Neuregulin 1–erbB2 signaling is required for the establishment of radial glia and their transformation into astrocytes in cerebral cortex

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Radial glial cells and astrocytes function to support the construction and maintenance, respectively, of the cerebral cortex. However, the mechanisms that determine how radial glial cells are established, maintained, and transformed into astrocytes in the cerebral cortex are not well understood. Here, we show that neuregulin-1 (NRG-1) exerts a critical role in the establishment of radial glial cells. Radial glial cell generation is significantly impaired in NRG mutants, and this defect can be rescued by exogenous NRG-1. Down-regulation of expression and activity of erbB2, a member of the NRG-1 receptor complex, leads to the transformation of radial glial cells into astrocytes. Reintroduction of erbB2 transforms astrocytes into radial glia. The activated form of the Notch1 receptor, which promotes the radial glial phenotype, activates the erbB2 promoter in radial glial cells. These results suggest that developmental changes in NRG-1–erbB2 interactions modulate the establishment of radial glia and contribute to their appropriate transformation into astrocytes.

Radial glial cells play a critical role in the construction of the mammalian brain by contributing to the formation of neurons and astrocytes and providing an instructive scaffold for neuronal migration (1–7). The establishment of radial glial cells from an undifferentiated sheet of neuroepithelium precedes the generation and migration of neurons in the cerebral cortex. During early stages of corticogenesis, radial glial cells can give rise to neurons (3–5). Subsequent neuronal cell movement in the developing mammalian cerebral cortex occurs mainly along radial glial fibers, although nonpyramidal neurons initially migrate into the cortex in a radial glial-independent manner (1, 2, 4–7).

During late stages of corticogenesis, as neurogenesis and migration dwindles, the radial glial scaffolding in the telencephalon is dismantled and transformed into type 1 astrocytes (8–10). Astrocytes contribute to the emergence and maintenance of mature brain circuitry through their function as modulators of neuronal activity (11), neurogenesis (12), and as potential neuronal precursors (13). Neurodevelopmental disorders affecting the development of radial glial cells lead to faulty neuronal and glial differentiation, thus resulting in gross CNS malformations characterized by neuronal misplacement and connectivity (14). Despite their significance, the molecular signals controlling the establishment, maintenance, and transformation of radial glial cells in the developing cerebral cortex are poorly defined.

Neuregulin-1 (NRG-1), a member of the NRG family of proteins, has been implicated in the differentiation of glial cells from both central and peripheral nervous systems (15, 16). In the developing CNS, NRG-1 is expressed in migrating neurons and oligodendrocyte precursors and is thought to positively influence radial glial cell function and oligodendrocyte development (17–21). NRG-1 mediates its effects via the activation of dimers of protein tyrosine kinase receptors, erbB2, erbB3, and erbB4. All three of these receptors are expressed in cerebral cortex during radial glial development (17, 22, 23). We thus sought to determine whether NRG-1-mediated signaling is involved in radial glial cell development and differentiation in the cerebral cortex.

We show that NRG-1 signaling, involving erbB2, may act in concert with Notch signaling to exert a critical influence in the establishment, maintenance, and appropriate transformation of radial glial cells in cerebral cortex.

Materials and Methods

Clonal Analysis to Study NRG's Role in the Initial Establishment of Radial Glial Cells. Embryonic day 9.5 (E9.5) telencepahalic vesicles from WT and NRG−/− mice (23) were dissociated and cultured at clonal density (500 cells per well) in eight-well chamber glass coverslides in neurobasal/N2/B27 medium. To test the influence of NRG-1, duplicate cultures were supplemented with 50 ng/ml NRG-1 [rhNRG-1 (glial growth factor 2); CeNes]. Location of single, isolated cells were noted 6 h after plating. After 6 days *in vitro*, cultures were fixed and colonies from single cells were labeled with neuron-specific TuJ-1 and radial glial-specific RC2 antibodies. The total number of clonal colonies present were counted for each experimental condition. Each of the colonies was assigned to one of three groups: colonies containing radial glia and other neural cells, colonies containing only radial glial cells, and colonies containing no radial glial cells. The number of radial glial cells in each group was also counted. Similarly, neuronal cells in clones were screened with TuJ-1 immunoreactivity. Results were from independent experiments with five different litters.

Explant Assays to Study NRG-1's Effect on Radial Glial Development. E9.5 telecephalic vesicles from WT and NRG-1−/− mice were sectioned into 50-μm-thick slices and cultured in MEM/10% FBS on poly-D-lysine-coated dishes for 4 days. Explants were fixed, immunolabeled with anti-RC2 and TuJ-1 antibodies, and nuclear-counterstained with bis benzimide. The ratio of RC2 or TuJ-1+ cells per number of total cells emigrated from explants (i.e., cell index) was quantified by placing the explants at the center of a 500 × 500-μm grid and calculating the total number of cells and RC2 or TuJ-1+ cells in the grid. Results were from independent experiments with three separate litters. WT and −/− E9.5 telencephalon from five different litters were used to analyze RC2 labeling *in vivo*.

RT-PCR. RT-PCR was carried out with the Superscript OneStep RT-PCR system (Invitrogen) using equal amounts of total RNA from E9.5–10 telencephalon. Each set of reactions was repeated thrice with RNA from different litters. See *Supporting Text*, which is published as supporting information on the PNAS web site, www.pnas.org, for details on primers and cycle parameters used.

Abbreviations: DN, dominant negative; E*n*, embryonic day *n*; GFAP, glial fibrillary acidic protein; NICD, Notch intracellular domain; NRG, neuregulin.

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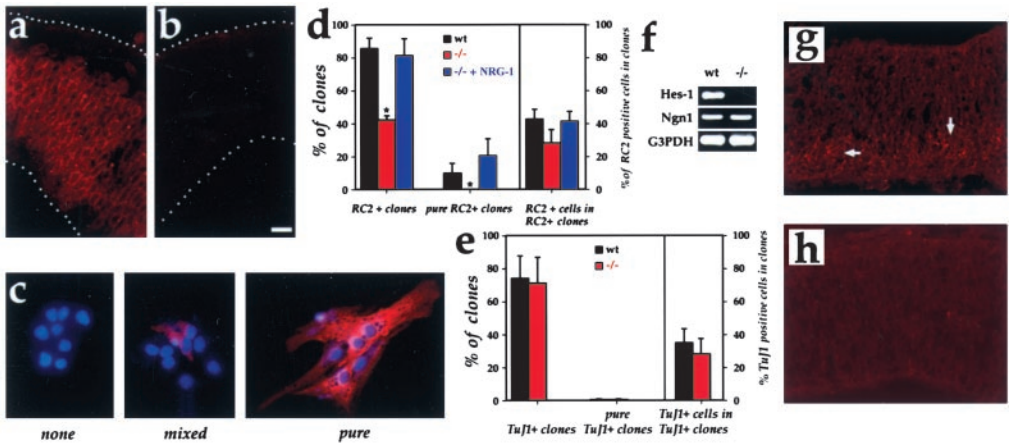

Fig. 1. NRG-1 promotes radial glial cell establishment. (*a* and *b*) Expression of NRG-1 in WT (*a*) and NRG−/− (*b*) E.9.5 telencephalon immunolabeled with anti-NRG1 antibodies. (*c*–*e*) Isolated neuroepithelial cells from WT and NRG−/− telencephalic vesicles were cultured at clonal density for 6 days. (*c*) Clones devoid of RC2-positive cells, clones containing some RC2-positive cells, and clones entirely made of RC2-positive cells were identified. (*d*) Significant reduction in the number of RC2+ cell-containing clones was noticed in the absence of NRG-1. This deficit was rescued by the addition of exogenous NRG-1. (*e*) No changes in the generation of neuronal cells were noticed in NRG−/− mice. (*f*) RT-PCR analysis of Hes1 and neurogenin expression in E9.5 WT and NRG−/− telencephalon. (*g*) RC2-positive processes of radial glial cells in E9.5 WT telencephalon. (*h*) Impaired RC2 labeling in littermate NRG−/− telencephalon. Data shown are mean ± SEM; *, $P < 0.01$, Student's *t* test. (Scale bar: *a*, *b*, *g*, and *h*, 10 μm; *c*, 20 μm.)

Luciferase Assays. E10.5 radial glia were cultured in neurobasal/B27 medium and FGF2 (20 ng/ml), at a density of 50,000 cells per cm^2 on polyornithine-laminin-coated plates. The next day, the cells were transfected with Notch intracellular domain (NICD), Nbl, ErbB2-luc, WT erbB2 pal-luc, or mut erbB2 pal-luc by using the calcium-phosphate method. The cells were harvested 48 h later, and luciferase activity was measured by using the Dual Luciferase Reporter Assay (Promega; ref. 24). See *Supporting Text* for details on the assay and gene constructs used.

Antibodies and ErbB2 Receptor Constructs. Full-length and truncated erbB2 constructs were obtained from D. Stern (Yale University; ref. 25). The truncated erbB2 was subcloned into pEGFP-N3 vector (CLONTECH) to generate the dominant negative (DN) erbB2-GFP plasmid. Full-length erbB2 was subcloned into pEGFP-N3 and pIRES-EGFP vectors (CLONTECH). The identities of these constructs were confirmed by sequencing. See *Supporting Text* for details on the list and source of antibodies used.

Generation and Analysis of Glial Fibrillary Acidic Protein (GFAP) Promoter–DNerbB2-GFP Mice. hGFAP promoter–DNerbB2-GFP transgene was used for pronuclear microinjection of fertilized eggs from FVB/N strain mice. For details on transgene construction and PCR or Southern blotting analysis of founder mice, see *Supporting Text*.

To analyze transgene expression in hGFAP–DNerbB2-GFP mice, cortices of E16 embryos were homogenized in 0.15 mM NaCl/0.05 mM Tris·HCl, pH 7.2/1% Triton X-100/1% sodium deoxycholate/0.1% SDS, immunoprecipitated with anti-GFP antibodies (Sigma), and immunoblotted with anti-GFP antibodies to detect the expression of GFP-tagged DNerbB2.

To assay the effect of DNerbB2 expression on tyrosine phosphorylation of erbB2 in hGFAP–DNerbB2-GFP mice, E16 cortical cells were challenged with NRG-1, and the phosphorylation status of erbB2 was evaluated in immunoprecipitation/blotting assays. See *Supporting Text* for details.

Bioassays. To assay the role of erbB2 receptors in radial glial development, radial glial cell cultures were established from E15 mouse cerebral cortices (17), plated at a density of 10,000 cells per well (eight-well chambers), and transfected with DNerbB2-GFP plasmid or GFP plasmid with Lipofectamine (GIBCO). After 48 h in neurobasal/N2 medium supplemented with NRG-1 (50 ng/ml), cells were fixed and labeled with RC2 or anti-GFAP antibodies. Radial glia and astrocytes were defined by their unique morphology and antigenic marker expression. RC2+ radial glial cells are unipolar or bipolar cells with elongated, pear-shaped cell soma and long, slender processes (>50 μm). In contrast, anti-GFAP+ astrocytes display a flat, stellate shape. Radial glial and astrocyte cells that are positive for both the reporter GFP and glial markers were quantified. Cells that are immunoreactive with both RC2 and anti-GFAP antibodies (8 ± 2.2% of astroglial population *in vitro*) generally have the multipolar morphology of astrocytes and are counted as astrocytes.

In separate assays, using radial glial cells from E16 rat cortices (plating density = 10,000 cells per well), erbB2 function was inhibited with anti-erbB2 receptor-specific mAb 7.6.4. As control, a mAb of the same isotype (Ig2A) was used. After 36 h of incubation in 10 μg/ml affinity-purified antibodies plus 50 ng/ml NRG-1, cultures were fixed in 4% paraformaldehyde and labeled with rat astroglial-specific rat-401 antibodies. Radial glial and astrocyte cell numbers were quantified by using the METAMORPH system (Universal Imaging, Media, PA).

Mature astrocytes from 4-week-old mice were cultured at low density (5,000 cells per well; 16-well chambers) as described (16) and transfected with erbB2-GFP or GFP plasmids. After 48 h *in vitro* in neurobasal/N2 medium plus NRG-1 (50 ng/ml), GFP-expressing cells that are positive for GFAP or RC2 were quantified as described. Quantitative analysis of the above bioassays were based on four to seven independent experiments.

To assay glial cell proliferation, 10 μm of BrdUrd was added to cultures 24 h before fixation and immunolabeling with anti-BrdUrd (Becton Dickinson) and GFAP or RC2 antibodies. Changes in cell death were measured by using terminal deoxynucleotidyltransferase-mediated dUTP end labeling assay (Roche Molecular Biochemicals).

Results

Role of NRG-1-Mediated Signaling in the Establishment of Radial Glial Cells. NRG-1 is expressed in the ventricular neuroepithelial cells, before the appearance of radial glial cells (Fig. 1 *a* and *b*). If NRG-1 is critical for the establishment of radial glia, the absence of NRG-1 should lead to altered radial glial development. To test this possibility, neuroepithelial cells from E9.5 telencephalic vesicles from WT and NRG-deficient mice were dissociated and plated at clonal density. Isolated cells were marked and allowed to proliferate, and after 6 days, the cellular composition of individual clones, generated from WT and NRG-efficient neuroepithelial cells, was analyzed (Fig. 1*c*). Eighty-six percent of the clones from WT neuroepithelial cells contained RC2-positive cells. In contrast, only 45% of the clones were RC2 positive in the absence of NRG (Fig. 1*d*). The percentage of RC2-positive cells per clone was also reduced in NRG mutants (WT, 43 ± 6%; −/−, 28 ± 9%). Purely RC2-positive clones were not generated from NRG-deficient neuroep-

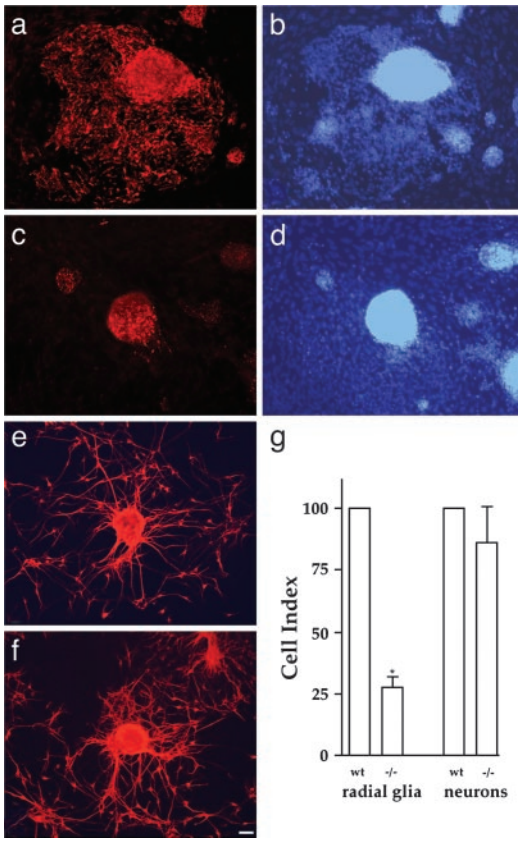

Fig. 2. Lack of NRG impairs radial glial cell generation. Explant cultures of telencephalon from E9.5 WT and NRG−/− mice were immunolabeled with RC2 (*a* and *c*) or TuJ-1 (*e* and *f*) antibodies. Compared with WT (*a* and *b*), significantly fewer RC2-positive cells were generated from NRG−/− explants (*c*, *d*, and *g*). Bis benzimide nuclear counterstain (*b* and *d*) indicates that deficiency in RC2+ cells in NRG−/− explants is not caused by general lack of cell generation. Neuronal generation was not affected in NRG−/− explants (*e*, WT; *f*, −/−; and *g*). (*g*) Number of radial glial or neuronal cells per total number of cells was calculated for each explant and used as cell index. Results from NRG−/− mice were normalized to measurements from littermate WT controls. Data shown are mean ± SEM; *, $P < 0.01$, Student's *t* test. (Scale bar: 60 μm.)

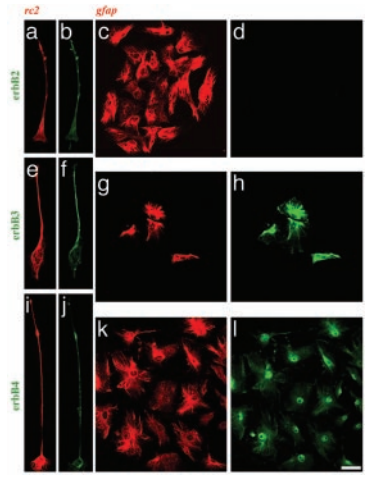

Fig. 3. Distribution of erbB receptors in radial glia and astrocytes. Embryonic radial glial cells (E15) and mature astrocytes (4 weeks) in culture were immunostained with polyclonal antibodies to erbB2 (*a* and *b*), erbB3 (*c* and *d*), and erbB4 (*e* and *f*). Radial glial cells and astrocytes were colabeled with RC2 and anti-GFAP antibodies, respectively. All erbB receptors are expressed in radial glia, whereas only erbB3 and erbB4 are highly expressed in astrocytes. (Scale bar: 60 μm.)

ithelial cells (WT, 10.5 ± 6%; −/−, 0%; Fig. 1*d*). The average numbers of cells per clone from WT and NRG mutant neuroepithelial cells were not significantly different (WT, 5.52 ± 1.26%; −/−, 4.3 ± 0.44%). The deficit in the establishment of RC2-positive radial glial cells in the absence of NRG can be rescued by the addition of exogenous NRG-1 to the NRG-deficient telencephalic neuroepithelial cells. In the presence of NRG-1, 82% of the clones were RC2 positive and the percentage of RC2-positive cells per clone was 42%, similar to that of WT cells (Fig. 1*d*).

To determine the specificity of the NRG effect on radial glial development, we tested the generation of neurons in these clones with a neuron-specific marker (TuJ-1). No significant difference was noticed in TuJ-1-positive clones between WT and NRG-1 mutant cells (WT, 77 ± 13.3%; −/−, 76.4 ± 12.3%), suggesting that NRG's effect is particular to the radial glial cell lineage and does not significantly influence early neuronal generation (Fig. 1*e*).

In addition to RC2, radial glial cells can be identified with markers such as brain lipid binding protein (BLBP) and glutamate transporter (GLAST). Compared with untreated controls, exposure to NRG-1 increased the number of BLBP- and GLAST-positive radial glial cells by 84 ± 11% and 70 ± 17%, respectively.

To further analyze the role of NRG-1 in the establishment of radial glial cells, E9.5 telencephalic vesicles were removed from WT and NRG−/− mice and cultured for 4 days, before immunolabeling with RC2 and neuron-specific Tuj1 antibodies. Both radial glial cells and neurons emigrated from WT explants, but 72 ± 3.8% fewer RC2-positive cells were generated from NRG−/− explants (Fig. 2). Neuronal generation appears not to have been significantly affected in NRG−/− explants. *In vivo*, in WT E9.5 telencephalon, RC2 labels strands of radial glia cell fibers (arrows, Fig. 1*g*), in agreement with previous studies (26). In contrast, a significant dearth of RC2+ cells was noticed in NRG mutant telencephalon (Fig. 1 *g* and *h*).

Because several basic-helix–loop–helix (bHLH) transcription factors are known to differentially influence neurogenesis and gliogenesis in the CNS, we next examined the effect of NRG-1 on transcription factors known to influence gliogenesis. The bHLH factor Hes1 plays a role in gliogenesis, whereas neurogenin 1 positively regulates neuronal differentiation (27, 28). *Hes1* also functions as an effector gene for Notch signaling, which is critical in the generation of radial glial cell identity (29). RT-PCR analysis of *Hes1* expression in telencephalon of E9.5 WT and NRG−/− mice indicated a lack of *Hes1* expression in NRG−/− neuroepithelial cells. In contrast, *neurogenin 1* was expressed normally in NRG−/− cells (Fig. 1*f*).

Together, these results suggest that NRG-1 plays a crucial role in the initial establishment of the radial glial cell scaffold before the onset of neuronal migration and layer formation in cerebral cortex.

Expression of ErbB2, ErbB3, and ErbB4 Receptors in Radial Glial Cells and Astrocytes. To map the distribution of erbB receptors during radial glial development freshly isolated embryonic cortical radial glia and mature cortical astrocytes were coimmunolabeled with affinity-purified anti-erbB antibodies and radial glial-specific RC2 or astrocyte-specific anti-GFAP antibodies, respectively. Radial glial cells are immunoreactive for erbB2, erbB3, and erbB4 (Fig. 3). In contrast, astrocytes from mature cerebral cortex primarily express erbB3 and erbB4 and significantly down-regulate erbB2 expression (Fig. 3).

The decrease in erbB2 expression, between E18 and P0, coincides with the radial glia to astrocyte transformation in the developing cerebral cortex (17, 22). We hypothesize that NRG-1 signaling involving the erbB2 receptor is crucial for the maintenance and function of radial glial cells during the early phases of cortical development. Down-regulation of this receptor and accompanying changes in NRG-1-induced signaling cascades may then trigger the

transformation of radial glia into astrocytes at the appropriate developmental stages during corticogenesis.

Role of ErbB2 Receptors in the Transformation of Radial Glial Cells into Astrocytes. To determine whether signaling involving erbB2 receptors plays a role in the maintenance and transformation of radial glial cells, we first blocked the radial glial cell surface expression and function of erbB2 with an affinity-purified mAb to erbB2, 7.6.4 (30). After 36 h *in vitro*, the distribution of radial glial and astrocyte cell populations was assessed by morphology and rat-401 immunofluorescence. Radial glial cells are unipolar or bipolar cells with elongated, pear-shaped cell soma and long, slender processes. In contrast, astrocytes display a flat, stellate shape. Blocking of erbB2 receptors on the radial glial cell surface led to the transformation of radial glial cells into astrocytes (Fig. 4). A greater proportion of glial cells cultured in the presence of anti-erbB2 antibodies expressed astrocyte morphology (radial glia/astrocyte = 21 ± 4%:79 ± 3%), whereas the cells cultured under control conditions were mainly of radial glial phenotype (radial glia/astrocyte = 71 ± 4%:28 ± 3%). These results support the hypothesis that signaling involving erbB2 receptors is crucial for the maintenance of radial glial cells and in the absence of erbB2 signaling radial glial cells transform into astrocytes.

To further test this idea, radial glial cells from E15 mouse cortices were transfected with DNerbB2-GFP or control GFP plasmids. The DNerbB2 receptor lacks the intracellular domain, including tyrosine kinase and phosphorylation sites, and thus does not transduce signals upon interactions with NRG-1. Expression of DNerbB2 in C6 glioma cells resulted in the inhibition of NRG-1-induced phosphorylation of erbB receptors (185 kDa). DNerbB2-GFP or GFP-transfected cells were maintained for 48 h in NRG-1 and then labeled with radial glial-specific RC2 and astrocyte-specific anti-GFAP antibodies. Transfected cells that were positive for GFP and glial markers were counted according to their phenotype. Glial cells expressing DNerbB2-GFP were mainly of astrocyte phenotype (radial glia/astrocyte ratio = 13.5 ± 2.1:85.65 ± 2). Cells transfected with control plasmids were mainly of radial glial phenotype (radial glia/astrocyte ratio = 70.31 ± 3:29.7 ± 3.34; Fig. 4). Cell death and cell division dynamics of DNerbB2 and GFP-transfected cells were analyzed with terminal deoxynucleotidyl-transferase-mediated dUTP end labeling and BrdUrd incorporation, respectively. Expression of DNerbB2 or GFP did not specifically affect the survival or division of embryonic cortical cells (Table 1, which is published as supporting information on the PNAS web site).

To test whether down-regulation of erbB2 receptor function triggers the transformation of radial glia into astrocytes *in vivo*, transgenic mice expressing DNerbB2 under the control human GFAP promoter were generated. Previous studies indicate that this promoter can drive gene expression in radial glia as early as E13 (31). A transgene encoding DNerbB2 linked to the GFP gene under the control of GFAP promoter was constructed and used for pronuclear microinjection (Fig. 5*a*). Seven founder lines were identified by Southern blot analysis of tail DNA and independent breeding lines were established from two (named DN-1 and DN-2; Fig. 5*b*). Immunoblotting and immunohistochemical analysis of embryonic cortices of these lines with anti-GFP antibodies indicated that the DNerbB2-GFP transgene was expressed in the developing cortex in astroglial cells (Fig. 5 *c* and *j*). Expression of DNerbB2 significantly attenuated NRG-1-induced phosphorylation of erbB2 receptors in these cortices (Fig. 5*d*). To analyze the effect of DNerbB2 expression on radial glial development *in vivo*, E16 cortices of DNerbB2 mice were removed and labeled with anti-GFAP antibodies. At E16, in WT cortices, only occasional GFAP-positive cells, primarily associated with blood vessels were noticed (Fig. 5 *e–g*). However, in both transgenic lines, a significant increase in GFAP-positive astroglial cells was evident in the developing cerebral wall (Fig. 5 *h–k*). Both multipolar astrocytes

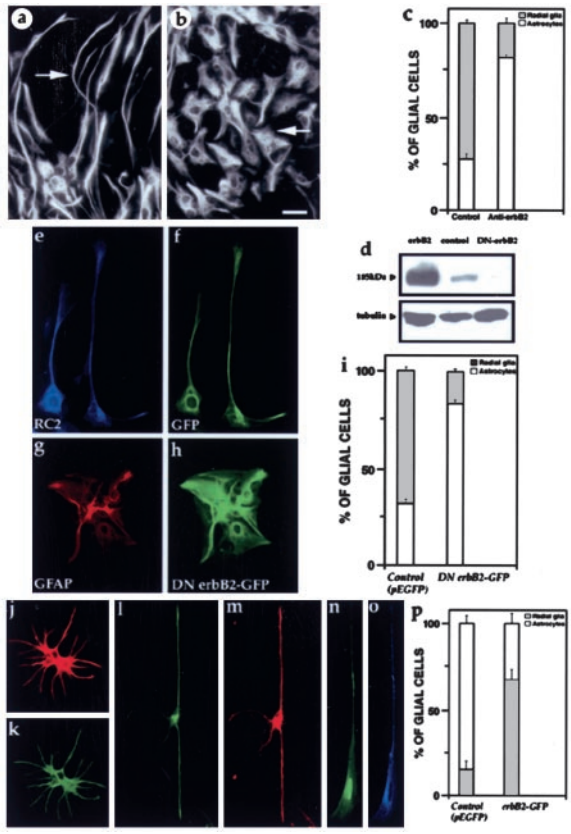

Fig. 4. Radial glia transform into astrocytes in the absence of signaling via erbB2, and erbB2 expression induces astrocyte to radial glia transformation. (*a–c*) Radial glial cells (E16) were cultured in the presence of anti-erbB2 mAb (*b*) or control (*a*) antibodies. Under control conditions, 71 ± 4% cells were radial glial cells (arrow, *a*); the rest were astrocytes (28 ± 3%). However, when erbB2 was blocked, only 21 ± 4% of the cells were of radial glial phenotype and 79 ± 3% of cells displayed the transformed phenotype of astrocytes (arrow, *b*; *c*). Cells were labeled with rat-401 antibodies. Data shown are mean ± SEM. (Scale bar = 30 μm.) (*d*) C6 cells were transfected with erbB2-GFP, GFP, or DNerbB2-GFP plasmids, treated with 50 ng/ml NRG-1 for 10 min, and immunoblotted with antiphosphotyrosine or acetylated tubulin antibodies. DNerbB2 drastically reduced NRG-1-induced tyrosine phosphorylation of ErbB receptors (185 kDa). (*e–i*) Radial glia from E15 mouse cortices were transfected with DNerbB2-GFP (*e* and *f*) or control GFP (*g* and *h*) plasmids. After 48 h, cells that are positive for GFP and the radial glial (RC2-blue; *e* and *f*) or astrocyte markers (anti-GFAP-red; *g* and *h*) were counted according to their phenotype. Glial cells expressing DNerbB2-GFP were mainly of astrocyte phenotype (radial glia/astrocyte ratio = 13.5 ± 2.1:85.65 ± 2; *e*, *f*, and *i*). Cells transfected with control plasmids were mainly of radial glial phenotype (radial glia/astrocyte ratio = 70.31 ± 3:29.7 ± 3.34; *g–i*). (Scale bar = 30 μm.) (*j–p*) Mature astrocytes from 4-week-old mouse cortices were transfected with GFP (*j* and *k*) or erbB2-GFP (*l–o*) plasmids. After 48 h, GFP-positive cells labeled with anti-GFAP (*k* and *m*; red) or RC2 (*o*; blue) antibodies were quantified. Cells expressing erbB2 were predominantly of radial glial phenotype (percentage of glial cells that are radial glia: GFP, 14 ± 4.6, erbB2-GFP, 65.7 ± 5.9), whereas cells transfected with GFP were mainly astrocytes (percentage of glial cells that are astrocytes: GFP, 85.3 ± 4.5; erbB2-GFP, 34.2 ± 6). (Scale bar = 30 μm.)

(arrows, Fig. 5*h*) and elongated, branching cells, characteristic of transforming radial glia (arrowheads, Fig. 5*h*) were seen. Both transgenic lines expressed similar patterns of premature radial glial differentiation, indicating the insertion site independent effect of DNerbB2 transgene. Taken together, these data suggest that down-regulation of erbB2 activity in radial glia leads to premature transformation of these cells into GFAP-positive astroglia in the developing cerebral wall. Regional differences in aberrant radial glial development, the overall effect on cortical patterning, and effects on neuronal or oligodendrocyte differentiation in these lines remains to be characterized.

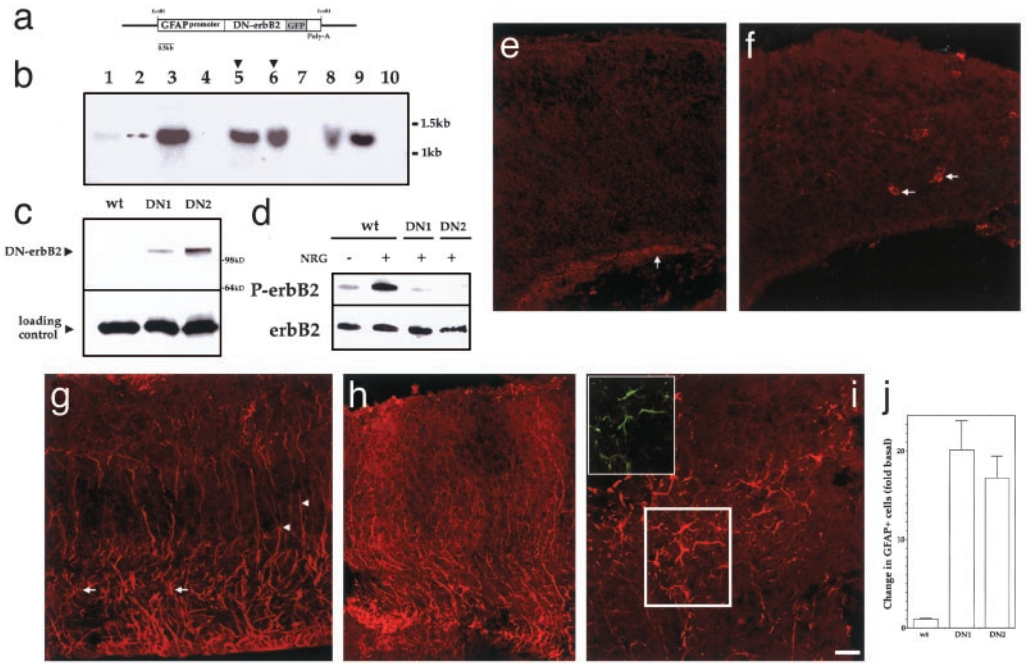

Fig. 5. Down-regulation of erbB2 function transforms radial glia into astrocytes *in vivo*. (*a*) The construct used for the generation of GFAP promoter-DNerbB2-GFP transgenic mice. (*b*) Southern blot analysis of founder mice. Of the seven founder lines, two (arrowhead) were used to establish breeding lines named DN1 and DN2. (*c*) DNerbB2-GFP transgene is expressed in the E16 cortices of DN1 and DN2 mice, but not in nontransgenic mice. DNerbB2-GFP protein expression was detected with anti-GFP antibodies. (*d*) NRG-1-induced tyrosine phosphorylation of erbB2 receptors is attenuated in E16 cortical cells from DN1 and DN2 mice. (*Upper*) Tyrosine phosphorylation of erbB2. (*Lower*) ErbB2 protein. (*e–g*) Minimal expression of GFAP-positive astrocytes in WT (E16) cortex. Arrows point to astrocytes found in association with blood vessels (*f*) or in the ventricular zone (*e*). (*h–j*) Increased number of GFAP+ astroglia were seen in DN1 (*h*) and DN2 (*i* and *j*) cortices. (*Inset*) DNerbB2-GFP transgene is expressed in astroglial cells. Transformed astrocytes in cortex (red) also label with anti-GFP antibodies (green). (*k*) An ≈17- to 20-fold increase in GFAP+ cells was found in DN1 and DN2 cerebral wall. Number of GFAP+ cells was counted in 10 sections of the cerebral wall from four embryos each of WT, DN1, and DN2. (Scale bar = *e–g*, 45 μm; *h–j*, 60 μm.)

Transformation of Astrocytes into Radial Glial Cells. To determine whether ectopic reexpression of erbB2 in mature astrocytes can induce them to revert to radial glial phenotype, mature astrocytes from 4-week-old mice cortices were transfected with GFP or erbB2-GFP, and the phenotypes of the GFP-positive transfected cells were monitored after 48 h with radial glial- and astrocyte-specific markers. The percentage of radial glial cells increased significantly after erbB2 expression (GFP 14 ± 4.6; erbB2-GFP 65.7 ± 5.9), whereas cells expressing control GFP were primarily astrocytes (radial glia/astrocyte ratio = 14.7 ± 4.6:85.3 ± 4.5; Fig. 4 *j–p*). ErbB2-expressing cells of intermediate morphology with fewer but elongated processes were also noticed (Fig. 4 *j–p*). These may represent cells that were in the process of transforming from astrocyte to radial glial phenotype. Terminal deoxynucleotidyl-transferase-mediated dUTP end labeling and BrdUrd incorporation analysis of GFP- or erbB2-GFP-transfected cells indicated no significant differences between these two groups (see Table 1). Together, these results indicate that reexpression of erbB2 in astrocytes can induce them to assume radial glial identity, and loss of erbB2 activity is a critical step in the transformation of radial glia into astrocytes.

Transactivation of the ErbB2 Promoter by Activated Notch1 in Radial Glial Cells. It has recently been shown that development of radial glia is regulated, in part, by Notch signaling (29, 32). Notch signaling is activated by binding of the DSL (Delta-Serrate-lag-2) family of ligands to the extracellular domain of Notch, releasing the intracellular domain, which can then migrate to the nucleus and regulate gene expression in concert with the CSL transcription factor, RBP-Jk or CBF-1 (33, 34). Truncated forms of Notch that consist solely of the intracellular domain (NICD) have constitutive signaling activity.

One likely downstream effector of Notch signaling in radial glial cells is erbB2. Multiple RBP-Jk binding sites have been identified in the human erbB2 promoter and NICD was shown to increase transcription of a reporter construct containing erbB2 RBP-Jk binding sites (35). Notch 1 receptors were expressed in radial glial cells (Fig. 6*a*) and both Notch1 and erbB2 were coexpressed in radial glia (data not shown). Transient transfection of radial glial cells with NICD was sufficient to activate the WT erbB2 promoter as well as an erbB2 reporter construct containing intact RBP binding sites but not a promoter containing mutated sites (Fig. 6). Furthermore, known inhibitors of Notch signaling, Numblike (Nbl; refs. 36 and 37) and a DN form of RBP-Jk (DN-RBP-Jk; ref. 29), were able to block erbB2 promoter activation by NICD (Fig. 6). Thus, these data demonstrate that the erbB2 promoter responds to Notch signaling in radial glial cells, suggesting that erbB2 is one of the effectors for the regulation of radial glial function by Notch signaling.

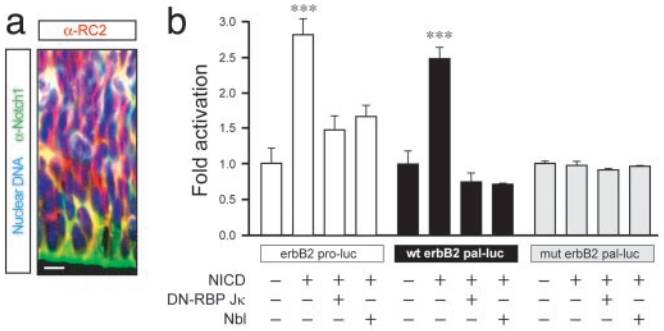

Fig. 6. Notch1 up-regulates the expression of erbB2 in radial glial cells. (*a*) Notch1 (green) is expressed in RC2-positive (red) radial glial cells of the E13.5 mouse cerebral cortex. Nuclei were detected by using bis benzimide (blue). (Scale bar = 10 μm.) (*b*) E10.5 radial glia were transfected with 250 ng per well of erbB2 pro-luc, WT erbB2 pal-luc, or mut erbB2 pal-luc. Cells were also transfected with 500 ng per well of NICD, DN-RBP Jk, or Nbl, where indicated. Transfection of NICD resulted in a stimulation of the erbB2 pro-luc and the WT erbB2 pal-luc reporter constructs. Activation of the WT erbB2 promoters was blocked by DN-RBP Jk and Nbl. No stimulation of the mutant version of the erbB2 promoter that lacks intact RBP Jk binding sites (mut erbB2 pal-luc) was observed. Data shown are mean ± SEM ($n = 3$). ***, $P \leq 0.005$, Student's *t* test.

Discussion

As a permissive and instructive scaffold for neuronal migration, and as precursors of neurons, radial glial cells play an essential role in the emergence of laminar organization in the mammalian cerebral cortex. A critical first step in this process is the establishment of radial glial cells from neuroepithelial cells in the ventricular zone. The generation of radial glial cells from neuroepithelial cells is impaired in NRG mutants and this defect can be rescued with exogenous NRG-1. The generation of radial glial phenotype and its derivatives is likely to be influenced by a hierarchy of diverse molecular cues, including ciliary neurotrophic factor, bone morphogenetic proteins, brain lipid binding protein, glutamate transporter, Musashi-1, *rax*, neurogenin-3, *Hes1*, and pax-6 (3, 38–42). The diversity of molecular cues that contribute to the establishment of radial glial cells is consistent with the possibility that there are multiresponsive precursors in the ventricular neuroepithelium. This diversity may also contribute to the generation of heterogeneity of radial glial cells within different regions of the developing brain.

Radial glial cells in the cerebral cortex may concurrently function as neuronal precursors and neuronal migratory guides, although the extent to which this occurs in the entire radial glial population at different stages of cortical development remains to be determined (3–5, 43). NRG-1 is known to modulate neuroepithelial cell proliferation and the reciprocal interactions between migrating cortical neurons and their radial glial guides (17, 18, 44). In the absence of NRG-1 signaling via erbB2 receptors, radial glial function and development is abnormal. NRG-1 has been identified as a susceptibility gene for schizophrenia (45), where abnormal generation, placement, and connectivity of neurons, resulting from aberrant radial glial development, may play a role in the etiology of the disease.

Recent studies indicate that Notch 1 signaling promotes the establishment of radial glial identity (29, 32). *Hes1*, an effector gene for Notch signaling, is down-regulated in NRG mutant telencephalon. Furthermore, NICD activates the erbB2 promoter in radial glial cells. This activation depended on the presence of RBP Jk binding sites and was sensitive to inhibition by Numblike and a DN form of RBP Jk. Thus our data suggests that Notch may act to up-regulate the expression of erbB2 and thus enhance the response of radial glial cells to NRG signals.

Whether NRG-1 actively biases neuroepithelial cells toward a radial glial fate at the expense of other cortical cell types remains to be determined. However, NRG's effect on Hes1 expression and the lack of significant effect on neurogenesis in NRG$-/-$ cells suggests that it may function to influence cortical precursors toward a glial-restricted or radial glial fate without directly acting on the generation of cortical neurons.

After the completion of neuronal migration in the cerebral cortex, radial glial cells transform into astrocytes or ependymal cells. They contribute to the emergence and maintenance of mature brain circuitry through their function as potential stem cells and as modulators of neuronal activity and neurogenesis (11–13). Down-regulation of erbB2 induces transformation of radial glia into astrocytes and ectopic expression of erbB2 causes astrocytes to revert to a radial phenotype. Such bidirectional developmental potential of radial glial cells and astrocytes also depends on soluble signals present in embryonic forebrain (10). A developmental increase in diffusible gliogenic signals is also thought to specify the generation of astrocytes in cerebral cortex (46–48). The role of erbB2 receptors in astrocyte development raises the possibility that cortical glial development is regulated, in part, by modulating the competence of the precursors to respond to diffusible gliogenic signals via developmental regulation of signaling receptor expression (also see *Supporting Text*).

Studies thus far indicate that radial glial cells exist in several different functional states: as precursors of neurons, guides for neuronal migration, and precursors of astrocytes. Similarly, astrocytes appear to exist in quiescent, as well as neural stem cell-like states. Astrocytes can revert to a radial glial-like phenotype and vice versa, in response to modulation of NRG-erbB2 signals. Functional induction of erbB2 in mature astrocytes may facilitate their ability to support cell migration or neurogenesis during ongoing maintenance of neural circuitry in mature CNS. Further characterization of NRG-erbB2-related mechanisms controlling the different stages of radial glial and astrocyte development will be essential to elucidate the role of radial glia and their derivatives in the emergence and maintenance of the cerebral cortex.

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Supporting Text

RT-PCR

Total RNA was extracted from fresh-frozen E9.5–10 telencephalon by using Trizol (Invitrogen). RT-PCR was carried out with the Superscript OneStep RT-PCR system (Invitrogen) by using equal amounts of RNA. Each set of reactions was repeated three times with RNA from different embryonic litters. The following primers and cycle parameters were used: G3PDH, 5'-accacagtccatgccatcac-3' and 5'-tccaccaccctgttgctgta-3', 15 s at 94ºC, 45 s at 58ºC, and 1 min at 72ºC for 40 cycles (PCR product size, 450 bp); HES1, 5'-cagccagtgtcaacacgacac-3' and 5'-tcgttcatgcactcgctgag-3', 15 s at 94ºC, 45 s at 58ºC, and 1 min at 72ºC for 40 cycles (PCR product size, 400 bp); NGN1, 5'-ccgacgacaccaagctcaccaagat-3' and 5'-gtggtatgggatgaaacagggcgtc-3', 15 s at 94ºC, 45 s at 60ºC, and 1 min at 72ºC for 40 cycles (PCR product size, 360 bp).

Antibodies

The following antibodies were used: TuJ-1 (Babco, Richmond, CA), polyclonal anti- GFAP (Dako), mouse anti-GFAP (Sigma), rat-401 and RC2 (Developmental Studies Hybridoma Bank, Iowa City, IA), polyclonal anti-brain lipid binding protein (N. Heintz, The Rockefeller University, New York), polyclonal anti-glutamate transporter (M. Watanabe, Hokkaido University, Sapporo, Japan), anti-NICD (A. Israel, Institut Pasteur, Paris), anti-phosphotyrosine (Chemicon), anti-acetylated tubulin (Sigma), anti-erbB2, erbB3, and erbB4 (Santa Cruz Biotechnology), anti-erbB2 mAb 7.6.4 (M. Greene, University of Pennsylvania, Philadelphia; ref. 1), and polyclonal anti-erbB4 (C. Lai, The Scripps Institute, La Jolla, CA).

Generation and Analysis of GFAP Promoter-DNerbB2-GFP Mice

hGFAP-DNerbB2-GFP transgene was constructed by removing the LacZ reporter coding region from the human GFAP promoter-LacZ reporter plasmid, gfa2-lac2 (2), by *Bam*HI digestion, followed by blunt-end ligation with the DNerbB2-GFP fragment. The latter was excised as a 2.9-kb *Nhe*I-*Not*1 fragment from DNerbB2-GFP plasmid. The identity of the hGFAP-DNerbB2-GFP was verified by restriction digest and sequencing. To generate transgenic mice, the injection fragment was excised from the hGFAP-DNerbB2-GFP plasmid with *Eco*RI, separated by agarose gel electrophoresis, purified, and used for pronuclear microinjection of fertilized eggs from FVB/N strain mice. Founder mice and their offspring were identified by PCR analysis or Southern blotting of DNA prepared from tail biopsies. For PCR analysis, 5' primer AGC AAG CCC TGT GCT CG and 3' primer CTG AAC TTG TGG CCG TTT ACG, complementary to the DNerbB2 and GFP sequences, respectively, were used to generate a 1.1-kb PCR product. For Southern blotting analysis, a [α-^{32}P]dCTP-labeled DNA probe was generated from a 650-bp *Fsp*I-*Nco*I fragment of DNerbB2-GFP plasmid, and used to probe the DNA blot (i.e., tail DNA digested with *Pvu*II, separated on a 0.8% agarose gel, and transferred to a Hybond+ nylon membrane).

To analyze transgene expression in hGFAP-DNerbB2-GFP mice, cortices of E16 embryos were homogenized in RIPA buffer (50 mM Tris, pH 7.4/150 mM NaCl/1% NP-40/0.25% Na-deoxycholate/0.1% SDS, plus Roche Complete protease inhibitor), centrifuged at 14,000 × *g* for 20 min at 4° C to remove debris, immunoprecipitated with anti-GFP antibodies (Sigma), and immunoblotted with anti-GFP antibodies to detect the expression of GFP-tagged DNerbB2.

To assay the effect of DNerbB2 expression on ErbB2 activation (i.e., tyrosine phosphorylation) in hGFAP-DNerbB2-GFP mice, E16 cortices were dissociated and cultured in DMEM/10%FBS at 2 × 10^6 cells per 60-mm dish for 2 days. Cells were then rinsed, maintained for 5 h in serum-free OptiMEM, and challenged with NRG-1 (50 ng/ml) for 10 min. Cells were then harvested in lysis buffer (50 mM Tris, pH 7.4/150 mM NaCl/1% NP-40, plus Roche Complete protease inhibitor mixture), centrifuged at 14,000 × *g* for 20 min at 4ºC to remove debris, immunoprecipitated with anti-ErbB2 antibodies (C-18, Santa Cruz Biotechnology), and Western blotted with anti-phoshotyrosine antibodies (Upstate Biotechnology, Lake Placid, NY). The same NC membrane was stripped and reprobed with polyclonal anti-ErbB2 antibodies. Immunohistochemical labeling of embryonic brain sections were carried out as described (3).

Luciferase Assays

Primary cultures of E10.5 radial glial cells/neural stem cells were cultured in neurobasal medium containing 2% B27, FGF2 (20 ng/ml, Upstate Biotechnology), 2 mM L-glutamine, 1 mM sodium pyruvate, and penicillin-streptomycin (100 units/ml) at a density of 50,000 cells per cm^2 on polyornithine-laminin-coated 24-well plates. The next day, the cells were transfected by using the calcium-phosphate method. The cells were harvested 48 h later and luciferase activity was measured by using the Dual Luciferase Reporter Assay System (Promega). The total amount of DNA in each well was equalized by the addition of pCDNA3. Transfection efficiency was normalized by using a control promoter, TK-renilla luciferase. Nbl is a gift from J. McGlade (Amgen Biologicals). ErbB2-luc contains –396 to –24 of the human erbB2 promoter cloned in to the pSOVA luciferase vector. WT erbB2 pal-luc and mut erbB2 pal-luc contain four copies of the RBPJ-binding palindrome (WT or mutant, respectively) found in the human erbB2 promoter, cloned upstream of the luciferase gene. All erbB2 reporter constructs were gifts from G. Gill (University of California, San Diego). NICD contains the intracellular domain of human Notch1 as described (4). DN-RBP Jk contains a point mutation in the DNA binding region (R218H) and was a gift from T. Honjo (Kyoto University, Kyoto).

NRG-erbB Signaling During Radial Glial Development

Lack of NRG-1 effect, after inhibition of erbB2 receptors in our studies, suggests that NRG-1 signaling via intact erbB3 and erbB4 receptors alone is not sufficient for radial glial differentiation. ErbB2 is a potent regulator of radial glial, Schwann cell, and oligodendrocyte differentiation, indicating that NRG-erbB2 interactions may function as a common regulatory signal of glial development in the nervous system (5, 6). The function of NRG-1-erbB interactions appears to depend critically on the specific developmental stage and cellular context (5, 7, 8). Furthermore, erbB receptors are phosphorylated in distinct tyrosine residues in response to different NRGs and activate different intracellular kinase signaling cascades. How the distinct neuregulin ligands and erbB receptors discriminate in their functional activity in the developing cerebral wall is yet to be fully defined (5, 7).

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Table 1. Effect of DNerbB2 and erbB2 on cell death and proliferation

Cells	Control	DNerbB2	erbB2
TUNEL-positive*	1 ± .0.05	-	0.93 ± 0.06
	1 ± 0.2	1.074 ± 0.2	-
BrdUrd-labeled	0.18 ± 0.04	-	0.23 ± 0.07
(radial glia/astrocyte)†	0.198 ± 0.05	0.21 ± 0.06	-

TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP end labeling.

*Data shown are mean ± SEM (*n* = 4). Results were normalized to GFP control measurements.

†BrdUrd was added to cultures 24 h before fixation and immunolabeling with anti-BrdUrd and radial glial- or astrocyte-specific antibodies. Data shown are mean ± SEM (*n* = 4).