). However, an in vivo role for the Trk receptors in cortical precursor biology has not yet been established.

Here, we have asked whether Trk signaling is important for embryonic cortical precursor development in vivo, by performing in utero electroporation with dominant-negative TrkB and TrkC or with TrkB shRNA to acutely, and in a cell-autonomous fashion, disrupt Trk signaling. In this regard, the TrkB receptor can be activated by BDNF, NT3 and NT4 (also known as Ntf5 – Mouse Genome Informatics) (Huang and Reichardt, 2003), and whereas previous work (Jones et al., 1994; Alcantara et al., 1997; Ringstedt et al., 1998; Xu et al., 2000; Lotto et al., 2001; Medina et al., 2004) has indicated that BDNF-mediated TrkB activation is important for cortical development in vivo, these studies concluded that any observed perturbations were a consequence of altered TrkB signaling in cortical neurons. The TrkC receptor is only activated by NT3, and previous work on *Nt3*$^{-/-}$ and *TrkC*$^{-/-}$ mice has primarily focused upon the profound deficits observed in the peripheral nervous system (Ernfors et al., 1994; Wilkinson et al., 1996; Klein et al., 1994; Tessarollo et al., 1994), or on perturbations in the biology of committed CNS glia or neurons (Minichiello and Klein, 1996; Martinez et al., 1998; Kahn et al., 1999; Ma et al., 2002; von Bohlen und Halbach et al., 2003). As both BDNF and NT3 are known to be expressed in precursor cells of the cortical neuroepithelium (Maisonpierre et al., 1990; Fukumitsu et al., 1998; Behar et al., 1997; Barnabé-Heider and Miller, 2003; Fukumitsu et al., 2006), and as cortical precursors express both of these receptors (Tessarollo et al., 1993; Behar et al., 1997; Barnabé-Heider and Miller, 2003), we have chosen to disrupt signaling via these two receptors both individually and together. These studies demonstrate that TrkB and TrkC receptor activation, presumably in response to BDNF and NT3, are necessary for the appropriate proliferation and differentiation of embryonic cortical precursors, and thus play an important early role in cortical development.

MATERIALS AND METHODS

Cultures and transfections

Cortical precursors were cultured as described previously (Barnabé-Heider and Miller, 2003; Barnabé-Heider et al., 2005; Gauthier et al., 2007). Cell density was 125,000 cells/well for four-well chamber slides. For

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transfections, 2 to 4 hours after plating, 1 μg DNA and 1.5 μl Fugene 6 (Roche, Welwyn Garden City, UK) mixed with 100 μl of Opti-MEM (Invitrogen) were incubated at room temperature for 40 minutes and then added to each well. The rat dnTrkB mutant consisted of a single mutation (K538N) in the ATP-binding site that rendered it kinase-dead (Atwal et al., 2000), whereas the rat dnTrkC mutant contained three mutated tyrosines (Y705N, Y709N and Y710N) within catalytic subdomain VIII (the kind gift of Pantelis Tsoulfas, University of Miami, Miami, FL). Trk constructs were subcloned into the pEF-GM expression vector (Paquin et al., 2005). The two TrkB shRNA constructs targeted two different regions on the TrkB mouse mRNA sequence. The sequence for TrkB shRNA1 was 5′-TTGTGGATTCCGGCTTAAATTCAAGAGATTTAAGCCGGAATCCA-CAA-3′, for TrkB shRNA2 was 5′-CCTTGTAGGAGAAGATCAATT-CAAGAGATTGATCTTCTCCTACAAGG-3′, and for the control shRNA was 3′-TTCTCCGAACGTGTCACGTTTCAAGAGAACGTGACACG-TTCGGAGAA-3′. This control shRNA was mismatched to known human and mouse genes (EZBiolab, Westfield, IN). The dnAkt mutant contains a point mutation (K179M) within its ATP-binding site (Songyang et al., 1997).

In utero electroporation

In utero electroporation was performed as described with the square electroporator CUY21 EDIT (TR Tech, Japan), delivering five 50 ms pulses of 40-50 V with 950 ms intervals (Barnabé-Heider et al., 2005; Paquin et al., 2005; Gauthier et al., 2007). E13/E14 CD1 mice were used, injecting a nuclear EGFP expression plasmid driven from the *Ef1α* (*Eef1a* – Mouse Genome Informatics) promoter (pEF-EGFP) (as above) at a 1:3 ratio, or in some cases as indicated, 1:2 ratio with pEF-GM (empty vector), pEF-dnTrkB, pEF-dnTrkC, shRNA negative control, TrkB shRNA-1 (shTrkB-1), TrkB shRNA-2 (shTrkB-2), dnAkt or pcDNA3.1-BDNF-HA (Hibbert et al., 2003) for a total of 4 or 3 μg DNA per embryo, and 0.05% Trypan Blue as a tracer. When both dnTrk constructs were co-electroporated, DNA was mixed at a ratio of 1 pEF-EGFP:2 pEF-dnTrkC:2 pEF-dnTrkB for a total of 5 μg DNA per embryo. In some experiments, amounts of dnTrkB and/or dnTrkC were varied, as specified in the Results.

Immunocytochemistry, biochemistry and antibodies

Immunocytochemistry of cultured cells and tissue sections was performed essentially as described (Gauthier et al., 2007). Neurotrophin stimulation, Trk immunoprecipitation and western blot analysis of freshly isolated cortical tissue was performed basically as described (Knusel et al., 1994), with the pan-Trk antibody (203b) (Hempstead et al., 1992). The primary antibodies used were rabbit anti-pTrk-Y490 (1:100; Santa Cruz Biotechnology), mouse anti-GFP (1:1000; Invitrogen), rabbit anti-GFP (1:500; Chemicon, Temecula, CA), rabbit anti-myc-Tag (1:200; Upstate, Lake Placid, NY), rabbit anti-cleaved caspase 3 (1:500; Cell Signaling Technology, Beverly, MA), mouse anti-Ki67 (1:200; PharMingen, Heidelberg, Germany), mouse anti-HuD (1:200; Invitrogen), mouse anti-NeuN (1:200; Chemicon), mouse anti-βIII-tubulin (1:800; Covance, Princeton, NJ), rabbit anti-GFAP (1:800; Chemicon), rabbit anti-GAD (1:200; Chemicon), rabbit anti-TrkB (1:500; Santa Cruz Biotechnology) and rabbit anti-phospho-histone-H3 (1:1000; Upstate). The secondary antibodies used for immunocytochemistry were indocarbocyanine (Cy3)-conjugated goat anti-mouse and anti-rabbit IgG (1:400; Jackson ImmunoResearch), FITC conjugated anti-mouse and anti-rabbit IgG (1:200; Jackson ImmunoResearch), dichlorotriazinyl amino fluorescein-conjugated streptavidin (1:1000; Jackson ImmunoResearch) and Cy3-conjugated streptavidin (1:1000; Jackson ImmunoResearch). For westerns, primary antibodies were monoclonal mouse anti-phosphotyrosine (4G10; 1:100; Upstate Biotechnology), rabbit anti-TrkBout or rabbit anti-TrkCout [1:5000 (Knusel et al., 1994; Hoehner et al., 1995)], and rabbit anti-GFP (1:500; Chemicon), mouse anti-HA (1:400; Boehringer Mannheim). Secondary antibodies for westerns were HRP-conjugated goat anti-mouse or anti-rabbit (1:10,000; Boehringer Mannheim).

Microscopy and confocal analysis

For quantification of immunocytochemistry on cultured cells, approximately 300 cells per condition per experiment were counted and analyzed. Digital image acquisition was performed with Northern Eclipse software (Empix, Mississauga, Ontario, Canada) using a Sony (Tokyo, Japan) XC-75CE CCD video camera. For quantification of immunocytochemistry on tissue sections, brains were chosen with a similar anatomical distribution and level of EGFP expression. Sections were analyzed using a Zeiss (Oberkochen, Germany) Pascal confocal microscope and the manufacturer's software. A mean of four scans taken with a 40× objective were computed for each image. Error bars indicate s.e.m., and the statistics were performed using either the Student's *t*-test or one-way ANOVA with Mann-Whitney post-hoc test, as appropriate.

RESULTS

TrkB and TrkC are essential for the development of cultured cortical precursors

To ask whether Trk receptors are important for the development of neural precursors, we examined primary murine embryonic day 12.5 (E12.5) cortical precursor cells, a system we have previously characterized in detail (Toma et al., 2000; Ménard et al., 2002; Barnabé-Heider and Miller, 2003; Barnabé-Heider et al., 2005; Gauthier et al., 2007). Upon plating in Fgf2, these cortical precursors are virtually all dividing, nestin-positive cells that first generate neurons at 1 day in vitro (DIV), and astrocytes and oligodendrocytes at 5-6 DIV. The increase in differentiated cells is accompanied by depletion of proliferating precursors.

We previously showed that freshly isolated cortical precursors express TrkB and TrkC, and respond to endogenously produced BDNF and NT3 in culture (Barnabé-Heider and Miller, 2003). To ask how these Trk receptors regulate cortical precursor biology, we inhibited their function using dominant-negative TrkB and TrkC receptors (dnTrkB and dnTrkC). The dnTrkB is a kinase-dead ATP-binding-site mutant we have previously characterized (Atwal et al., 2000). The dnTrkC has three mutated tyrosines within the catalytic domain and functions as a dominant-negative in TrkC-expressing PC12 cells (Pantelis Tsoulfas, personal communication). We confirmed the efficacy of these dnTrks by co-transfecting them with EGFP into freshly plated cortical precursors and immunostaining 2 days later with an antibody for activated Trk phosphorylated at Y490. In cells transfected with dnTrkB and/or dnTrkC, only approximately 40% of the GFP-positive cells expressed readily detectable phosphoTrk levels, compared with 70% in controls.

We next asked whether Trk receptor inhibition affected survival. Precursors were co-transfected with EGFP and dnTrkB and/or dnTrkC, and survival was assessed at 2 DIV by counting EGFP-positive cells with condensed, apoptotic nuclei, or by immunostaining for cleaved caspase 3. Inhibition of TrkB, TrkC or both caused an approximately two- to threefold increase in condensed nuclei (Fig. 1A,B), and a two- to fourfold increase in cleaved caspase-3-positive cells (Fig. 1C). Thus, Trk signaling is important for cortical precursor survival in culture, consistent with our previous work showing that endogenously produced neurotrophins in these cultures support cell survival (Barnabé-Heider and Miller, 2003).

We also asked whether Trk signaling was important for cell proliferation by performing similar culture experiments, and immunostaining cells at 2 DIV for the proliferation marker, Ki67 (Fig. 2A); inhibition of TrkB and/or TrkC caused a significant decrease in the number of proliferating cells (Fig. 2B). However, as this decrease could be a secondary effect of enhanced cell death rather than a direct effect on cell division, we performed similar experiments in the presence of the pan-caspase inhibitor, ZVAD. ZVAD completely abolished the enhanced death seen in cells transfected with dnTrkB and/or dnTrkC, and decreased cell death to levels below those seen in basal conditions (Fig. 2C,D). Immunostaining for Ki67 revealed that, even when cell death was

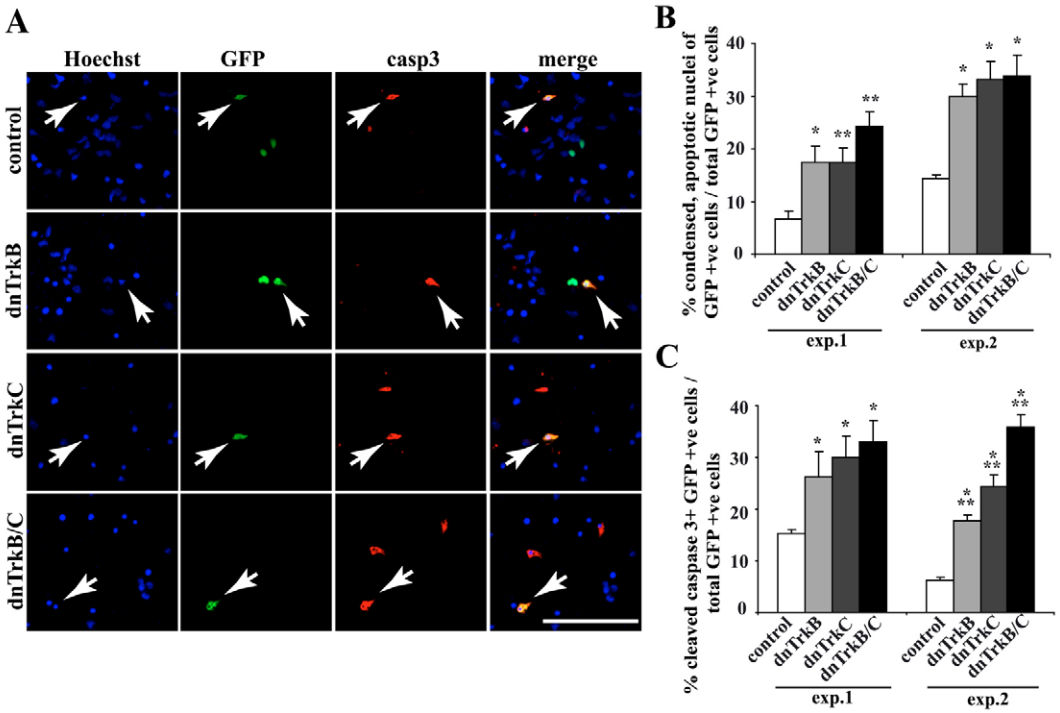

Fig. 1. TrkB and TrkC signaling are essential for the survival of cultured murine embryonic cortical precursor cells. (**A**) Fluorescence micrographs of cortical precursor cultures co-transfected with plasmids encoding EGFP and the empty vector (control), dnTrkB, dnTrkC or both (dnTrkB/C), and then analyzed at 2 days for EGFP (green, GFP), and cleaved caspase 3 (red, casp 3). Cells were counterstained with Hoechst 33258 (blue) to show all nuclei. Arrows denote double-labeled cells. (**B**,**C**) Quantification of the percentage of EGFP-positive cells (B) with condensed, fragmented nuclei, and (C) positive for cleaved caspase 3 in experiments similar to A. In each panel, two experiments of four are shown. Error bars denote the s.e.m. Scale bar: 100 μm. *P<0.05, **P<0.01, ***P<0.001 relative to control-transfected cultures.

prevented with ZVAD, inhibition of TrkB and/or TrkC caused a decrease in precursor proliferation (Fig. 2E). Thus, Trk signaling, presumably activated by endogenously produced BDNF and NT3, directly promotes proliferation of cultured cortical precursors.

Trk signaling in cortical precursors is essential for normal cortical development in vivo

To ask whether Trk signaling was necessary for cortical precursor development in vivo as well as in culture, we performed in-utero electroporation to transfect precursors of the VZ/SVZ of the embryonic cortex (Barnabé-Heider et al., 2005; Paquin et al., 2005; Gauthier et al., 2007). We have previously demonstrated that 1 day following electroporation at E14/15, all of the transfected cells reside in the VZ/SVZ and most of them are proliferating (Paquin et al., 2005). Many of these transfected cells differentiate into neurons over the next few days, which migrate out of the VZ/SVZ and into the cortical plate, where they ultimately become principal neurons of layers 2 and 3. Later in development, at early postnatal periods, some of the transfected cells that remain in the VZ/SVZ adopt an astrocytic fate.

Initially, we confirmed that TrkB and TrkC were expressed in the E14.5 cortex in vivo, as previously reported (Tessarollo et al., 1993; Behar et al., 1997). Cortical tissue was isolated at E14.5, postnatal day 3 (P3) or from adults, and was triturated and exposed to either 100 ng/ml BDNF or 200 ng/ml NT3 for 5 minutes to maximally activate Trk receptors. Full-length Trk receptors were immunoprecipitated using a panTrk antibody, and visualized by western blots with anti-phosphotyrosine. Blots were then re-probed for total TrkB or TrkC. This analysis confirmed that full-length TrkB was present in the E14.5 cortex, albeit at lower levels than at P3 or in the adult (Fig. 3A), as we have previously shown (Knusel et al., 1994), whereas full-length TrkC levels were constant from E14.5 to adulthood. Interestingly, tyrosine phosphorylation of TrkB and TrkC were lower in the adult brain, probably as a consequence of increased truncated Trk receptors (Knusel et al., 1994). Thus, signal transducing full-length TrkB and TrkC are both present within the E14.5 cortex.

We therefore used in-utero electroporation to ask whether Trk signaling was essential for cortical precursor development. We electroporated plasmids encoding a nuclear EGFP and dnTrkB, dnTrkC or both at E13/14, and analyzed the embryos 3 days later at E16/17. To confirm that cells were appropriately co-transfected, we performed double-label immunocytochemistry for EGFP and the myc tag on dnTrkB (Atwal et al., 2000); 77±6% (n=5 brains) of the GFP-positive cells expressed detectable dnTrkB (Fig. 3B). We then analyzed cell migration by immunostaining for EGFP and the neuronal marker HuD (Elavl4 – Mouse Genome Informatics) to help define cortical morphology (Fig. 3C). This analysis revealed that, relative to control EGFP-positive cells, 30-50% fewer dnTrkB- or dnTrkC-expressing cells had migrated to the cortical mantle (which includes both the intermediate zone of migrating neurons and the cortical plate) (Fig. 3D).

To confirm these findings, we also knocked down TrkB mRNA using two different TrkB shRNAs. To establish the efficacy of these shRNAs in cortical precursors, we co-transfected them with EGFP into cultured precursors, and immunostained them for TrkB 3 days later. Approximately half of the control cells expressed TrkB at high levels, and the TrkB shRNAs reduced this percentage by two- to

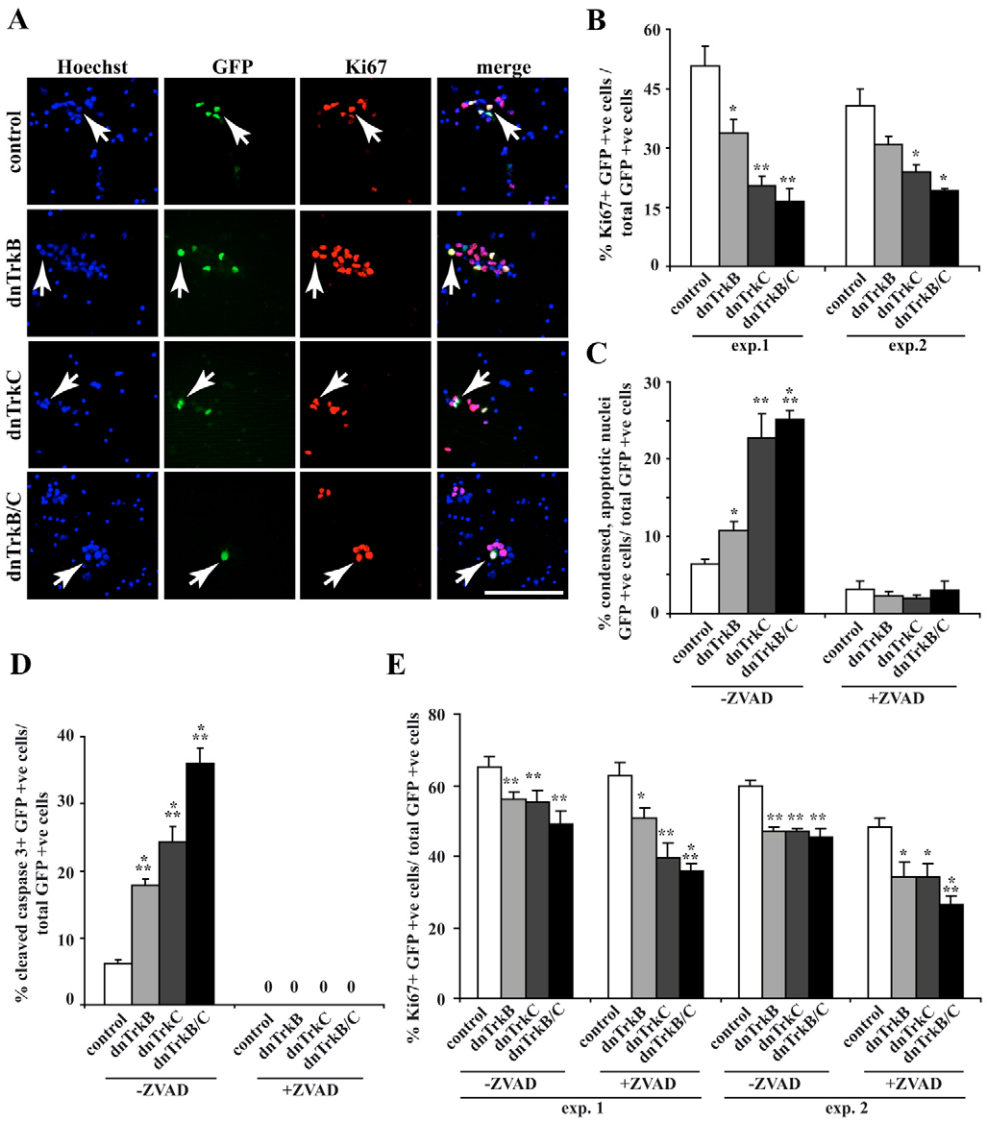

Fig. 2. TrkB and TrkC are essential for proliferation of cultured murine embryonic cortical precursor cells. (**A**) Immunostaining for EGFP (green, GFP) and Ki67 (red) for precursor cells transfected with EGFP and empty vector (control), dnTrkB, dnTrkC or both (dnTrkB/C) and cultured for 2 days. Cells were counterstained with Hoechst 33258 (blue). Arrows denote double-labeled cells. (**B**) Quantification of the percentage of transfected, Ki67-positive cells in experiments similar to A. Two experiments of four are shown. (**C**,**D**) Quantification of the percentage of apoptotic cells in experiments similar to A, except that cells were cultured with or without 100 μM ZVAD-FMK. Apoptosis was assessed by (C) analysis of condensed, fragmented nuclei and by (D) immunostaining for EGFP and cleaved caspase 3. In both panels, one experiment of two is shown. (**E**) Quantification of the percentage of transfected, Ki67-positive cells in experiments similar to A, except that cells were cultured with or without 100 μM ZVAD-FMK for 2 days. Error bars indicate s.e.m. Scale bar: 100 μm. **P*<0.05, ***P*<0.01, ****P*<0.001 relative to control-transfected cultures.

threefold (Fig. 3E). We then utilized these shRNAs to knockdown TrkB in vivo; E13/14 cortices were co-electroporated with EGFP and one of the two TrkB shRNAs, and then were analyzed 3 days later for cell migration (Fig. 3F). As seen with the dnTrks, the TrkB shRNAs reduced the percentage of transfected cells in the cortical mantle by approximately 50% (Fig. 3G). Thus, TrkB and TrkC signaling are necessary for normal development of cortical precursors in vivo.

Trk signaling is essential for cortical precursor cell proliferation but not survival in the embryonic cortex

To determine the cellular basis for the perturbed cortical development observed when TrkB or TrkC were inhibited, we characterized cell survival and proliferation 1 and 3 days post-electroporation. Initially, we examined survival by immunostaining for cleaved caspase 3. Only very small numbers of EGFP-positive, cleaved caspase-3-positive cells were observed (Fig. 4A). Numbers were similar in control and experimental brains at both timepoints, and ranged from 0 to 3 cells per section, with never more than 11 double-labeled cells within the 6 sections/brain that were analyzed. Moreover, the total numbers of EGFP-positive cells 3 days post-electroporation were similar between control and dnTrk groups (Fig. 4B; 1250±202 EGFP-positive control cells and 1256±146 dnTrk transfected cells when all groups were combined), supporting the argument that that there was no loss of dnTrk-expressing cells. Thus, unlike cultured precursors, cortical precursors in vivo do not require Trk signaling for survival, perhaps because there are alternative survival factors within the cortical neuroepithelium.

We next asked whether Trk signaling was important for precursor cell proliferation. Confocal microscopy of sections immunostained for Ki67 showed that virtually all transfected, Ki67-positive cells were located within the VZ/SVZ in all groups (Fig. 4C). At 1 and 3 days, approximately 70-80% and 8-13%, respectively, of control transfected cells within the VZ/SVZ were Ki67-positive, and inhibition of TrkB and/or TrkC signaling reduced this proliferation at both timepoints (Fig. 4D,E).

We obtained similar results when we electroporated cortices with the TrkB shRNAs. As seen with the dnTrks at 3 days, only very small numbers of cells were positive for EGFP and cleaved caspase 3 (normally 0-2 cells/section, and never more than 7 cells within the 5-6 sections/brain quantified). By contrast, knockdown of TrkB reduced the number of proliferating, transfected precursors, as monitored by immunostaining for either Ki67 or for the mitosis

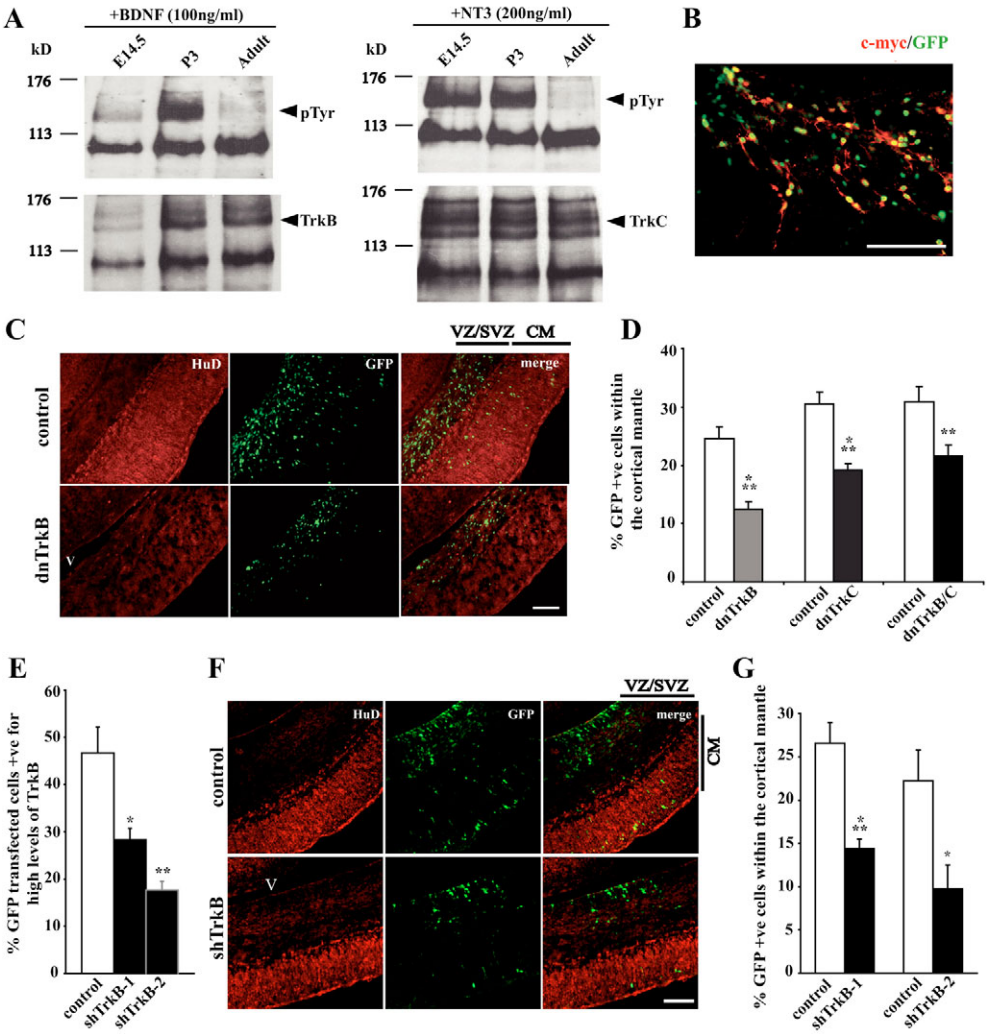

Fig. 3. In mouse, Trk signaling is necessary for development of neural precursors within the embryonic cortex. (**A**) Western blots for phosphotyrosine (pTyr) in full-length, panTrk immunoprecipitates of freshly isolated E14.5, P3 and adult cortical tissue stimulated for 5 minutes with BDNF or NT3. Blots were re-probed for TrkB (left panel) or TrkC (right panel). Arrowheads in upper panels indicate phosphotyrosine-positive full-length TrkB or TrkC, and in lower panels full-length TrkB and TrkC. Size markers are indicated to the left. (**B**) Fluorescence micrograph of a coronal section of a cortex co-electroporated with EGFP and myc-tagged dnTrkB and immunostained at 3 days for EGFP (green) and the myc-tag (red). Double-labeled cells are yellow in this merged image. (**C**) Fluorescence micrographs of coronal sections through cortices co-transfected with EGFP and the empty vector (control; top panels) or dnTrkB (bottom panels) and analyzed at 3 days. Sections were immunostained for HuD (red, left panels), and EGFP (GFP, green, middle panels; right panels are the merge). (**D**) Quantification of sections as in C for the percentage of total EGFP-positive transfected cells within the cortical mantle. *n*=at least ten animals each, five to six sections/embryo. (**E**) Quantification of the percentage of transfected precursors expressing high levels of TrkB 3 days after co-transfection with EGFP and control shRNA or one of two TrkB shRNAs (shTrkB-1, shTrkB-2). (**F**) Fluorescence micrographs of coronal sections through cortices electroporated with EGFP and control shRNA (control; top panels) or TrkB shRNA (shTrkB; bottom panels) and analyzed at 3 days. Sections were double-labeled for HuD (red, left panels) and EGFP (GFP, green, middle panels; right panels are the merge). (**G**) Quantification of sections as in F for the percentage of total EGFP-positive cells within the cortical mantle. *n*=six controls and four TrkB shRNA brains, five to six sections/embryo. **P*<0.05, ***P*<0.01, ****P*<0.001 relative to control-transfected sections. Error bars indicate s.e.m. Scale bars: 100 μm. CM, cortical mantle; V, ventricle; VZ/SVZ, ventricular zone/subventricular zone.

marker, phospho-histone H3 (Fig. 4F,G). The magnitude of this reduction was similar to that obtained with the dnTrks at 3 days (Fig. 4E).

These data showed that Trk signaling is required for precursor proliferation. Two Trk signaling cascades that might mediate this proliferative response are the MEK-ERK and the PI3-kinase-Akt (Pik3-Akt1 – Mouse Genome Informatics) pathways. As we previously showed that MEK is essential for cortical precursor neurogenesis, but not proliferation (Paquin et al., 2005), we asked whether Akt might be the relevant downstream effector, using an HA-tagged form of a previously characterized dominant-negative Akt (Songyang et al., 1997). Transfection of this construct into 293 cells demonstrated expression of an appropriately sized HA-tagged protein (Fig. 5A). Electroporation of this construct into E13/14 cortices, and analysis 2 days later revealed only very few transfected, cleaved caspase-3-positive cells (from 0-2 cells/section, with no more than 10 cells within the 6 sections/brain quantified), indicating that this was not a major

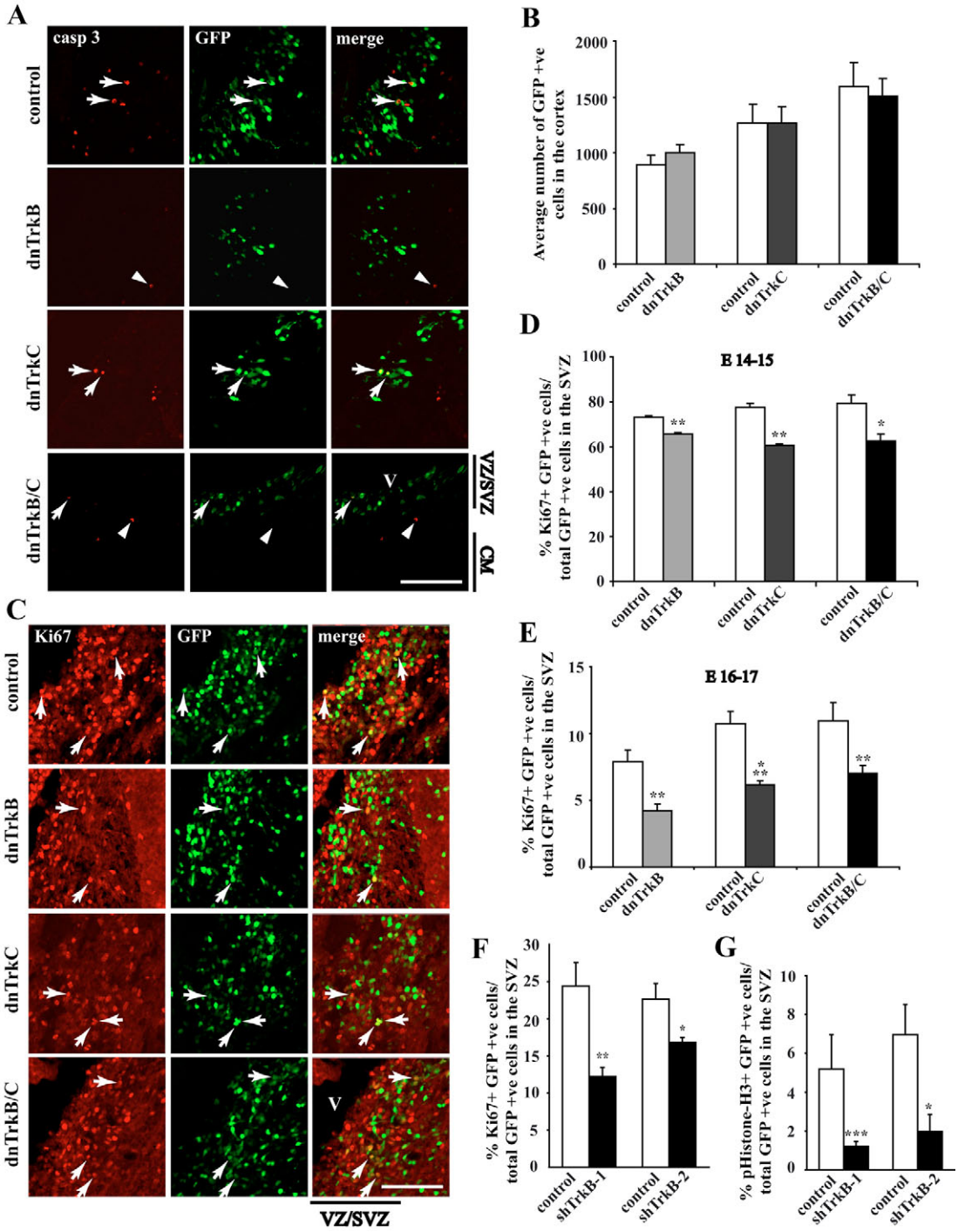

Fig. 4. In mouse, Trk receptor signaling regulates embryonic cortical precursor cell proliferation but not survival in vivo. (**A-E**) Cortices were electroporated with EGFP and the empty vector (control), dnTrkB and/or dnTrkC and analyzed 1-3 days later. (A) Confocal micrographs of coronal sections through cortices immunostained for EGFP (GFP, green) and cleaved caspase 3 (casp 3, red) at 1 day. The right panels show the merges. Arrows indicate transfected, cleaved caspase-3-positive cells, and arrowheads cells that only express cleaved caspase 3. (B) Quantification of the total number of EGFP-positive cells in six sections through electroporated cortices at 3 days. *n*=at least nine animals per group. (C) Confocal micrographs of coronal VZ/SVZ sections immunostained for EGFP (GFP, green) and Ki67 (red) at 3 days. The right panels show the merges. Arrows indicate transfected, Ki67-positive cells. (D,E) Quantification of the percentage of transfected, Ki67-positive cells in sections like C, at 1 or 3 days post-electroporation. For D, *n*=at least three embryos. For E, *n*=at least ten embryos, four to five sections/embryo at each timepoint. (**F**,**G**) Quantification of the percentage of transfected, Ki67-positive (F) or phospho-histone-H3-positive (G) cells in the VZ/SVZ of cortices transfected with EGFP and control shRNA (control) or one of two TrkB shRNAs (shTrkB-1, shTrkB-2) at E13/14 and analyzed 3 days post-electroporation. *n*=at least four, five to six sections/embryo. Error bars indicate s.e.m. Scale bars: 100 μm. **P*<0.05, ***P*<0.01, ****P*<0.001 relative to control-transfected sections.

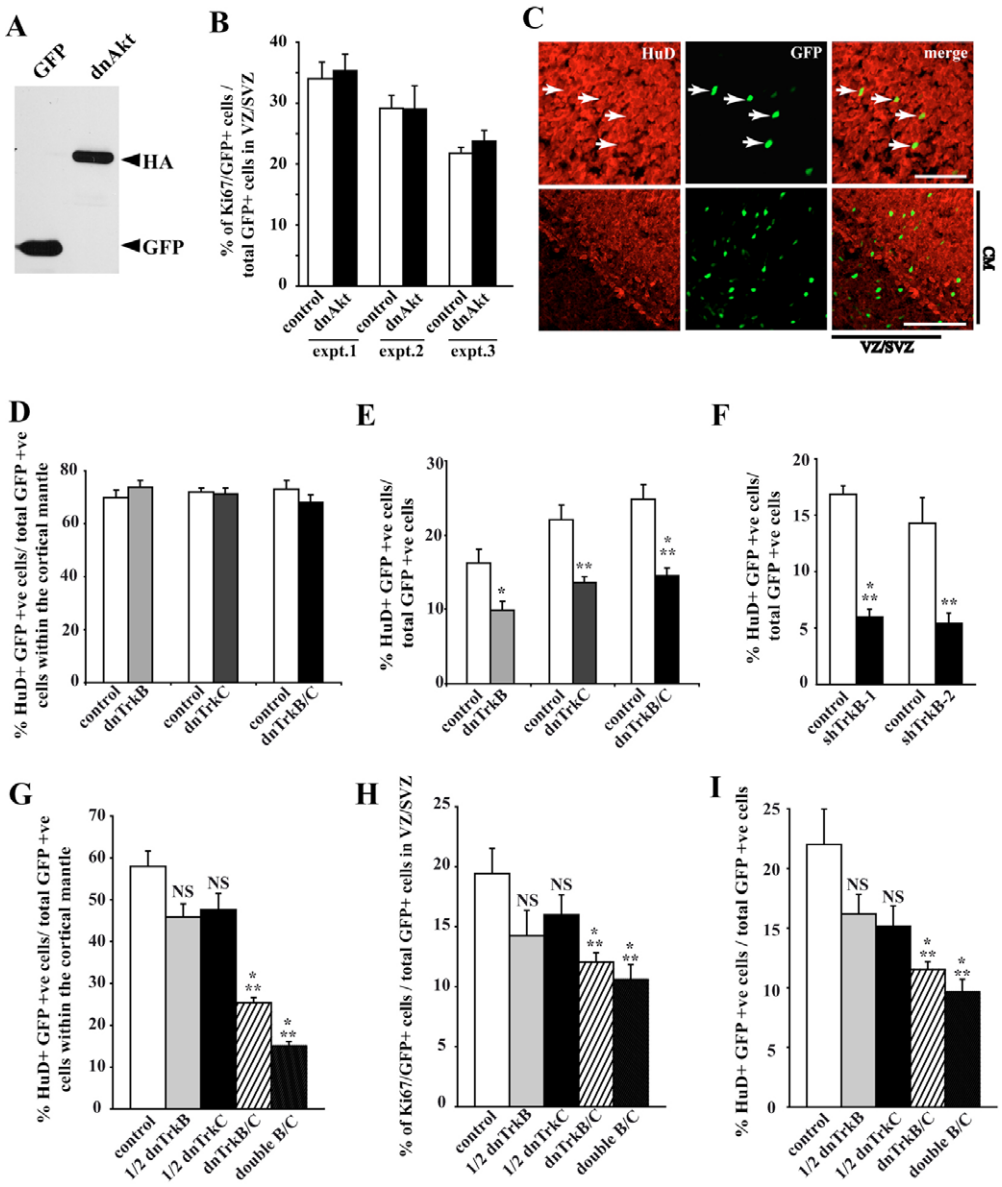

Fig. 5. Inhibition of Trk, but not Akt, signaling regulates murine cortical precursors in vivo. (**A**,**B**) Akt is not essential for survival or proliferation of cortical precursors in vivo. (A) Western blot of HEK 293 cells transfected with HA-tagged dnAkt or GFP and probed for the HA-tag or GFP. The upper arrow denotes an appropriately sized HA-positive band. (B) Quantification of the percentage of transfected, Ki67-positive cells in the VZ/SVZ of cortices electroporated with EGFP and empty vector (control) or dnAkt and analyzed at 2 days. *n*=six each, five to six sections/embryo. (**C-F**) Trk receptor signaling is necessary for appropriate embryonic neurogenesis. Cortices were electroporated with EGFP and the empty vector (control), dnTrkB, dnTrkC or both (dnTrkB/C) and analyzed at 3 days. (C) Confocal micrographs of coronal sections immunostained at 3 days for HuD (red) and EGFP (GFP, green). Upper panels are higher magnification images through the cortical plate, and the lower panels show both the VZ/SVZ and part of the cortical mantle (CM). Arrows indicate double-labeled cells. (D,E) Quantification of micrographs similar to C analyzing the percentage of cells co-expressing EGFP and HuD (D) within the cortical mantle or (E) the entire cortex. *n*=at least ten mice, four to five sections/embryo. (F) Quantification of the percentage of transfected, HuD-positive cells in cortices electroporated with EGFP and control shRNA (control) or one of two TrkB shRNAs (shTrkB-1, shTrkB-2) at 3 days. *n*=six controls and four TrkB shRNA brains, five to six sections/embryo. (**G-I**) Trk receptors collaborate to regulate precursor proliferation and neurogenesis. Quantification of the percentage of (G) EGFP-positive cells in the cortical mantle, (H) EGFP, Ki67-positive cells in the VZ/SVZ and (I) EGFP, HuD-positive neurons in cortices electroporated with EGFP and 1.5 μg of dnTrkB or dnTrkC alone (1/2 dnTrkB, 1/2 dnTrkC), 1.5 μg dnTrkB plus dnTrkC (dnTrkB/C) or 3 μg dnTrkB plus dnTrkC (double B/C) at 3 days. *n*=six controls, four dnTrkB, five dnTrkC, ten dnTrkB/C and ten double B/C, four sections/embryo. Error bars indicate s.e.m. Scale bar: 100 μm. **P*<0.05; ***P*<0.01; ****P*<0.001; NS, nonsignificant.

survival pathway. Moreover, similar numbers of transfected, Ki67-positive precursors were seen in control and dnAkt-transfected cortices (Fig. 5B). Thus, Trks probably signal via targets other than Akt and MEK to mediate cortical precursor survival and proliferation.

Trk signaling is essential for appropriate neurogenesis

We previously showed that one downstream Trk pathway, the SHP-2-MEK-ERK pathway, is essential for genesis of neurons from cortical precursors (Ménard et al., 2002; Barnabé-Heider and Miller,

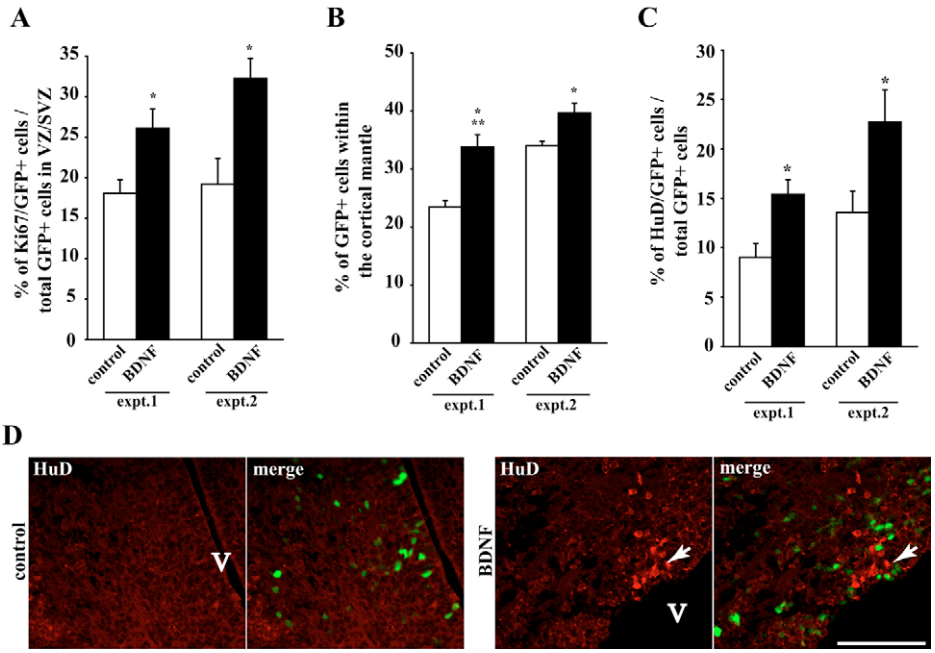

Fig. 6. Increased BDNF expression in the cortical neuroepithelium promotes precursor proliferation and enhances neurogenesis in mouse. Cortices were electroporated with EGFP and BDNF or the empty vector, and analyzed at 3 days post-electroporation. (**A-C**) Quantification of the percentage of (A) EGFP, Ki67-positive cells in the VZ/SVZ, (B) EGFP-positive cells within the cortical mantle and (C) EGFP, HuD-positive cells. *n*=eight each, five to six sections/embryo. (**D**) Confocal micrographs of coronal sections immunostained for HuD (red) and EGFP (green). The left panels of each pair are HuD staining, and the right panels are merges of HuD and EGFP staining. Arrows indicate a cluster of EGFP-negative, HuD-positive cells. Error bars indicate s.e.m. Scale bar: 100 μm. **P*<0.05; ****P*<0.001. V, ventricle.

2003; Paquin et al., 2005; Gauthier et al., 2007). Our finding (Fig. 3C,D,F,G) that dnTrks inhibited cells from moving from the VZ/SVZ into the cortical mantle suggested that they either: (1) inhibited neurogenesis; or (2) inhibited migration of newly born cortical neurons to the cortical plate. To distinguish these two possibilities, we performed immunocytochemistry for the early neuronal markers HuD and βIII-tubulin to ask if newly born neurons were trapped within the VZ/SVZ. Confocal microscopy demonstrated, in all groups, almost all transfected, newly born neurons were in the cortical mantle, with only very few in the VZ/SVZ (Fig. 5C). Thus, even when TrkB/C signaling is inhibited, newly born neurons migrate out of the VZ/SVZ.

These data imply that the decrease in transfected cells within the cortical mantle reflects decreased neurogenesis. To confirm this, we calculated neuron number at 3 days by multiplying the number of transfected cells in the cortical mantle by the percentage of transfected, HuD-positive cortical mantle neurons (75% in both control and dnTrk groups, Fig. 5C,D). This analysis showed that approximately 17-25% of control-transfected cells were neurons, and that dnTrks reduced this percentage (Fig. 5E). We also counted the number of HuD-positive transfected cells in TrkB shRNA electroporated cortices at 3 days; TrkB knockdown decreased the percentage of newly born neurons by two- to threefold (Fig. 5F). Thus, TrkB is essential for normal cortical neurogenesis.

TrkB and TrkC collaborate to regulate proliferation and neurogenesis in the cortical neuroepithelium

The previous experiments did not show increased effects on cortical development when dnTrkB and dnTrkC plasmids were electroporated together, potentially because less of each construct was used in the co-electroporations (2 μg of each plasmid when used together versus 4 μg of each when used individually). We therefore performed electroporations with 1.5 μg of dnTrkB or dnTrkC plasmids alone or together to ask whether TrkB and TrkC signaling cooperated. We also co-electroporated 3 μg of each to ensure that we obtained maximal inhibition of Trk signaling. These experiments showed that when 1.5 μg dnTrkB or dnTrkC were electroporated individually, there was a trend to fewer GFP-positive cells in the cortical plate (Fig. 5G), and decreased proliferation (Fig. 5H), and neurogenesis (Fig. 5I), but that none of these reached significance. However, co-electroporation of 1.5 μg each of dnTrkB plus dnTrkC led to a highly significant decrease in all of these parameters (Fig. 5G-I; $P<0.001$). Even more pronounced decreases were obtained when 3 μg dnTrkB plus dnTrkC were co-electroporated together (Fig. 5G-I). Thus, TrkB and TrkC signaling cooperate to promote cortical precursor proliferation and neurogenesis.

Overexpression of BDNF in cortical precursors promotes proliferation and enhances neurogenesis

These data suggest that BDNF and/or NT3 made within the cortical neuroepithelium regulate cortical precursor cell development. To directly test this idea, we co-transfected E13/14 cortices with plasmids encoding BDNF and EGFP, and performed immunocytochemistry 3 days later. This analysis revealed an increase in the percentage of transfected, Ki67-positive cells in the VZ/SVZ (Fig. 6A), indicating that BDNF promoted proliferation. Moreover, the percentage of cells that migrated to the cortical mantle and the percentage of transfected cells that expressed HuD were both higher (Fig. 6B,C), indicating increased neurogenesis. Interestingly, we also observed clusters of HuD-positive neurons in the VZ/SVZ, something that was never seen in control brains (Fig. 6D). Some of these HuD-positive neurons were EGFP-positive, but many others were EGFP-negative and were located in close proximity to transfected cells (Fig. 6D, right panels), suggesting that BDNF secreted from transfected cells promoted the premature genesis of neurons in their vicinity. Thus, BDNF levels are normally limiting, and increasing BDNF within the cortical epithelium increases proliferation and neurogenesis.

Perturbation of Trk signaling does not alter astrocyte formation, but leads to a depletion of postnatal precursors within the VZ/SVZ

These data demonstrated that inhibition of Trk signaling perturbed appropriate neurogenesis, with more undifferentiated, but less proliferative, cells remaining in the VZ/SVZ from E14 to E17. We

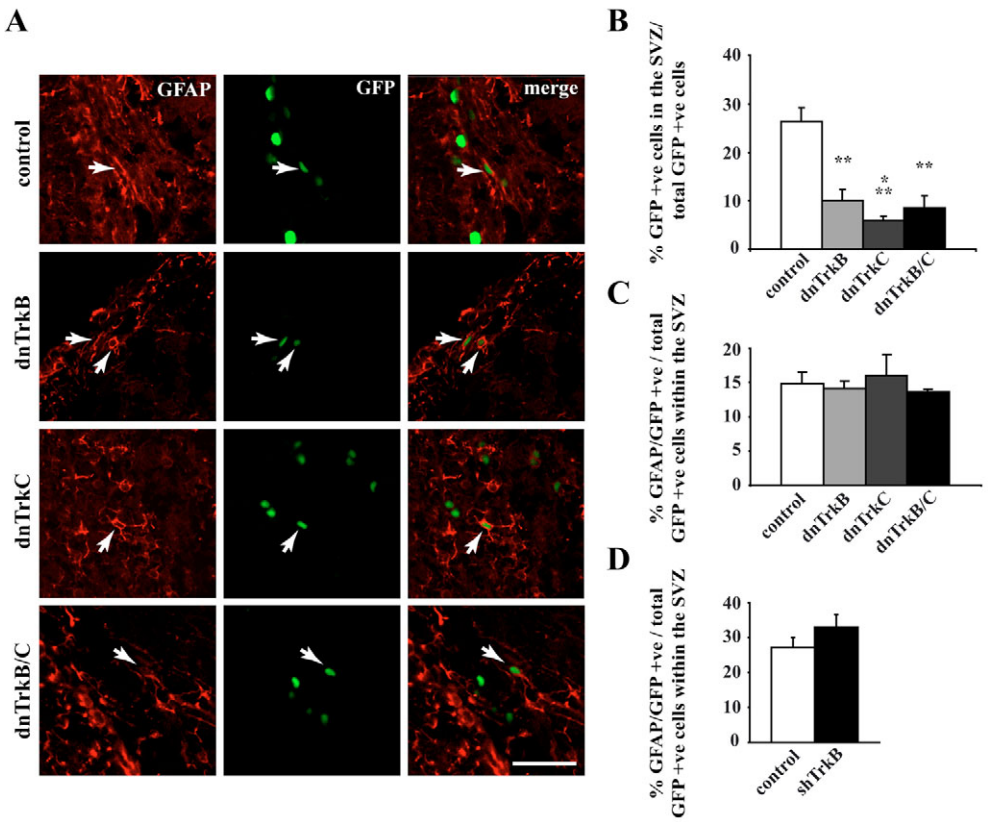

Fig. 7. In mouse, Trk signaling in embryonic precursors is necessary for maintenance of normal numbers of precursors in the postnatal SVZ, but not for astrocyte formation. Cortices were electroporated with EGFP and the empty vector (control), dnTrkB, dnTrkC or both (dnTrkB/C), and analyzed at P3. (**A**) Confocal micrographs of coronal sections through the electroporated VZ/SVZ immunostained for EGFP (GFP, green) and GFAP (red). The right panels show the merges. Arrows indicate double-labeled cells. (**B**,**C**) Quantification of micrographs similar to those in A for the percentage of (B) EGFP-positive cells and (C) EGFP, GFAP-positive cells in the VZ/SVZ. *n*=five, five sections/cortex. (**D**) Quantification of confocal micrographs similar to A where cortices were co-electroporated with EGFP and control shRNA (control) or TrkB shRNA (shTrkB) to determine the percentage of EGFP, GFAP-positive cells within the VZ/SVZ. *n*=four control and three TrkB shRNA brains. Error bars indicate s.e.m. Scale bar: 100 μm. ***P*<0.01; ****P*<0.001 relative to control-transfected sections.

therefore asked whether it also caused more long-term perturbations by examining electroporated brains at P3. Immunostaining revealed that in controls at P3 all of the transfected neurons were present within the cortical layers (Fig. 8A), and all of the transfected, GFAP-positive astrocytes were located within the VZ/SVZ (Fig. 7A), as we have previously published (Barnabé-Heider et al., 2005; Paquin et al., 2005; Gauthier et al., 2007). A similar pattern was observed in brains transfected with dnTrkB and/or dnTrkC (Fig. 7A, Fig. 8A). However, the percentage of transfected cells in the VZ/SVZ (which contains only undifferentiated precursors and newly differentiated glial cells at this stage) was decreased from approximately 30% to 5-10% (Fig. 7B).

To confirm this apparent depletion of cells within the VZ/SVZ, we determined the total number (as proposed to proportion) of cells within the SVZ versus cortical layers. The total number of transfected, EGFP-positive cells in the entire cortex was approximately similar for all groups (in the five sections/animal that were quantified, the means ± s.e.m. were control, 606±110; dnTrkB, 664±68; dnTrkC, 663±68; dnTrkB/C, 739±103; $P>0.4$ for all comparisons relative to the control; *n*=at least four per group). Transfected cell numbers within the cortical layers were also statistically similar (means±s.e.m. were control, 458±102; dnTrkB, 594±51; dnTrkC, 625±66; dnTrkB/C, 682±108; $P>0.15$ for all comparisons relative to the control). By contrast, the number of transfected cells in the SVZ was significantly reduced in dnTrk-transfected brains (means±s.e.m. were control, 148±8; dnTrkB, 70±24; dnTrkC, 38±5; dnTrkB/C, 57±9; $P<0.002$ for all comparisons relative to control). Thus, inhibition of Trk signaling caused depletion of cells within the postnatal SVZ.

This selective depletion of cells within the SVZ could be due to decreased precursor proliferation during embryogenesis, and/or to a perturbation in astrocyte formation in the VZ/SVZ. However, immunostaining for GFAP demonstrated that approximately 15% of the EGFP-positive cells in the VZ/SVZ co-expressed GFAP in both control and dnTrk-expressing cortices (Fig. 7A,C). Moreover, a similar percentage of GFAP-positive cells was seen in cortices electroporated with control versus TrkB shRNAs (Fig. 7D). Thus, inhibition of Trk receptor signaling did not alter neonatal gliogenesis, but probably depleted cells within the postnatal SVZ by decreasing precursor cell proliferation.

Acute inhibition of Trk receptor signaling in cortical precursors leads to long-term effects on cortical neuron development

We also characterized the development of cortical neurons in these transfected, neonatal brains by immunostaining for HuD and for a second neuronal marker, NeuN (Neuna60 – Mouse Genome Informatics) (Fig. 8A); in all groups, approximately 60-70% and 75% of the GFP-positive cells within the cortical layers expressed NeuN and HuD, respectively (Fig. 8B,C). However, the location of these transfected neurons differed between groups. Virtually all control transfected cells (>90%) were located within the upper part of layers 2/3, but approximately 30-40% of the dnTrkB and/or dnTrkC-transfected cells were instead located within the lower portion of layers 2/3 (Fig. 8D,E).

These data indicate: (1) that inhibition of Trk signaling during embryogenesis delayed but did not permanently inhibit precursors from becoming neurons (although the transient nature of the transfections also makes it possible that dnTrk levels had decreased sufficiently by later timepoints to allow neurogenesis to proceed); and (2) that these later-born neurons did not migrate to a location appropriate for their birthdate. To ask whether this perturbed migration was due to a requirement for TrkB/TrkC signaling in cortical interneuron migration (Polleux et al., 2002),

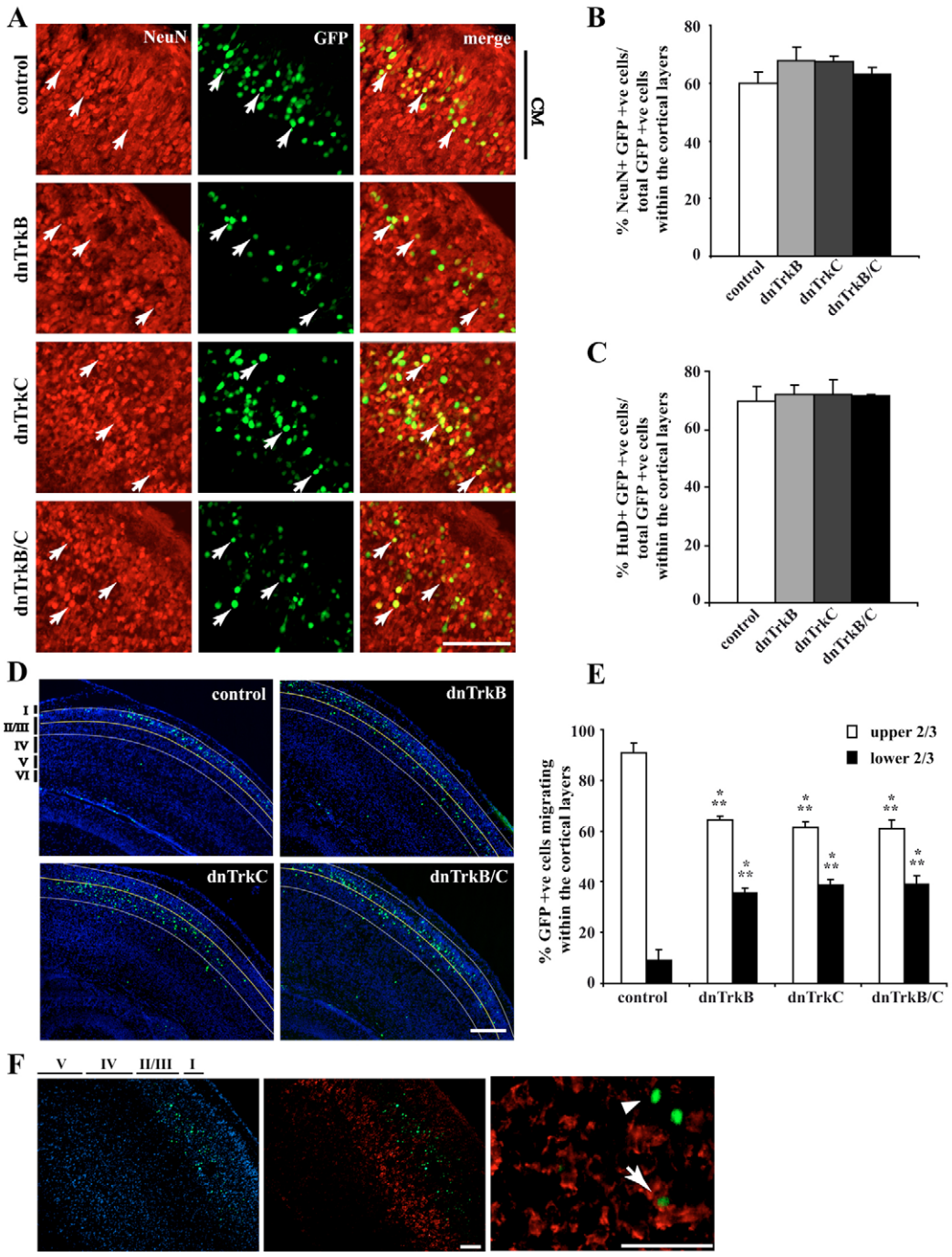

Fig. 8. Inhibition of Trk signaling in embryonic cortical precursors leads to postnatal perturbations in cortical neurons in mouse. Cortices were electroporated with EGFP and the empty vector (control), dnTrkB, dnTrkC or both, and analyzed at P3. (**A**) Confocal micrographs of coronal sections through cortices immunostained for EGFP (GFP, green) and NeuN (red). The right panels show the merges. Arrows indicate double-labeled cells. (**B**,**C**) Quantification of micrographs similar to A for percentage of (B) EGFP, NeuN or (C) EGFP, HuD-positive cells within the cortical layers. *n*=five, five sections/brain. (**D**) Photomicrographs of coronal sections immunostained for EGFP (green) and counterstained with Hoechst (blue). Cortical layers are denoted on the left, and regions defined as the upper and lower parts of layers II/III are demarcated by the white lines. (**E**) Quantification of the percentage of EGFP-positive cells in the upper (white columns) and in the lower (black column) parts of layers II/III in sections similar to (D). *n*=five, five sections/brain. (**F**) Photomicrographs of coronal sections immunostained for EGFP (green) and GAD67 (red) and counterstained for Hoechst (blue). Shown are EGFP and Hoechst (left) or EGFP and GAD67 (middle). The right panel is a higher-magnification view showing a rare, double-labeled cell (arrow), and several EGFP-positive, GAD67-negative cells (arrowhead). Error bars indicate s.e.m. Scale bars: 100 μm in A; 200 μm in D; 50 μm in F. ***P<0.001 relative to control-transfected sections.

we immunostained cortices for the GABAergic interneuron marker GAD67 (Gad1 – Mouse Genome Informatics). This analysis demonstrated that only a very small number of transfected cortical precursors developed into GAD67-positive interneurons (Fig. 8F), and that this number was unchanged by expression of dnTrkB, dnTrkC or both (one to two EGFP-positive/GAD67-positive cells per section in all groups). This result is consistent with the finding that most rodent cortical interneurons derive from the medial ganglionic eminence, and not the cortical neuroepithelium (reviewed by Xu et al., 2003). Thus, the perturbations seen here are probably due to a more general requirement for TrkB/TrkC signaling in migration of principal cortical neurons (Medina et al., 2004).

DISCUSSION

The studies described here support a number of major conclusions. First, experiments acutely inhibiting TrkB and TrkC and overexpressing BDNF in vivo demonstrate that neurotrophin-mediated activation of these two receptors is essential for the proliferation of embryonic cortical precursors, an unexpected role for Trk signaling within the cortical neuroepithelium. Second, we show that inhibition of TrkB and TrkC signaling in vivo causes a delay in the generation of new neurons, and ultimately perturbs postnatal localization of principal cortical neurons. Conversely, BDNF overexpression enhances neurogenesis, indicating that Trk signaling regulates the timing and potentially the extent of cortical neurogenesis. By contrast, TrkB and TrkC receptor signaling are not

required for cortical astrocyte formation, at least within the first few days postnatally. Finally, these studies demonstrate that inhibition of Trk signaling in embryonic cortical precursors causes a decrease in the number of postnatal SVZ precursors, possibly as a direct consequence of decreased embryonic proliferation. Thus, neurotrophin-mediated TrkB and TrkC activation regulates the proliferation, maintenance and differentiation of embryonic cortical precursors, suggesting that this family of growth factors may play a more general role in the regulation of CNS neural precursor cells.

The neurotrophin receptors TrkB and TrkC are activated in response to their preferred ligands; BDNF, NT3 and NT4 in the case of TrkB, and NT3 in the case of TrkC (Huang and Reichardt, 2003). BDNF and NT3 are both expressed within the developing cortical neuroepithelium (Maisonpierre et al., 2000; Behar et al., 1997; Fukumitsu et al., 1998; Fukumitsu et al., 2006), and TrkB and TrkC are coincidently expressed on embryonic cortical precursors (Tessarollo et al., 1993; Behar et al., 1997; Barnabé-Heider and Miller, 2003) (data shown here). Moreover, we and others have shown that, in cultured cortical precursors, BDNF and/or NT3 can regulate survival, proliferation and differentiation (Ghosh and Greenberg, 1995; Lukaszewicz et al., 2002; Barnabé-Heider and Miller, 2003). However, although cortical perturbations have been observed in *Bdnf*$^{-/-}$ and *TrkB*$^{-/-}$ mice (Jones et al., 1994; Alcantara et al., 1997; Ringstedt et al., 1998; Xu et al., 2000; Lotto et al., 2001; Medina et al., 2004), these changes were attributed to the lack of BDNF-mediated TrkB signaling in cortical neurons. Similarly, any CNS deficits observed in *Nt3*$^{-/-}$ and *TrkC*$^{-/-}$ mice have been attributed to perturbations in survival and connectivity of central, including cortical, neurons (Minichiello and Klein, 1996; Martinez et al., 1998; Ma et al., 2002; von Bohlen und Halbach et al., 2003), or to alterations in glial cell development (Kahn et al., 1999). Here, we report that TrkB and TrkC are necessary for development of cortical precursors in vivo, and that they act together, presumably in response to BDNF and NT3 in the cortical neuroepithelium.

The results we obtained were similar for TrkB and TrkC, and our DNA titration experiments indicated that the two receptors function together to promote proliferation and neurogenesis, playing complementary roles during this developmental window. This, and the acute, cell-autonomous nature of the perturbations made here, may explain why previous studies examining single neurotrophin and/or receptor knockouts have not observed such changes. In this regard, a previous study (Medina et al., 2004) examining a nestin-driven conditional TrkB knockout demonstrated that a higher proportion of *TrkB*$^{-/-}$ cells were maintained within the embryonic VZ/SVZ, a phenotype very similar to that seen here with acute inhibition of TrkB. These *TrkB*$^{-/-}$ cells within the SVZ, which were not characterized phenotypically, were interpreted as being newly born neurons that did not migrate. However, on the basis of data presented here, we propose that this perturbation at least partially reflected the necessity for TrkB in the appropriate timing/extent of cortical neurogenesis, as we have documented here using both dominant-negative Trk receptors and an acute shRNA-mediated knockdown of TrkB.

How do receptor tyrosine kinases such as the Trks signal to regulate the biology of cortical precursor cells? We have previously demonstrated (Barnabé-Heider and Miller, 2003) that PI3-kinase but not the MEK-ERK pathway is essential for survival of cultured cortical precursors, findings similar to those recently reported for embryonic stem cells (Pyle et al., 2006). Moreover, a second study demonstrated that overexpression of the PI3-kinase target protein Akt1 led to enhanced cortical precursor cell survival, proliferation and self-renewal (Sinor and Lillien, 2004). However, data presented here inhibiting Akt in cortical precursors in vivo showed that Akt was not necessary for either the survival or proliferation of these cells, at least within the time frame we examined. Perhaps PI3-kinase mediates cortical precursor cell survival and potentially proliferation via other targets such as Ilk (Hannigan et al., 2005) or Pdk1 (Mora et al., 2004). With regard to cortical precursor differentiation, we previously demonstrated that the MEK-ERK pathway is essential for neurogenesis, acting to phosphorylate and activate the C/EBP family of transcription factors (Ménard et al., 2002; Paquin et al., 2005). In addition, we have recently shown that Shp2 (Ptpn11 – Mouse Genome Informatics), a protein tyrosine phosphatase that is necessary for maximal activation of the MEK-ERK pathway downstream of TrkB (Easton et al., 2006), is essential for the differentiation, but not survival or proliferation, of cortical precursor cells (Gauthier et al., 2007). Interestingly, evidence indicates that this same Shp2-MEK-ERK-C/EBP pathway inhibits astrocyte formation while it promotes neurogenesis (Ménard et al., 2002; Paquin et al., 2005; Liu et al., 2006; Gauthier et al., 2007), providing a mechanism that would allow growth factors like the neurotrophins to bias precursors to make only neurons during the neurogenic period.

Although our experiments in the embryonic cortex indicate that inhibition of Trk signaling functions to decrease and/or delay neurogenesis, this effect was no longer apparent by P3. One explanation for this finding is that these transfections were transient, and that the level of Trk inhibition decreased over time so that these precursors could ultimately generate equal numbers of neurons. A second explanation is that other extrinsic cues also promote neurogenesis, potentially via the same Shp2-MEK-ERK-C/EBP pathway, and that ultimately neurogenesis 'catches up', even when Trk signaling is inhibited. While our data do not distinguish these alternatives, they do show that a perturbation of Trk signaling in embryonic precursors ultimately caused mislocalization of principal cortical neurons. If this was simply due to a delay in neurogenesis, then the delayed neurons should be resident in more superficial cortical layers, as was previously seen with transient Notch pathway activation (Mizutani and Saito, 2005). Instead, we document here that these later-born neurons are located in deeper cortical layers, providing support for previous studies showing that neurotrophin signaling regulates cortical neuron migration (Gates et al., 2000; Polleux et al., 2002; Medina et al., 2004) and that BDNF can regulate the phenotype of newly born cortical neurons (Fukumitsu et al., 2006).

Little is known about how environmental cues such as growth factors regulate the survival, proliferation and differentiation of multipotent neural precursors in the embryonic CNS. Here, we provide evidence that the neurotrophins, growth factors previously thought to primarily regulate the development of differentiated CNS neurons and glial cells, also regulate the proliferation and differentiation of cortical precursors in vivo. Moreover, as many other extrinsic cues converge on to the same signaling pathways, this may provide one way in which the complex extracellular environment of the neuroepithelium can be integrated to dynamically regulate precursor cell survival, proliferation and ultimately cell genesis during development.

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