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Gde2 regulates cortical neuronal identity by controlling the timing of cortical progenitor differentiation

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SUMMARY

The mammalian cortex is a multilaminar structure consisting of specialized layer-specific neurons that form complex circuits throughout the brain and spinal cord. These neurons are generated in a defined sequence dictated by their birthdate such that early-born neurons settle in deep cortical layers whereas late-born neurons populate more superficial layers. Cortical neuronal birthdate is partly controlled by an intrinsic clock-type mechanism; however, the role of extrinsic factors in the temporal control of cell-cycle exit is less clear. Here, we show that Gde2, a six-transmembrane protein that induces spinal neuronal differentiation, is expressed in the developing cortex throughout cortical neurogenesis. In the absence of Gde2, cortical progenitors fail to exit the cell cycle on time, remain cycling, accumulate and exit the cell cycle en masse towards the end of the neurogenic period. These dynamic changes in cell-cycle progression cause deficits and delays in deep-layer neuronal differentiation and robust increases in superficial neuronal numbers. *Gde2*$^{-/-}$ cortices show elevated levels of Notch signaling coincident with when progenitors fail to differentiate, suggesting that abnormal Notch activation retains cells in a proliferative phase that biases them to superficial fates. However, no change in Notch signaling is observed at the time of increased cell-cycle exit. These observations define a key role for Gde2 in controlling cortical neuronal fates by regulating the timing of neurogenesis, and show that loss of Gde2 uncovers additional mechanisms that trigger remaining neuronal progenitors to differentiate at the end of the neurogenic period.

KEY WORDS: Gde2, Cortex, Differentiation, Mouse

INTRODUCTION

The cytoarchitecture of the mammalian cortex is formed during embryonic and postnatal development through the synchronization of progenitor proliferation and specification, neuronal differentiation and neuronal migration (reviewed by Shoemaker and Arlotta, 2010; Molyneaux et al., 2007; Marin and Rubenstein, 2003). These tightly regulated events lead to a multilaminar structure containing layer-specific classes of pyramidal neurons that form local networks and long-range connections throughout the brain and spinal cord (Rakic, 2007). Lineage tracing and transplantation experiments suggest that the time when progenitors exit the cell cycle defines their eventual fate such that neurons that contribute to deep cortical layers are generated first, whereas later-born cortical neurons occupy more superficial positions (Luskin et al., 1988; Bayer and Altman, 1991; McConnell and Kaznowski, 1991; Reid et al., 1995). This ordered sequence of neuronal differentiation is consolidated further by a progressive restriction in progenitor competence whereby progenitors later in development preferentially give rise to superficial cortical neurons (Desai and McConnell, 2000; Frantz and McConell, 1996). Thus, the timing of cortical neurogenesis is a key regulatory nexus to ensure cortical neuronal diversity; however, the molecular mechanisms that control this process are not well understood.

Studies in the *Drosophila* embryonic nervous system have provided major insight into the mechanisms that link birthdate and neuronal fate. In *Drosophila*, neural progenitors (neuroblasts) express a set of transcription factors in a characteristic sequence that correlates precisely with the ordered generation of particular sets of neurons (reviewed by Pearson and Doe, 2004; Brody and Odewald, 2002; Jacob et al., 2008). This sequence of events is recapitulated in vitro using isolated neuroblasts and is linked to the number of cell cycles that have progressed, suggesting that the derivation of different neuronal cell types from multipotent progenitors depends primarily upon an intrinsic cell-cycle clock mechanism. One interpretation of these observations is that neuronal diversity is controlled by the extent of cell-cycle progression rather than the simple passage of time (Isshiki et al., 2001; Pearson and Doe, 2004). In the mammalian cortex, layer-specific neuronal fates are ultimately shaped by a combination of intrinsic mechanisms and environmental signals; however, recent experiments suggest that a similar intrinsic timing mechanism operates to ensure the sequence of layer-specific pyramidal neuron generation (Qian et al., 2000; Shen et al., 2006; Gaspard et al., 2008). Clonal analyses of isolated cortical progenitors show that neurogenesis in vitro proceeds as observed in vivo. Specifically, Cajal-Retzius cells are generated first, followed by deep-layer and superficial cortical neurons. Moreover, this sequence of differentiation is accompanied by a corresponding restriction in competence (Shen et al., 2006). Studies aiming to understand the molecular mechanisms involved in this regulation suggest that the cell-intrinsic mechanisms that control the timing of neurogenesis are phylogenetically conserved; for example, the transcription factors *hunchback* (*hb*) and *seven up* regulate the ratio of early- to late-born neurons in the fly nervous system, and their vertebrate counterparts Ikaros (Ikzf1 – Mouse Genome Informatics) and CoupTFI/II (Nr2f1 and Nr2f2 – Mouse Genome Informatics) play similar roles in the mammalian cortex and retina (Elliott et al., 2008; Maurange et al., 2008; Faedo et al., 2008; reviewed by Okano and Temple, 2009).

We considered the possibility that, in addition to cell intrinsic mechanisms, regulatory pathways that directly stimulate progenitor cell-cycle exit would constitute important control modules that could affect progenitor competence and the generation of neuronal

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diversity and number during development. One such pathway has been described in the developing spinal cord. Here, the six-transmembrane protein Gde2 (Gdpd5 – Mouse Genome Informatics) regulates the timing of motor neuron progenitor differentiation and the generation of different motor neuron subtypes through the downregulation of Notch signaling (Rao and Sockanathan, 2005; Nogusa et al., 2004; Yan et al., 2009; Sabharwal et al., 2011). Gde2 is expressed in early-born medial motor column (MMC) motor neurons, where it acts non-cell-autonomously to inhibit Notch signaling in adjacent motor neuron progenitors using extracellular glycerophosphodiester phosphodiesterase (GDPD) activity (Sabharwal et al., 2011). Genetic ablation of Gde2 results in deficits in alpha motoneurons of the limb-innervating lateral motor column (LMC); namely, early-born LMC motor pool formation is delayed and late-born LMC motor pools fail to differentiate (Sabharwal et al., 2011; Kanning et al., 2010). Based on these observations, we investigated the possibility that Gde2 constitutes a regulatory mechanism that plays important roles in other systems where the timing of neurogenesis is a key factor in controlling neuronal fate and numbers, such as the developing cortex.

We show here that Gde2 is expressed in postmitotic cortical neurons throughout the period of cortical neurogenesis. By analyzing conditional and conventional *Gde2*$^{-/-}$ mutant mice, we find that Gde2 is required for the generation of cortical neurons of all layers; however, removal of Gde2 prior to the onset of neurogenesis causes progenitor cells to remain in the cell cycle and delays their exit to generate postmitotic neurons. This phenomenon results in the reduction of deep-layer cortical neurons and the expansion of superficial neurons. Mechanistically, Gde2 ablation increases Notch signaling at earlier developmental time points, suggesting that elevated levels of Notch causes cells to remain proliferative for longer, thereby changing their competence in terms of neuronal fate. We also observe a burst of cell-cycle exit of cortical progenitors in *Gde2*$^{-/-}$ animals towards the end of neurogenesis although Notch signaling appears to be unchanged. These observations suggest that Gde2 in postmitotic cortical neurons forms a feedback mechanism that works in concert with cell-intrinsic mechanisms to control the timely progression of cell-cycle exit of cortical progenitors. Moreover, loss of Gde2 uncovers a second mechanism that triggers the differentiation of existing neuronal progenitors prior to the initiation of gliogenesis. Taken together, our data suggest that cell extrinsic regulators are key components of the regulatory networks that ensure the generation of appropriate numbers and subtypes of cortical neurons during development.

MATERIALS AND METHODS

Animals and genotyping

Generation of *Gde2*$^{-/-}$ and *Gde2*^{lox} mice and genotyping was performed as described previously (Sabharwal et al., 2011). All animal procedures were carried out according to Johns Hopkins University IACUC guidelines. Wild-type (WT) and mutant *Gde2*$^{-/-}$ alleles were identified using the following primers: 5′-CAGTCTCTGCCCAAAGCTACTGT-3′, 5′-TCCACATACTTCCCGCATCCTCAT-3′. The 1.4-kb WT and 700-bp *Gde2* mutant products were resolved on high resolution agarose gels. The *Gde2*^{lox} and WT alleles were identified using the following primers: 5′-TCAGTCTCTGCCCAAAGCTACTGT-3′, 5′-TCTGGCTCCTTTGTGG-AGTTGCTA-3′.

Neuronal counts

Quantitative analyses of cortical excitatory neurons were performed on 25-30 μm-thick sections. Cell counts were performed on 200 × 200 μm fields of six to nine sections from four to six embryos in each instance using ImageJ software (NIH). The areas of quantification were stratified as medial or lateral, rostral or caudal, and the respective numbers of neurons quantified in each panel were averaged.

In situ hybridization

In situ hybridization experiments were carried out as described (Rao and Sockanathan, 2005). Bright-field images were captured on a Zeiss Axioskop2 microscope.

Immunofluorescence

Immunofluorescence experiments were carried out as described (Rao and Sockanathan, 2005). Confocal images were acquired with a Zeiss LSM 5 PASCAL microscope. The ApopTag fluorescein in situ apoptosis detection kit (Chemicon S7110) was used for terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) analysis. Rabbit C-terminal Gde2 polyclonal antibodies were generated against an 82 amino acid C-terminal His-fusion protein (Covance, 1:1000). Primary antibodies used were as follows: rabbit anti-Brn2 (1:500, Santa Cruz sc28594), rabbit anti-Cux1 (1:500, Santa Cruz sc13024), rat anti-Ctip2 (1:500, AbCam ab18465), rabbit anti-Tbr1 (1:500, AbCam ab31940), rabbit anti-Tbr2 (1:500, AbCam ab23345), rat anti-BrdU (1:100, AbCam ab6326), mouse anti-BrdU/IdU (1:50, BD Bioscience, 347580), rabbit anti-Ki67 (1:1000, AbCam, ab15580), rat anti-Pdgfr-α (BD-Bioscience 558774), guinea pig anti-Olig2 (1:40,000, T. M. Jessell, Columbia University, NY, USA), rabbit anti-Er81 (1:32,000, T. M. Jessell).

Cell-cycle analyses

Bromodeoxyuridine (BrdU; 100 mg/kg body weight) was injected intraperitoneally (i.p.) into pregnant female mice 1 hour prior to embryo harvest for estimation of S phase. To assess S phase, we calculated proliferation index as ($BrdU^+Ki67^+$)/$Ki67^+$ cells where Ki67 marks all cycling cells. To assess cell cycle exit, we injected BrdU i.p. to pregnant dams 18 hours prior to embryo harvest, and the cell-cycle exit index was calculated as ($BrdU^+Ki67^-$)/($BrdU^+Ki67^+$) (Siegenthaler et al., 2009).

EdU birthdating

Birthdating experiments were performed using 5-ethynyl-2′-deoxyuridine (EdU), a novel thymidine analog (Chehrehasa et al., 2009). A single pulse of EdU (50 mg/kg body weight) was injected i.p. into pregnant female mice at two relevant time points of the cortical neurogenic period [embryonic day (E) 12.5 or E15.5] and embryos were harvested at postnatal day (P) 5 for analyses. Detection of EdU was performed as described (Zeng et al., 2010) in conjunction with immunostaining using antibodies against specific laminar markers to estimate the birthdates of these neuronal populations.

4-OHT injections

4-Hydroxytamoxifen (4-OHT; Sigma) was dissolved in ethanol at a concentration of 20 mg/ml and was stored in aliquots at –80°C. Aliquots were emulsified in five volumes of sunflower seed oil, centrifuged under vacuum to remove the ethanol and delivered as a single i.p. injection (Badea et al., 2003).

Western blots

Cortices of E12.5-13.5 *Gde2*$^{-/-}$ mice and their WT littermates were dissected and lysed in lysis buffer (2% SDS in PBS). Proteins were quantified using the BCA Kit (Pierce) and separated by 10% SDS-PAGE in Tris-glycine buffer. The ratio of Notch1 intracellular domain (NICD1) to total Notch1 of *Gde2*$^{-/-}$ cortices was normalized to WT controls (detection of NICD1, 1:750, Cell Signaling Technology; Notch, 1:1000, Cell Signaling Technology). Blots were quantified using ImageJ (NIH) and statistical analysis of multiple litters was performed using paired one-tailed Student's *t*-test.

In utero electroporation

Procedures were performed as described by Mizutani and Saito (Mizutani and Saito, 2005). Briefly, timed pregnant dams (E13.5) were anesthetized by i.p. injection of avertin prior to injection of 0.5 μl of pCAGGS-eGFP or pCAGGS-dnMAML.eGFP (2.5 μg/μl) into the lateral ventricle of embryos. Electric pulses of 35 V for 50 mseconds were

delivered five times with 950-msecond intervals. Mice were sacrificed at E18.5 and embryonic brains were analyzed.

RESULTS

Gde2 is expressed in the developing cortex

To define the temporal and spatial expression of Gde2 in the developing cortex, we examined the distribution of Gde2 transcripts and protein from E11.5 to E17.5 when layer-specific pyramidal neurons are generated (Molyneaux et al., 2007). Throughout this period, low levels of *Gde2* mRNA were detected throughout the cortical plate; however, *Gde2* transcripts were highly enriched in the preplate and in postmitotic cortical neurons (Fig. 1A-C; data not shown). Gde2 protein expression was analyzed in WT mice using polyclonal antibodies directed against the C-terminal portion of the protein and specificity was confirmed by loss of antibody reactivity in *Gde2*$^{-/-}$ littermates (Fig. 1A′-C′,A″-C″) (Sabharwal et al., 2011). Gde2 protein showed a similar temporal and spatial distribution pattern to *Gde2* transcripts with enrichment of Gde2 in postmitotic neurons (Fig. 1A′-C′,D). Expression in the ventricular zone (VZ) is non-specific at E11.5 as antibody staining detected reactivity in this region in *Gde2*$^{-/-}$ cortices (Fig. 1A″); nevertheless, Gde2 protein is present in the VZ from E12.5 (Fig. 1B′-C″). At this later stage, the presence of Gde2 in the VZ area is likely to be a consequence of the distribution of Gde2 within axons of pyramidal neurons. Thus, the pattern of Gde2 expression in the developing cortex appears to be similar to that in the spinal cord in that Gde2 is excluded from VZ progenitors but is enriched within postmitotic neuronal cell bodies and axons.

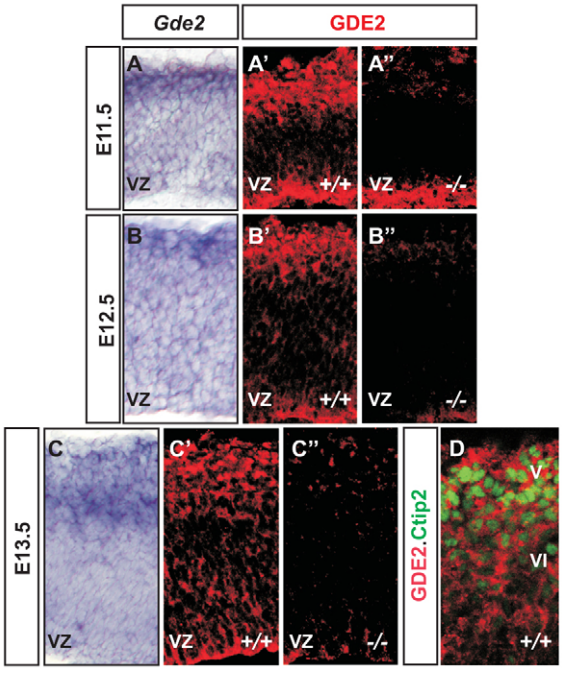

Fig. 1. *Gde2* expression in the cortex. (**A-C**) Sectioned mouse embryonic cortices showing the distribution of *Gde2* transcripts. (**A′-C″**,**D**) Sectioned mouse embryonic cortices from WT (+/+) and *Gde2*$^{-/-}$ (–/–) animals showing distribution of Gde2 protein in cortical neuronal cell bodies and processes using antibodies specific to Gde2. (D) Gde2 colocalizes with layer V and VI cortical neurons expressing high and low levels of Ctip2, respectively. Signal observed in the VZ in A″ is non-specific to Gde2.

Gde2 is required for the generation of deep-layer cortical neurons

Laminar-specific cortical neurons can be distinguished through their expression of distinct transcription factors that control cortical neuronal identities and their properties, such as cell body settling positions and axonal trajectory patterns (Molyneaux et al., 2007; Leone et al., 2008; Shoemaker and Arlotta, 2010). We utilized these molecular markers to gain insight into the requirement for endogenous Gde2 in layer-specific cortical neuronal differentiation by comparing their expression in *Gde2*$^{-/-}$ mice and control WT littermates. Animals were analyzed at two different developmental stages that approximate initial and late periods of cortical neuronal differentiation. At E13.5, *Gde2*$^{-/-}$ mutants showed an ~50% decrease of layer VI Tbr1^{+} neurons during the peak period of layer VI neurogenesis (Fig. 2A,A′,G). By E17.5, however, the numbers of Tbr1^{+} neurons had recovered to WT levels (Fig. 2G). At this stage, a subset of layer V neurons marked by ER81 (Etv1 – Mouse Genome Informatics) expression was found to be reduced in *Gde2*-null cortices by 20%, although overall numbers of Ctip2^{+} (Bcl11b–Mouse Genome Informatics) layer V neurons was unchanged, whereas there was a dramatic loss of *RORb*$^{+}$ layer IV neurons (Fig. 2B,B′,C,C′,H). Strikingly, *Gde2*$^{-/-}$ animals showed a robust expansion of layer II/III superficial neurons in both the marginal and intermediate zones (MZ and IZ; Fig. 2D,D′,E,E′,I). No gross changes in the overall radial thickness of the cortex at E17.5 were detected (*Gde2*$^{+/+}$ 523.88±27 μm; *Gde2*$^{-/-}$ 512.04±21 μm; Student's *t*-test P=0.645; n=5); however, the density of cells appeared to be marginally increased (Fig. 2D-F′). These observations suggest that Gde2 is required for the differentiation of deep-layer cortical neurons and raise the possibility that the expansion of layer II/III neurons is a consequence of delayed differentiation of cortical progenitors.

To test this hypothesis, we injected pregnant dams with BrdU prior to the peak periods of layer VI to layer II/III neurogenesis, and analyzed WT and *Gde2*$^{-/-}$ littermates 18 hours later to quantify the number of cycling progenitors that had exited the cell cycle in each case (Siegenthaler et al., 2009). Calculation of the proportion of BrdU-labeled cells that were postmitotic (see Materials and methods) showed that the number of cells exiting the cell cycle in *Gde2*$^{-/-}$ mutants at E12.5 was markedly decreased compared with WT controls, but no changes in cell-cycle exit were apparent at E13.5 or E14.5 (Fig. 3A-C′,E). However, at E15.5, there was a striking increase in the number of cells that had exited the cell cycle in *Gde2*$^{-/-}$ embryos relative to control littermates (Fig. 3D-E); this observation is consistent with the expansion of layer II/III neurons observed at E17.5. Similar results were obtained using Tbr2 (Eomes – Mouse Genome Informatics) expression to demarcate the subventricular zone (SVZ; supplementary material Fig. S1).

To relate the changes in cell-cycle exit with cell fate, we injected the nucleotide analog EdU into pregnant dams at either E12.5 or E15.5 to label cycling cells, and subsequently analyzed the identity of EdU^{+} neurons at P5 using molecular markers specific for the cortical layers. Consistent with the observed reduction in cell-cycle exit at E12.5, we found a substantial decrease of EdU^{+} Tbr1^{+} and EdU^{+} Ctip2^{+} deep-layer neurons in *Gde2*$^{-/-}$ animals injected at E12.5 compared with WT littermates (Fig. 3F-G′,H,L). By contrast, we detected a robust expansion in the number of EdU^{+} Cux1^{+} and EdU^{+} Brn2^{+} (Pou3f2 – Mouse Genome Informatics) layer II/III neurons in animals injected at E15.5, reflecting the increased levels of cell-cycle exit in *Gde2*-null animals observed at this time point (Fig. 3I-L).

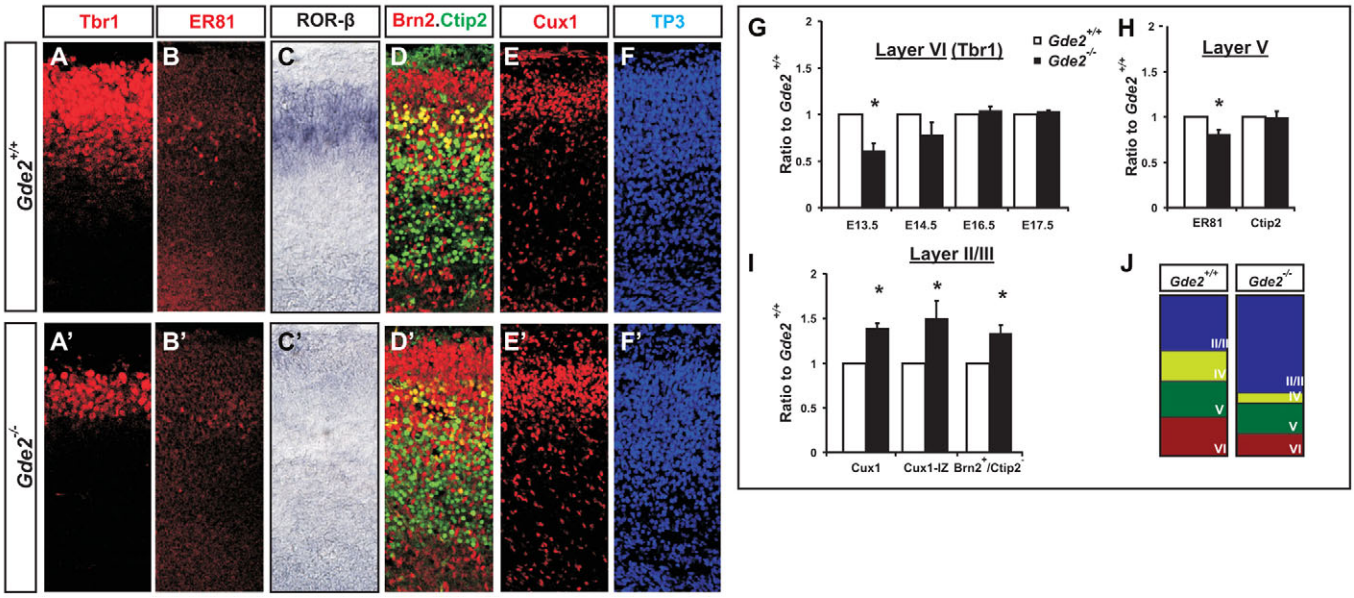

Fig. 2. Gde2 ablation results in layer-specific changes in cortical neurons. (**A-F′**) Sectioned mouse embryonic cortices showing representative images of layer-specific marker expression using immunohistochemistry, in situ hybridization (C,C′) and the nuclear stain TOPRO3 (TP3; F,F′). (**G-I**) Graphs of the ratio of deep and superficial neurons in *Gde2*$^{-/-}$ cortices and that of WT littermates from E13.5-17.5 (G) and at E17.5 (H,I). All graphs mean ± s.e.m. *n*=4-6. (G) **P*=0.04. (H) **P*=0.0034. (I) Cux1, **P*=0.0003; Cux1-IZ, **P*=0.041; Brn2^{+}/Ctip2^{-}, **P*=0.006. Student's two-tailed *t*-test. (**J**) Schematic of the changes in cortical neurons observed in WT and *Gde2*$^{-/-}$ animals with a different color representing each layer.

Taken together, these observations suggest that loss of Gde2 disrupts the timing of cortical progenitor differentiation such that there is a deficit in deep-layer cortical neurons and abnormal expansion of superficial layer II/III neuronal numbers. The marked increase in cell-cycle exit at E15.5 further implies that a separate Gde2-independent mechanism operates to drive the differentiation of cortical progenitors at later stages of development.

Gde2 ablation increases the cortical progenitor pool

To determine whether the decrease in deep-layer neurons followed by the expansion of superficial-layer neurons arises from changes in cell death, we performed TUNEL analyses in *Gde2*$^{-/-}$ mice and compared them with WT siblings. No changes in TUNEL were observed in *Gde2*$^{-/-}$ animals from E12.5 to E17.5, suggesting that the decrease in deep-layer neurons in these mutants arises from a delay or failure of neuronal differentiation and that the later increase in superficial neurons is due to increased cell-cycle exit of cortical progenitors (supplementary material Fig. S2). One prediction of this model is that progenitors that fail to exit the cell cycle on time remain cycling prior to their en masse differentiation initiating at E15.5. To test this possibility, we administered a 1-hour BrdU pulse to pregnant dams to label all cells in S phase, and quantified the total number of BrdU^{+} cells from E12.5 to E16.5 in *Gde2*-null and WT cortices (Chenn and Walsh, 2002). We observed a marked increase in the total number of BrdU^{+} cells in *Gde2*$^{-/-}$ cortices at E13.5 and E14.5 but no changes at either E12.5 or E16.5 (Fig. 4A-E). Tbr2^{+} BrdU^{+} cells were similarly elevated in these animals, suggesting that the total numbers of intermediate progenitor cells were also expanded at E13.5 and E14.5 in the absence of Gde2 (supplementary material Fig. S3) (Sessa et al., 2008). To determine whether progenitor cells showed increased proliferation in the absence of Gde2, we measured the ratio of cycling cells that were in S phase in *Gde2*$^{-/-}$ animals compared with WT animals (BrdU^{+}Ki67^{+}/Ki67^{+}). No changes in the S-phase index were observed at E13.5, but we detected an increase of the S-phase ratio in *Gde2*$^{-/-}$ animals at E14.5 indicative of increased proliferation of cortical progenitors (Fig. 4F).

Taken together, these results suggest that the loss of Gde2 leads to an increase in the overall number of cortical progenitors in the VZ and SVZ in the period between E12.5 and E15.5, when we observe decreased and increased cell-cycle exit respectively. Consistent with this observation, Ki67^{+} progenitors accumulate at E14.5 and are moderately elevated at E16.5 in *Gde2*$^{-/-}$ animals but appear comparable to WT littermates at E12.5 (supplementary material Fig. S4). As cortical progenitors differentiate into astrocytes after neurogenesis is complete, we examined whether Gde2 ablation affected postnatal astrocyte formation; however, no discernible changes in astrocyte numbers were detected between *Gde2*$^{-/-}$ and WT littermates at P0 (supplementary material Fig. S5).

Gde2 is required for superficial cortical neuron differentiation

Our results show that in the absence of Gde2, cortical progenitors accumulate at the time of deep-layer neuronal differentiation prior to initiating their belated differentiation during the time of superficial corticoneurogenesis. However, it is possible that progenitors that would normally give rise to superficial neurons also require Gde2 for their differentiation, but that this requirement is masked by the early phenotype of cortical progenitor accumulation.

To determine potential roles for Gde2 in the formation of layer II/III cortical neurons, we utilized a conditional ablation approach in which we administered 4-hydroxytamoxifen (4-OHT) to induce Cre-dependent removal of the Gde2 GDPD domain and 3′coding sequences in *R26CreER; Gde2*$^{lox/-}$ mice (Badea et al., 2003; Sabharwal et al., 2011). We injected pregnant dams with 4-OHT at E14.5 such that Cre-dependent removal of Gde2 occurs after the

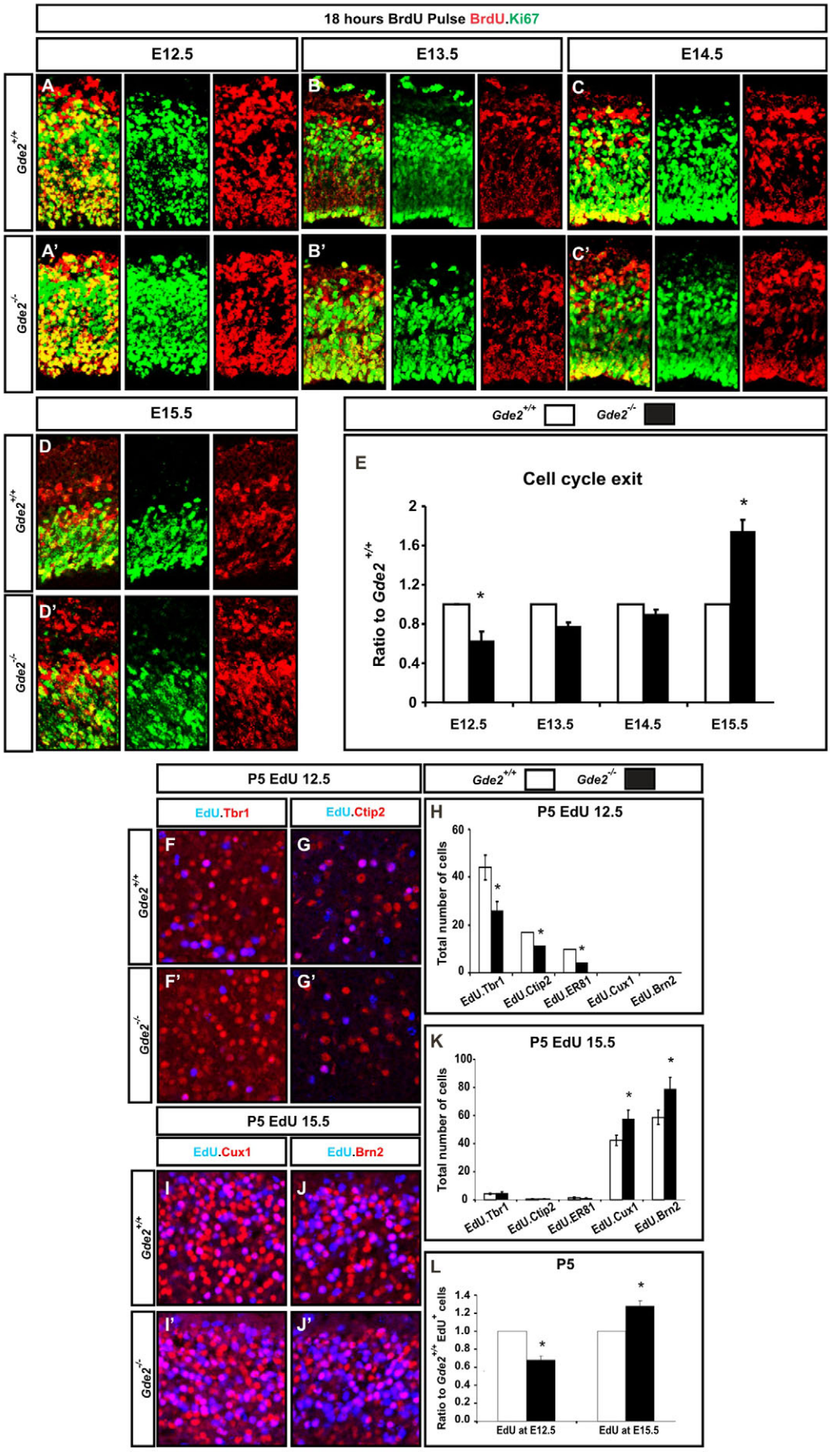

Fig. 3. Loss of Gde2 changes cell-cycle exit of prospective deep and superficial cortical neuronal progenitors. (**A-D'**) Representative confocal images of mouse embryonic cortices labeled by BrdU injection at different developmental stages and analyzed 18 hours later. (**E**) Graphs quantifying cell-cycle exit indices of *Gde2*$^{-/-}$ animals compared with WT littermates showing a reduction at E12.5 but an increase at E15.5. E12.5. **P*=0.02; E15.5, **P*=0.022, Student's *t*-test. (**F-G'**,**I-J'**) Confocal images of P5 cortices showing co-expression of EdU with layer-specific markers after EdU injection at either E12.5 or E15.5, to label deep and superficial cortical neurons, respectively. (**H**,**K**) Total number of EdU-labeled deep-layer and superficial-layer cortical neurons/section showing a decrease in deep-layer neurons born at E12.5 and an expansion of superficial neurons born at E15.5 in *Gde2*$^{-/-}$ animals. (H) EdU.Tbr1^{+}, **P*=0.0018; EdU.Ctip2^{+}, **P*=0.0026; EdU.ER81^{+}, **P*=0.00053; Student's *t*-test. (K) EdU.Cux1^{+}, **P*=0.012; EdU.Brn2^{+}, **P*=0.033; Student's *t*-test. (**L**) Graph of the ratios of EdU-labeled cells in *Gde2*$^{-/-}$ P5 cortices compared with WT showing overall decrease of neurons born in *Gde2*$^{-/-}$ animals at E12.5, but an increase of neurons born in these animals at E15.5. EdU E12.5, **P*=0.04; EdU E15.5, **P*=0.038; Student's *t*-test. All graphs are mean±s.e.m., *n*=4-6.

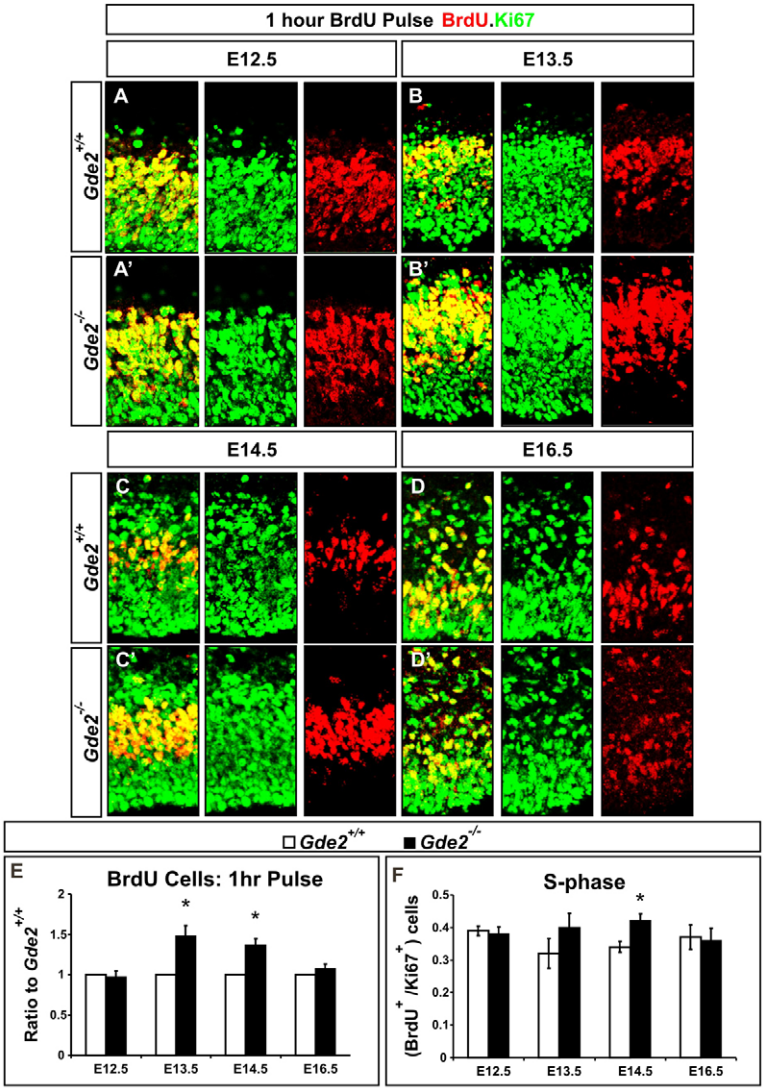

Fig. 4. *Gde2*$^{-/-}$ mutants have increased numbers of cycling cortical progenitors. (**A-D′**) Representative images of mouse embryonic cortices labeled for 1 hour with BrdU showing increased numbers of cycling progenitors in the absence of Gde2. (**E**) Graph of the ratio of BrdU$^+$ cycling cells in *Gde2*$^{-/-}$ mutants compared with WT littermates shows an increase of BrdU$^+$ cycling progenitors at E13.5 and E14.5. E13.5, **P*=0.023; E14.5, **P*=0.0092; Student's *t*-test. (**F**) Calculation of S-phase indices (BrdU$^+$Ki67$^+$/Ki67$^+$) show an increase in the proportion of cycling cells in S-phase at E14.5. **P*=0.034; Student's *t*-test. The lack of significance at E13.5 might reflect the overall increase in the number of cycling cells observed in *Gde2*$^{-/-}$ mutants, which could potentially obscure moderate changes. All graphs are mean±s.e.m., *n*=4-6.

differentiation of deep-layer cortical neurons but prior to the peak period of layer II/III cortical neurogenesis. 4-OHT administration effectively ablated Gde2 as very little Gde2 protein could be detected in *R26CreER; Gde2*$^{lox/-}$ cortices at E17.5 compared with *R26CreER; Gde2*$^{+/-}$ controls (Fig. 5A,A′). No changes in layer VI and layer V neurons were detected, consistent with the timed ablation of Gde2 after the bulk of deep-layer neuronal differentiation has transpired (Fig. 5D-E). However, we observed a 40-50% decrease of Cux1$^+$ and Brn2$^+$ layer II/III neurons in *R26CreER; Gde2*$^{lox/-}$ cortices compared with controls (Fig. 5B-C′,E). These observations suggest that Gde2 is required to regulate the differentiation of neurons that contribute to all cortical layers.

Gde2 ablation results in elevated Notch signaling in cortical progenitors

To investigate the potential signaling pathways that Gde2 regulates to control the timing of neuronal differentiation in the cortex, we focused on the Notch pathway for the following reasons. First, Notch signaling plays central roles in maintaining undifferentiated cortical progenitor pools (Corbin et al., 2008; D'Souza et al., 2010) and second, transient expression of constitutively active Notch at early neurogenic stages causes an increase of superficial-layer neurons, similar to the *Gde2*$^{-/-}$ phenotype (Mizutani and Saito, 2005). To determine whether Gde2 controls the differentiation of cortical progenitors through inhibition of Notch signaling, we analyzed the expression of *Hes5* and *Blbp*, two direct targets of Notch that are induced upon binding of the Notch intracellular domain (NICD) and CSL transcription factors (Iso et al., 2003; Anthony et al., 2005). Examination of *Gde2*$^{-/-}$ and WT cortices at E11.5 prior to the initiation of cortical neurogenesis showed no differences in *Hes5* and *Blbp* (*Fabp7* – Mouse Genome Informatics) expression (Fig. 6A-B′). However, we observed a robust upregulation of *Hes5* and *Blbp* at E12.5, and of *Blbp* in E13.5 *Gde2*$^{-/-}$ mutants compared with WT controls, corresponding to the peak of layer VI neuronal generation and the onset of differentiation of layer V and IV neurons (Fig. 6C-F′). No changes were detected in *Hes5* and *Blbp* expression from E14.5 to E16.5 (Fig. 6G-H′; supplementary material Fig. S6). Our results showed that Gde2 is indeed required for the differentiation of superficial-layer neurons, a requirement that is obscured by the accumulation of cycling progenitors in *Gde2*$^{-/-}$ animals (Fig. 5). Analysis of *R26CreER; Gde2*$^{lox/}$ cortices at E17.5 after 4-OHT injection at E14.5 revealed elevated levels of *Blbp* expression in these animals compared with *R26CreER; Gde2*$^{+/-}$ controls (supplementary material Fig. S6). These collective observations indicate that loss of Gde2 results in elevated Notch signaling in cortical progenitors.

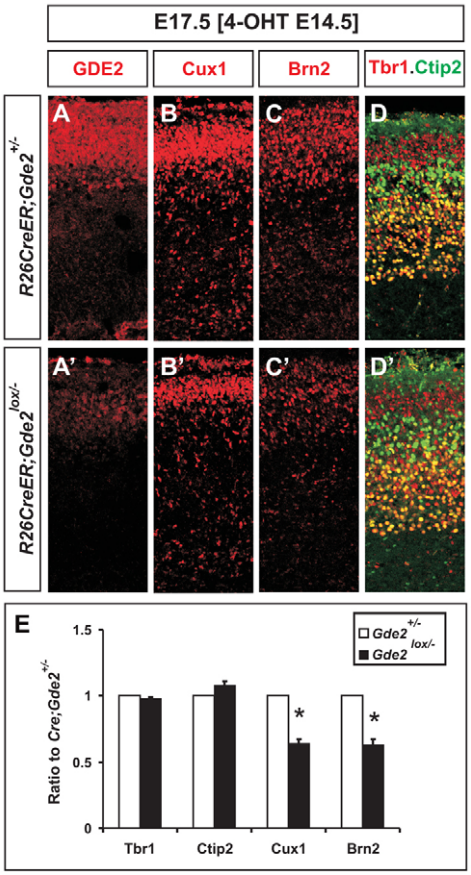

Fig. 5. Gde2 is required for the generation of superficial-layer neurons in the cortex. (**A-D′**) Representative confocal images of mouse embryonic cortices showing effective ablation of Gde2 protein by tamoxifen (4-OHT) administration and specific decreases in superficial neuronal numbers. (**E**) Graph quantifying layer-specific cortical neurons after timed ablation of Gde2 from E14.5. No changes in neurons born before tamoxifen administration were detected in *R26CreER;Gde2*$^{lox/-}$ animals compared with controls; however, substantial decreases in layer II/III neurons were detected. Cux1, **P*=0.00039; Brn2, **P*=0.0038; Student's *t*-test. Graphs show mean±s.e.m., *n*=4-6.

However, the lack of Notch perturbation in the absence of Gde2 after E15.5 is consistent with the model that the increase in cell-cycle exit in conventional *Gde2*$^{-/-}$ mutants does not occur through Gde2-mediated changes in Notch signaling but instead transpires through separate mechanisms.

Binding of ligand to the Notch receptor triggers a series of cleavage events that releases NICD from the membrane. The NICD is transported to the nucleus where it interacts with co-factors, such as Maml, to regulate transcription of its target genes (Corbin et al., 2008; D'Souza et al., 2010). Western blot analysis of cortical extracts derived from *Gde2*$^{-/-}$ animals compared with extracts prepared from WT littermates showed that *Gde2*$^{-/-}$ cortices contained higher levels of NICD than did WT animals, consistent with elevated Notch signaling (Fig. 6I). We reasoned that blockade of Notch signaling in *Gde2*$^{-/-}$ cortices should prevent the delay of cell-cycle exit of deep-layer neurons and the subsequent expansion of layer II/III neurons observed in the absence of Gde2. We therefore carried out in utero electroporation using constructs expressing either GFP or a dominant-negative version of Maml (dnMAML) to block Notch signaling in *Gde2*$^{-/-}$ cortices at E13.5 and examined the distribution of GFP^{+} neurons at E18.5. Cells expressing GFP alone in *Gde2*$^{-/-}$ cortices were located in layers superficial to Ctip2^{+} layer V neurons, whereas a small number of GFP^{+} cells remained in the VZ (Fig. 6J). These observations are consistent with our earlier results showing that in the absence of Gde2, progenitor cells remain proliferative and exit later to form layer II/III neurons. By contrast, many GFP^{+} cells expressing dnMAML in *Gde2*$^{-/-}$ cortices were positioned in layers V and VI, and some co-expressed Ctip2, implying that they had exited the cell cycle at appropriate times to acquire deep-layer neuronal identities. Reinforcing this notion, few, if any, dnMAML-expressing cells were found in the VZ of *Gde2*$^{-/-}$ cortices (Fig. 6K). These collective observations suggest that blockade of Notch signaling by dnMAML restores deep-layer neuronal generation and prevents the expansion of superficial-layer neurons in *Gde2*$^{-/-}$ animals.

DISCUSSION

Cortical neuronal diversity and number is regulated at the time of cell-cycle exit, and arises in part from cell-intrinsic clock-type mechanisms (Shoemaker and Arlotta, 2010; Qian et al., 2000; Shen et al., 2006). We show here that extrinsic regulators such as Gde2 are key players in the temporal control of neurogenesis, suggesting that they work in concert with intrinsic mechanisms to ensure timely cell-cycle exit and the generation of appropriate numbers of layer-specific neurons. Our study suggests a model in which Gde2 expressed in newly born cortical neurons forms a feedback signal to downregulate the level of Notch signaling in adjacent cortical progenitors, thereby inducing their differentiation (Fig. 7). We show by timed ablation of Gde2 that Gde2 is required for the generation of neurons that occupy all layers of the cortex; however, loss of Gde2 at early developmental stages causes a buildup of cycling progenitors that ultimately differentiate to generate increased numbers of superficial neurons at the expense of deep-layer neurons. Our observations suggest that a second mechanism operates to force the differentiation of remaining cycling neuronal progenitors prior to gliogenesis; however, this mechanism appears to be independent of Gde2-mediated downregulation of Notch signaling (Corbin et al., 2008).

Gde2 and the temporal control of cortical neurogenesis

Signals emanating from cortical neurons are known to influence progenitor differentiation primarily during the neuronal to glial switch; for example, cardiotrophin and Notch ligands are expressed in cortical neurons but regulate the temporal initiation of gliogenesis in progenitor cells relative to the cessation of neurogenesis (Barnabé-Heider et al., 2005; Namahira et al., 2009). More recent studies show that the neuron-specific transcription factor Sip (Sipd1 – Mouse Genome Informatics) regulates cortical neuronal fates by preventing premature initiation of neurogenesis and gliogenesis; however, loss of Sip does not alter cell-cycle parameters (Seuntjens et al., 2009). Our study now shows that cortical neurons can regulate progenitor cell differentiation by controlling the timing of cell-cycle exit, which directly impacts the generation and numbers of layer-specific cortical neurons. Specifically, analyses of *Gde2*$^{-/-}$ and *Gde2*$^{lox/-}$ conditional mice in which Gde2 is ablated after deep-layer neurons have formed, show that Gde2 is required for the differentiation of neurons that occupy both deep and superficial layers of the cortex. Interestingly, the

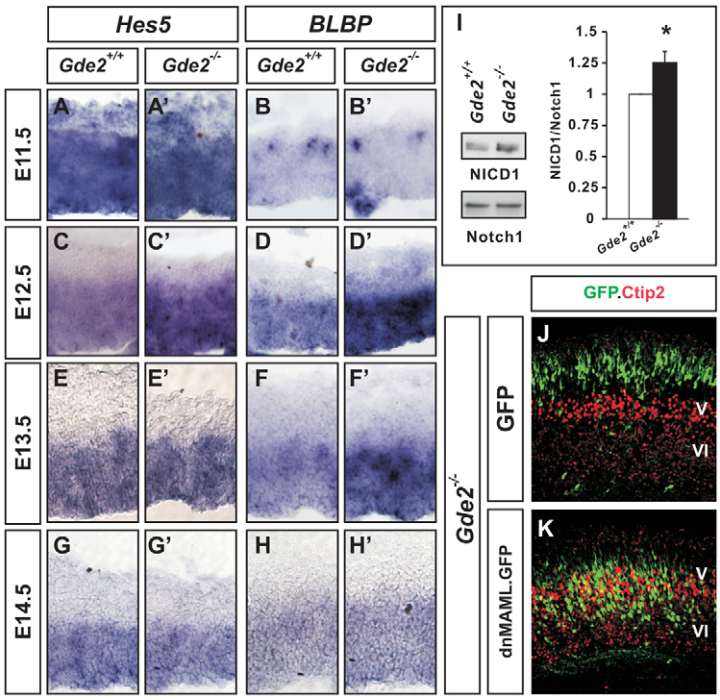

Fig. 6. Loss of Gde2 leads to increased Notch target gene expression. (**A-H′**) Representative images of sectioned mouse cortices showing distribution of *Hes5* and *Blbp* transcripts throughout development. Increased Notch target gene expression is observed in *Gde2*$^{-/-}$ cortices at E12.5 and E13.5. (**I**) Representative western blot showing increase of NICD in *Gde2*$^{-/-}$ cortices. Graph shows densitometric quantification of NICD levels normalized to total Notch in *Gde2*-null and WT cortices as detected by western blot. **P*=0.039, one-tailed Student's *t*-test; mean±s.e.m., *n*=3 litters (5-6 embryos total). (**J**,**K**) Representative images of sectioned E18.5 *Gde2*$^{-/-}$ cortices that were electroporated with plasmids expressing GFP alone (J) or dnMAML.GFP (K) at E13.5 during the peak period of deep-layer neuronal differentiation. Ctip2^{+} cells (red) mark layers V and VI.

earliest born neurons that populate layer VI appear the least affected by the loss of Gde2; we observe that some Tbr1^{+} neurons are generated normally in the absence of Gde2 although the differentiation of subsets of Tbr1^{+} neurons is delayed in *Gde2*$^{-/-}$ animals. This observation parallels that of the spinal cord in which loss of Gde2 is not required to generate early-born MMC neurons but is necessary for the timing and generation per se of LMC motor pools (Sabharwal et al., 2011). Thus, the logic behind Gde2 function appears to be conserved between the cortex and spinal cord in that early-born neurons differentiate by Gde2-independent mechanisms to establish a neuronal source of Gde2 that in turn acts non-cell-autonomously to regulate subsequent progenitor differentiation. Consistent with this model, we observe reduced numbers of later-born layer V and IV neurons in *Gde2*$^{-/-}$ animals. These deficits arise from the reduced cell-cycle exit that we observe at E12.5 followed by an accumulation of cycling progenitor cells, which occurs during the peak periods of layer V and layer IV neuronal differentiation between E12.5 and E15.5. Our efforts to determine whether the length of the cell cycle is altered in the absence of Gde2 using a well-established paradigm involving spaced consecutive injections of IdU and BrdU showed no differences in cell-cycle length between *Gde2*$^{-/-}$ and WT animals (M.R., J.C., S.P. and S.S., unpublished observations) (Georgala et al., 2011). A caveat of this approach is that it assumes that all progenitors share uniform cell-cycle parameters, which might not be the case in *Gde2*$^{-/-}$ cortices given the changes in cell-cycle exit in these animals that are superimposed on normal variations of cell-cycle length over time. Nevertheless, the changes in cell-cycle kinetics resulting from the loss of Gde2 ultimately results in the altered composition of layers V, IV and II/III, and although layer VI neurons recover in numbers by E17.5, the delay in their formation might negatively impact the formation of connections with neighboring pyramidal neurons or incoming interneurons (Rakic, 2007; Miyoshi and Fishell, 2011). Our study thus shows that Gde2 is important for the generation of the cytoarchitecture of the cortex through its function in regulating the timing of cortical neurogenesis. The identification of molecular markers that discriminate between subpopulations of layer-specific cortical neurons will help determine whether Gde2 regulates the differentiation of specific subclasses of neurons within a particular layer.

Notch downregulation, progenitor competence and neuronal fates

In cortical neurons, Gde2 is localized in cell bodies and along neuronal processes that contact cells in the VZ and SVZ, thereby facilitating non-cell-autonomous downregulation of Notch signaling in cortical progenitors. Gde2 ablation results in increased Notch signaling in progenitors at E12.5 and E13.5, which coincides precisely with when we observe a decrease in cell-cycle exit and an accumulation of cycling cortical progenitors in *Gde2* mutant animals. These observations suggest that abnormally high levels of Notch signaling at these time points retain progenitors in a proliferative state, and delay or prevent their differentiation (Corbin et al., 2008; Gaiano and Fishell, 2002). Instead, cortical progenitors continue cycling, biasing their competence towards superficial neuronal fates. This model is strongly supported by in utero electroporation studies in which constitutively active versions of Notch (caNotch) were transiently expressed in cortical progenitors from E13.5 to E15.5 (Mizutani and Saito, 2005). Progenitors that expressed caNotch lost the ability to generate deep-layer neurons but instead differentiated into layer II/III neurons when caNotch was removed. This phenotype is strikingly similar to the phenotypes observed in *Gde2*$^{-/-}$ mutants. Does elevated Notch signaling arising from Gde2 ablation during early corticoneurogenesis have other consequences on cortical development? Exposure of early cortical progenitors to Notch is necessary for the expression of NF1A (Nfia – Mouse Genome Informatics), which specifies astroglial fates, raising the possibility that there is increased astrocyte development in *Gde2*$^{-/-}$ animals (Namahira et al., 2009; Deneen et al., 2006; Gaiano and Fishell, 2002). However, we do not observe any differences in Gfap or

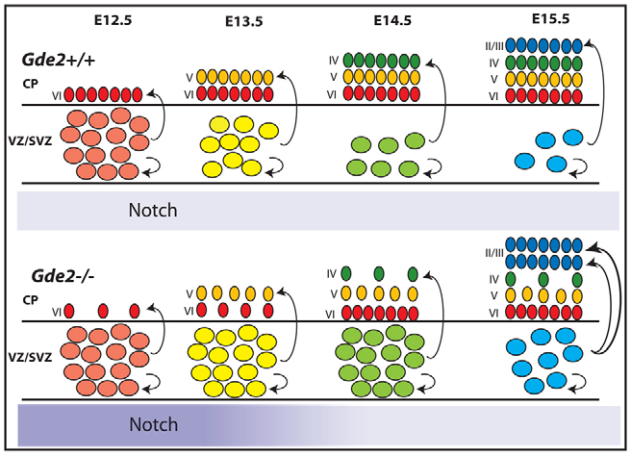

Fig. 7. Model of Gde2 function in cortical differentiation. In WT animals (*Gde2*$^{+/+}$), progenitors in the ventricular and subventricular zones (VZ/SVZ) proliferate and exit the cell cycle to generate deep and superficial neurons in sequence. Late-born neurons migrate past early-born neurons to settle in the superficial layers such that the laminar structure of the cortex is generated in an 'inside-out' manner. In the absence of Gde2 (*Gde2*$^{-/-}$), the generation of layer VI neurons is delayed as a consequence of elevated Notch signaling, leading to an accumulation of cycling progenitors. The increase in the number of cell cycles experienced by cycling progenitors as a consequence of elevated Notch results in altered competence to generate subsequent layers such that there is a deficit in early-born cortical neurons. However, progenitors are able to exit the cell cycle at the time of layer II/III neurogenesis such that there is a corresponding expansion of layer II/III neurons. We note that progenitors that normally give rise to layer II/III neurons also require Gde2 for timely exit of the cell cycle. These cells probably exit later than normal to generate layer II/III cortical neurons.

S100β staining in *Gde2* mutant cortices at early postnatal stages, suggesting that Notch-dependent astrogliogenesis operates through mechanisms independent of Gde2.

We observe that cortical progenitors exhibit a burst in cell-cycle exit towards the end of the neurogenic period in *Gde2*$^{-/-}$ mutants, which leads to a dramatic expansion of layer II/III neurons. Interestingly, this increase in differentiation is not accompanied by changes in Gde2-mediated Notch signaling reflected by *Hes5* and *Blbp* expression. Why is neurogenesis increased at these time points and what are the mechanisms that are involved? We speculate that normally, progenitors that are specified towards a neuronal fate are forced to exit the cell cycle at the end of the neurogenic period by a mechanism that as yet remains undefined. We suggest that such a control mechanism is unmasked in *Gde2* mutants owing to the abnormal accumulation of cycling cortical progenitors in these animals, which allows changes in cell-cycle exit to be detected by conventional means. The mechanistic nature of the burst in cell-cycle exit is unclear but could involve modulation of extrinsic signaling pathways or, alternatively, could arise if progenitors normally undergo a finite number of cell cycles once specified down a neuronal lineage.

Conclusions

Our study suggests that cortical neurons can regulate cortical neuronal diversity through feedback mechanisms that regulate the timing of neurogenesis. Our observations identify Gde2 as a crucial regulator of cortical neuronal differentiation by its ability to control the timing of neurogenesis through downregulation of Notch signaling in cortical progenitors. Loss of Gde2 alters progenitor cell-cycle parameters such that they ultimately lead to changes in progenitor competence and, accordingly, to imbalances in layer-specific cortical neurons. Defining the targets of Gde2 function will be essential for deciphering the pathways that directly link extrinsic control of cell-cycle exit with intrinsic programs that regulate neurogenesis.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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