Development 129, 5029-5040 (2002)
Printed in Great Britain © The Company of Biologists Limited 2002
DEV9867

Modulation of the notch signaling by *Mash1* and *Dlx1/2* regulates sequential specification and differentiation of progenitor cell types in the subcortical telencephalon

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Accepted 12 August 2002

SUMMARY

Notch signaling has a central role in cell fate specification and differentiation. We provide evidence that the *Mash1* (bHLH) and *Dlx1* and *Dlx2* (homeobox) transcription factors have complementary roles in regulating Notch signaling, which in turn mediates the temporal control of subcortical telencephalic neurogenesis in mice. We defined progressively more mature subcortical progenitors (P1, P2 and P3) through their combinatorial expression of MASH1 and DLX2, as well as the expression of proliferative and postmitotic cell markers at E10.5-E11.5. In the absence of *Mash1*, Notch signaling is greatly reduced and 'early' VZ progenitors (P1 and P2) precociously acquire SVZ progenitor (P3) properties. Comparing the molecular phenotypes of the delta-like 1 and *Mash1* mutants, suggests that *Mash1* regulates early neurogenesis through Notch- and Delta-dependent and -independent mechanisms. While *Mash1* is required for early neurogenesis (E10.5), *Dlx1* and *Dlx2* are required to downregulate Notch signaling during specification and differentiation steps of 'late' progenitors (P3). We suggest that alternate cell fate choices in the developing telencephalon are controlled by coordinated functions of bHLH and homeobox transcription factors through their differential affects on Notch signaling.

Key words: *Mash1*, *Dlx2*, *Dll1*, Telencephalon, Notch signaling, LGE, Striatum, Radial glia, Neurogenesis, Mouse

INTRODUCTION

Early development of the central nervous system (CNS) involves specification of progenitor cells to generate the anlagen of CNS subdivisions and precursors of distinct cells types (Jessell, 2000; Briscoe and Ericson, 2001; Marin and Rubenstein, 2002). From a given progenitor zone, different types of neurons and glia are generated over time. Temporal control of this process regulates the sequential production of different classes of neurons that migrate to the mantle zone where they contribute to forming layered or nuclear neuronal assemblies. The time when a progenitor cell produces a neuron is tightly linked to the properties of that neuron. For example, in the cerebral cortex, early-born neurons form the deep cortical layers whereas late-born neurons form the superficial cortical layers (Chenn et al., 1997). In the nuclear structures of the subcortical telencephalon (subpallium), such as the striatum, neuronal birthrate is coupled to the production of different subdivisions such as the patches and matrix (van der Kooy and Fishell, 1987).

Several lines of evidence suggest that the timing of cell fate specification and differentiation in the vertebrate nervous system is regulated through a process of lateral inhibition mediated by Notch signaling (Chitnis and Kintner, 1996; Henrique et al., 1997; Lewis, 1996). Notch is a cell-surface receptor that is activated by contact with a DSL ligand (*Delta, Serrate, Lag2*). In mammals, there are four Notch genes (*Notch1-Notch4*) and five DSL ligand genes (*Dll1, Dll3, Dll4, Jag1* and *Jag2*) (Lindsell et al., 1996). Ligand-induced Notch signaling involves proteolytic cleavage of Notch, which releases its intracellular domain (Notch-IC) and allows its translocation to the nucleus. Notch-IC directly modulates the function of a transcription factor known as CSL [*CBF1* (*Rbpsuh* – Mouse Genome Informatics) *Suppressor of Hairless* (*SuH*), *Lag1*]. During neurogenesis, Notch activation of CSL induces the expression of WRPW-bHLH transcription factors that inhibit neuronal differentiation (e.g. HES genes or *Drosophila Enhancer of Split*) and represses the expression of proneural bHLH transcription factors (e.g. *Mash1* or *Achaete-Scute* in

Drosophila) (Artavanis-Tsakonas et al., 1995; de la Pompa et al., 1997; Robey, 1997). Thus, an increase in Notch signaling within a progenitor cell biases it not to differentiate, whereas a decrease in Notch signaling facilitates its maturation.

There are several classes of proneural bHLH genes expressed in the mammalian telencephalon, including an *Achaete-Scute* homolog *Mash1* (*Ascl1* – Mouse Genome Informatics), *atonal* homologs neurogenin 1, neurogenin 2 and *Olig2* (Ma et al., 1997; Fode et al., 2000; Takebayashi et al., 2000; Nieto et al., 2001). *Mash1* is primarily expressed in the progenitor zones of the subcortical telencephalon (Lo et al., 1991; Guillemot and Joyner, 1993; Porteus et al., 1994), where in mice it is required for the generation of early-born neurons (Casarosa et al., 1999; Horton et al., 1999). Casarosa et al. (Casarosa et al., 1999) showed that *Mash1* is required for the expression of *Dll1* and *Dll3* at E12.5, and therefore Notch signaling (and *Hes5* expression) is reduced in the *Mash1* mutants. Their BrdU birthdating analysis demonstrates that early-born neurons (postmitotic at ~E10.5) are reduced in the subcortical telencephalon. However, Horton et al. (Horton et al., 1999) found that the subcortical telencephalon in *Mash1* mouse mutants exhibits precocious expression of a marker of early neurogenesis (β-III-tubulin) at E11.0. These apparently contradicting observations could reflect cell-autonomous and non cell-autonomous affects of *Mash1* functions. To test this possibility, we have compared the phenotypes of the *Mash1* and *Dll1* mutants at E10.5.

Although *Mash1* is required for early neurogenesis in the subcortical telencephalon (Casarosa et al., 1999; Horton et al., 1999), the *Dlx1* and *Dlx2* homeobox genes are required in mice for late neurogenesis in the same tissue (Anderson et al., 1997a). DLX1 and DLX2 are co-expressed in subsets of progenitor cells (Eisenstat et al., 1999) where they have partially redundant functions in controlling the differentiation of a secondary proliferative zone called the subventricular zone (SVZ) (Anderson et al., 1997a). In the *Dlx1/2* mutants, late-born neurons fail to mature fully; however, when the mutant SVZ cells are dissociated and grown in culture, they are able to proceed at least partially along their differentiation program (Anderson et al., 1997a). These observations point to the possibility that the block in neurogenesis may involve misregulation of cell-cell communication, perhaps through the Notch signaling pathway.

To gain insights into the temporal regulation of progenitor cell fate specification in the developing telencephalon at the cellular level, we have examined the effect of *Mash1*, *Dll1* and *Dlx1/2* mutations on Notch signaling and differentiation. We provide evidence that: (1) *Dll1* mediates Notch signaling prevents precocious differentiation of progenitors; (2) *Mash1* has a cell-autonomous function in the development of a subset of early telencephalic progenitors and a non-cell autonomous function in mediating lateral inhibition through positively regulating Notch signaling; (3) *Dlx1* and *Dlx2 n*egatively regulate Notch signaling to properly specify a later subset of neuronal progenitors and promote their terminal differentiation.

MATERIALS AND METHODS

Mice

Wild-type and mutant mice were cared for according to procedures of the UCSF Committee on Animal Research. Animals were sacrificed using cervical dislocation. Wild-type (CD1 and C57Bl6), *Mash1* mutant (Guillemot et al., 1993) and *Dlx1/2* mutant (Qiu et al., 1997) mice were bred at UCSF. The mutant mice were out-crossed with C57Bl/6 mice. *Dll1* mutants (Hrabe de Angelis et al., 1997) were bred in the laboratory of M. Hrabe de Angelis. The morning of the vaginal plug was considered E0.5. Embryos were collected in phosphate-buffered saline (PBS) and fixed overnight in 4% paraformaldehyde. Embryos were then run through a sucrose/PBS gradient (10% and 20%) and embedded in OCT (Tissue-Tek) for cryostat sectioning. Sections were collected at 10 μm and adjacent sections were placed on different slides for comparing gene expression patterns.

In situ RNA hybridization

cDNA plasmids used for in situ hybridization analysis were obtained from the following people: Gerry Weinmaster (*Notch1*, *Notch3*, *Dll1*, *Jag1*, *Hes1* and *Hes5*), Brian Crenshaw (*Brn4*; *Pou3f4* – Mouse Genome Informatics), Ming Tsai *(COUP-TF1)*, Brian Condie (*Gad67*; *Gad1* – Mouse Genome Informatics), Charles Gerfen [dopamine receptor 2 (*D2r*; *Drd2* – Mouse Genome Informatics)], Francois Guillemot (*Mash1*), Steve Potter (*Gsh2*), Heiner Westphal (*Lhx2*). *Dlx2*, *Dlx5* and *Dlx6* are from the Rubenstein laboratory (Liu et al., 1997). In situ RNA hybridization on frozen sections was carried out using ^{35}S-labeled antisense-riboprobes as described (Sussel et al., 1999). Photographs were taken using darkfield optics on an Olympus SZH10 microscope.

Immunofluorescence

Production and characterization of the DLX2 antibody is described (Eisenstat et al., 1999; Porteus et al., 1994). The mouse monoclonal MASH1 antibody was a gift from Dr David Anderson and was also purchased from Pharmingen (Cat #556604). Rabbit polyclonal MASH1 antibody was generated in Dr Jane Johnson's laboratory.

Phosphorylated histone 3 (PH3) (rabbit IgG; Upstate Biotech), MAP2 (mouse IgG; Sigma: #M1406), GABA (rabbit IgG; Sigma #A2052), RC2 (mouse IgM; Developmental Studies Hybridoma Bank), PCNA (mouse IgG; Novocastra), GAD65(mouse IgG; Pharmingen # 69221A), β-III-tubulin (mouse IgG; Promega: #G7121), GFAP (rabbit IgG; Sigma). For MASH1 and PCNA doubling-labeling, the sections were first treated in boiling 10 mM sodium citrate before blocking in 5% normal goat serum (Gibco)/phosphate buffered saline (PBS)/0.1%TritonX-100. Primary antibodies were diluted in the same buffer and incubated overnight at 4°C. The sections were rinsed in PBS, then incubated with secondary antibodies at 1:200-300 dilution for 1 hour at room temperature, then rinsed in PBS and finally were mounted with Vectashield mounting medium with DAPI (Vector Labs #H1200). The secondary antibodies were conjugated with either Alexa-488 or Alexa-596 (Molecular Probes). The fluorescent images were photographed or electronically captured with Spot II imaging camera using a Nikon Optiphot 2 microscope.

Primary cell culture

For acute dissociation of E10.5 or E11.5 wild type (CD1 and C57B6) subcortical cells, embryos were dissected in cold PBS, the mesenchyme surrounding the telencephalon was carefully removed and only subcortical region of the telencephalon was isolated (note it also includes some ventral and lateral pallial mantle cells). The tissue was trypsinized for 30 minutes at 37°C in 500 μl; an equal volume of neurobasal medium (Gibco) containing 10% fetal bovine serum (FBS; UCSF Cell Culture Facility) was added to arrest the trypsinization. The cells were then dissociated by tituration, rinsed twice in neurobasal medium with 1% FBS and plated at concentration of 1×10^5 cells/50 μl in a well coated with poly-D-lysine (Tissue-Tek II slide chambers, Nalgene). After the cells were allowed to adhere for 30-60 minutes, the medium was withdrawn and the cells were fixed in 4% paraformaldehyde for 10 minutes at room temperature. The cells were then rinsed in PBS and analyzed with antibodies as described above.

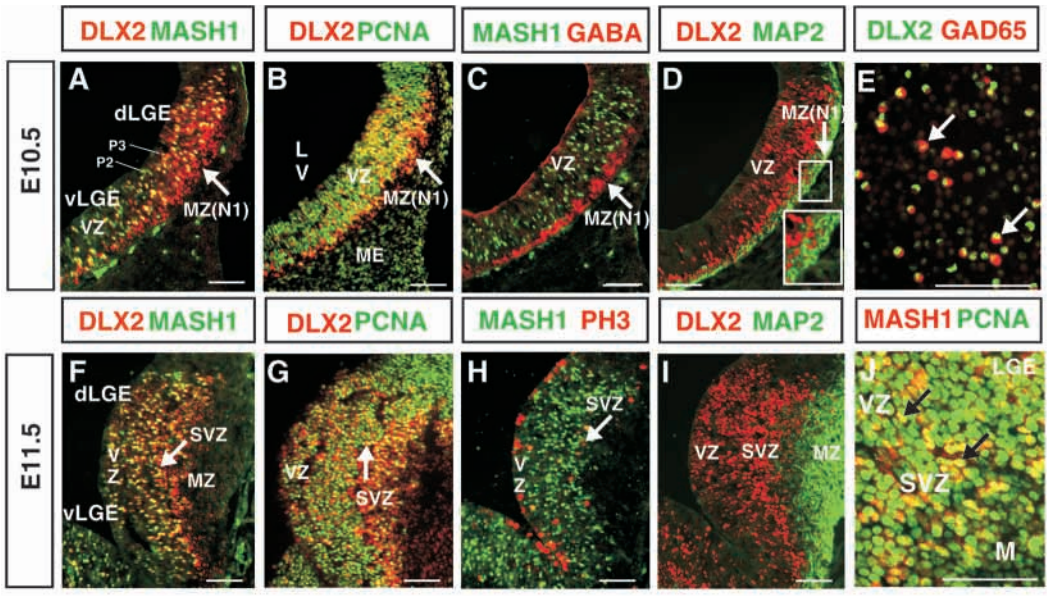

Fig. 1. Combinatorial expression of MASH1, DLX2 and markers of proliferation and differentiation provide evidence for distinct stages of cellular maturation in the lateral ganglionic eminence (LGE). Fluorescence microscopic images of wild-type subcortical cells stained with a pair of antibodies (differentially labeled with green or red fluorophors) that bind to the proteins indicated above each panel; yellow cells are double labeled. Coronal sections (10 μm) from E10.5 embryos (A-D) and E11.5 embryos (F-J) show the primordium of the LGE. (E) Dissociated cells prepared from E10.5 subcortical telencephalon (arrows indicate double-positive cells). The boxed areas in Fig. 2 show the approximate regions of the LGE shown in Fig. 1. PCNA labels proliferating cells, and thereby defines the location of the progenitor zones, whereas MAP2 and GABA label postmitotic neurons (arrow in C,D), and thereby define the position of the mantle zones. PH3 labels M-phase cells in the VZ and the SVZ (arrow in H). P1 progenitors (MASH1–/DLX2–) and P2 progenitors (P2, MASH1+/DLX2–) are only in the VZ. P3 progenitors (MASH1+/DLX2+) are in the VZ and SVZ. In A, P1 progenitors are unlabeled, P2 progenitors are green and P3 progenitors are yellow. N1 cells are the earliest born neurons; we propose that they are generated from P2 progenitors (see Fig. 7). N1 neurons are MAP2+ and continue to express DLX2 (see inset in D). The LGE progenitor zone can be divided into at least two parts along the dorsoventral axis: the dorsal LGE (dLGE) and the ventral LGE (vLGE) (Yun et al., 2001). dLGE neurogenesis precedes that of vLGE, and therefore may contribute to a major portion of the N1 population. Some MASH-/DLX2+ cells are observed in the VZ and SVZ (particularly in the dLGE); these are the red cells in the VZ in A. These cells might correspond to another type of progenitor. LV, lateral ventricle; ME, mesenchyme; MZ, mantle zone; VZ, ventricular zone; SVZ, subventricular zone. Scale bars: 100 μm. Arrows in J indicate examples of PCNA/Mash1 double-positive cells.

RESULTS

Combinatorial expression of MASH1 and DLX2 identify different populations of subcortical progenitors and early-born neurons

To gain insights into the temporal regulation of progenitor cell fate specification in the developing telencephalon, we characterized the molecular phenotypes of MASH1- and DLX2-expressing cells in neural progenitors and differentiating neurons of the LGE (lateral ganglionic eminence, the primordium of the striatum) at E10.5 and E11.5. At these ages, we detect three cell layers: (1) the pseudostratified epithelium of the ventricular zone (VZ) that contains progenitor cells and radial glia; (2) the subventricular zone (SVZ), which contains progenitors that are not organized as a pseudostratified epithelium; and (3) the mantle zone (MZ), which contains postmitotic neurons.

At E10.5, the VZ and MZ are apparent, whereas the SVZ has not appeared as a distinct layer. By E11.5, a layer of subventricular PH3+ cells (a M-phase marker) (Hendzel et al., 1997) are more coherently arranged, and can be distinguished as a SVZ (Fig. 1H), which persists as development proceeds (Sheth and Bhide, 1997).

Based on the expression of MASH1 and DLX2 with proliferative (PCNA and PH3), and neuronal (MAP2, GAD65) markers, we have defined different cell types in the VZ, SVZ and MZ (Fig. 1; also see legend to Fig. 7). Prior to E9.5, neuroepithelial cells in the LGE are MASH1–/DLX2–; we define these as Type 1 progenitors (P1).

Then, by ~E9.75 MASH1and DLX2 expression in the LGE is detectable in the VZ (Bulfone et al., 1993) (data not shown). MASH1+/DLX2– progenitors only appear in the VZ (Fig. 1A,F); we have named these Type 2 progenitors (P2).

MASH1+/DLX2+ cells are also only apparent in the progenitor layers (Fig. 1A,F); the MASH1+/DLX2+ nuclei are scattered within the VZ and are concentrated in the position of the SVZ (Fig. 1A,F). We have named these Type 3 progenitors (P3).

At E10.5, DLX2+ postmitotic cells (MAP2+, PCNA–) cells are found superficial to the VZ; we refer to these early born neurons as N_1 cells (Fig. 1A-D). All GAD65-expressing cells at E10.5 express DLX2 but not MASH1 (Fig. 1E, and data not shown). From E11.5, there are many postmitotic neurons in the MZ that are DLX2– and we refer to these late-born neurons as N2 cells (Fig. 1I).

In the following sections, we use this descriptive analysis as a foundation to interpret the roles of *Mash1* and *Dlx2* during early steps of cell fate specification and differentiation in the subcortical telencephalon.

Premature acquisition of late progenitor properties and a reduction in early neurogenesis in the subcortical telencephalon of *Mash1* mutants

A previous study reported that neurons born around E10.5 were reduced in the subcortical telencephalon of E18.5 *Mash1* mutants (Casarosa et al., 1999). However, Horton et al. (Horton et al., 1999) found precocious expression of a differentiating

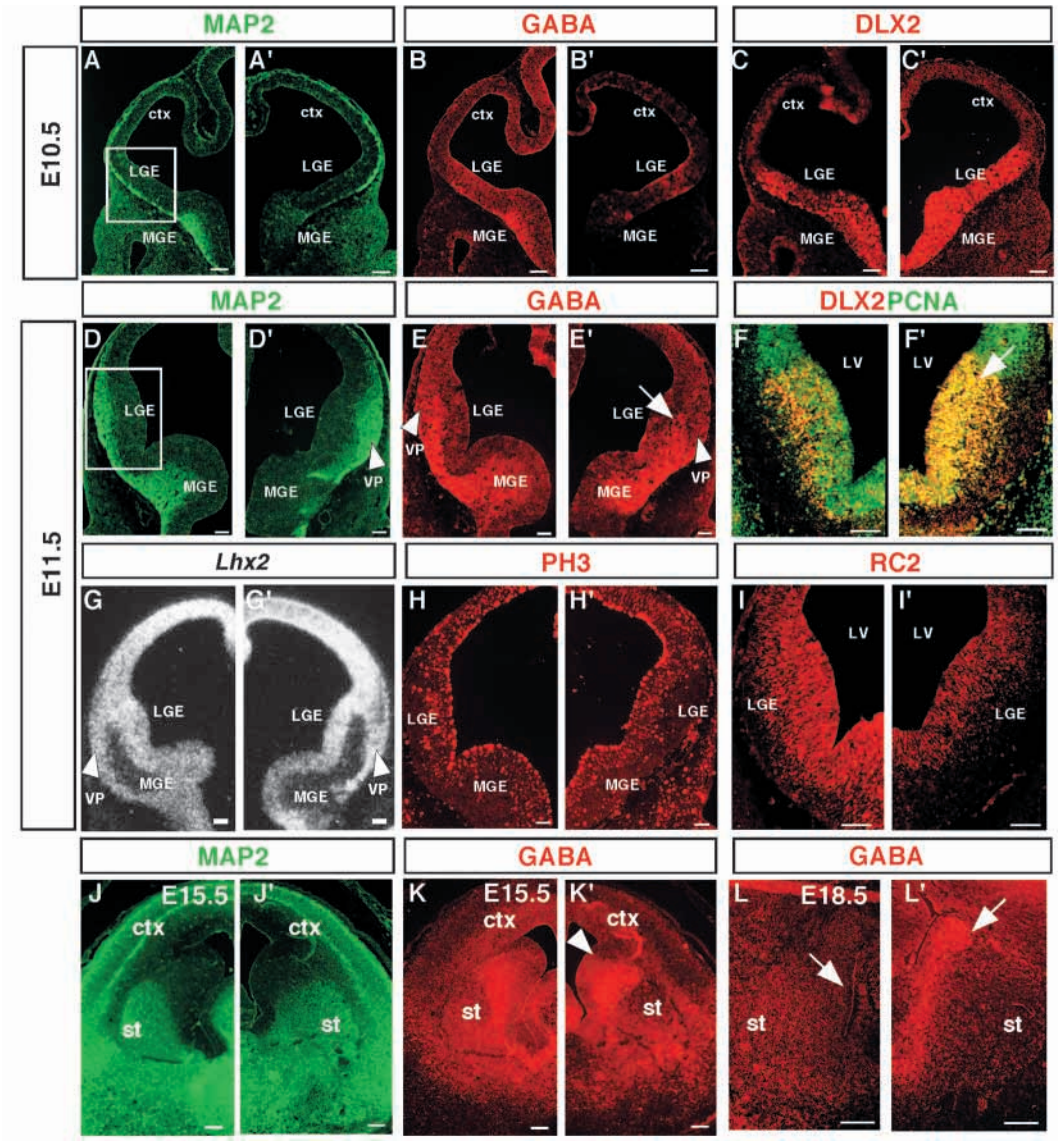

Fig. 2. *Mash1* mutants have a defect in the production of early-born (N1) neurons and their VZ cells precociously acquire SVZ-like properties. Fluorescence and darkfield microscopic images of coronal hemi-sections of control (left side) and *Mash1* mutant (right side) telencephalons at E10.5 (A-C′), E11.5 (D-I′, E15.5 (J-K′) and E18.5 (L,L′). The sections were stained with antibodies directed to the antigens, or ^{35}S-labeled probe, listed above each panel. Yellow cells are double-labeled. While *Mash1* mutants produce few MAP2+ cells at E10.5 (N1 cells; A-B′), their progenitor zone appears to mature precociously based on the increased number of cells that express DLX2 (C,C′,F,F′). These DLX2 cells are mitotically active precursors, based on co-expression with PCNA (arrow in F′). At E11.5, the mutant LGE produces many GABA+ cells (arrow E″), but most of these do not express MAP2. We suggest that a substantial number of the MAP2-expressing cells adjacent to the LGE in D′ (arrowhead) are not produced by the LGE, but rather correspond to ventral pallial cells (see arrowhead in G′ for pattern of *Lhx2* expression, which marks the mantle of the ventral pallium, VP). Note the paucity of MAP2 expression in the MGE mantle (D′) while GABA expression on the same section is abundant (E′). *Mash1* also has later functions in subcortical development. At E15.5 and E18.5 there is ectopic expression of GABA in the progenitor zone of the LGE. Arrowhead in K′ indicates ectopic GABA expression; arrows in L,L′ indicate LGE proliferative zones. ctx, cortex; LV, lateral ventricle; MGE, medial ganglionic eminence; st, striatum. Scale bars: ~100 μm.

neuronal marker (β-III-tubulin) in the ventricular zone of the *Mash1* mutants at E11. These apparently contradicting observations may reflect cell-autonomous and non cell-autonomous aspects of *Mash1* function. To evaluate this possibility, we re-examined the phenotype of *Mash1* mutants at E10.5 and compared these results with the phenotype of *Dll1* mutants.

At E10.5, *Mash1* mutants show molecular defects in both the proliferative and postmitotic zones. The mutant subcortical telencephalon has a hypoplastic mantle zone based on the reduction MAP2+ and GABA+ cells (Fig. 2A,A′,B,B′). These defects were not associated with increased levels of apoptosis (not shown). By E11.5 subcortical neurogenesis is detectable in the LGE, but remains abnormal in the MGE, based on the low level of MAP2 expression (Fig. 2D,D′). Note that although MAP2 expression is low in the mutant MGE at E11.5, GABA expression is now present (Fig. 2E,E′). GABA expression appears to mark an earlier step in differentiation than MAP2 expression.

Concurrent with the defect in MZ maturation (lack of N1 neurogenesis), the VZ shows a molecular defect suggesting an acceleration of differentiation at E10.5 and E11.5. In the *Mash1* mutant, DLX2 is expressed in the majority of VZ cells, whereas in the wild type, DLX2 expression is less extensive (Fig. 2C,C′). These ectopic DLX2+ cells have properties of proliferating precursors as they are PCNA+ (Fig. 2F,F′). In addition, there is a reduction in RC2 expression (Fig. 2I,I′).

The VZ also expresses SVZ markers such as *Gad67*, *Dlx5*, *Dlx6*, *Brn4* and *Six3* (Fig. 3E,E′,G,G′,H,H′, and data not shown), consistent with the results at E12.5 of Casarosa et al. (Casarosa et al., 1999). These observations suggest that in the absence of *Mash1*, there is premature maturation of early progenitors (P1 and P2) to later progenitors (P3). However, the precocious acquisition of later progenitor cell fate is

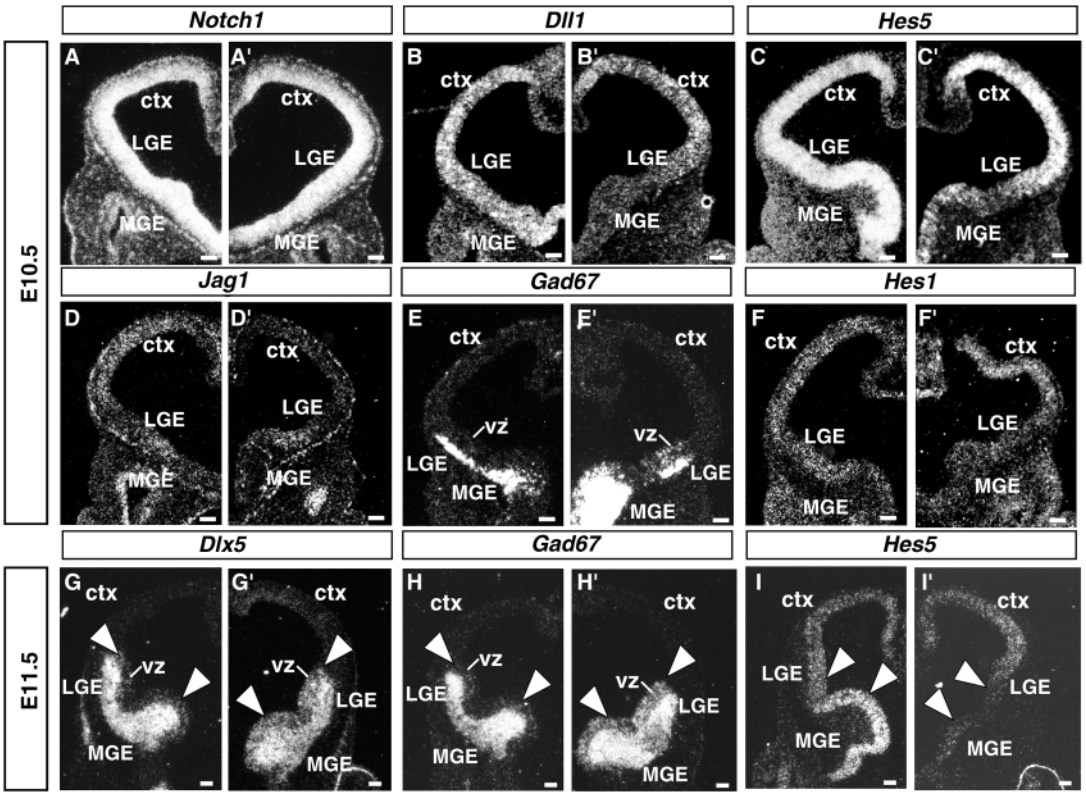

Fig. 3. Notch signaling is compromised in the absence of *Mash1* function as early as E10.5. Dark-field photomicroscopic images of coronal hemi-sections of control (left side) and *Mash1* mutant (right side) telencephalons at E10.5 (A-F′) and E11.5 (G-I′) studied using ^{35}S-labeled radioactive in situ hybridization. The cDNAs encoding the riboprobes used in the analysis are listed above each normal/mutant pair of images. The VZ in the *Mash1* mutant has molecular characteristics of the SVZ, based on the increased expression of *Dlx5*, *Gad67* and the reduced expression of *Hes5* (arrowheads in G-I′). ctx, cortex; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; VZ, ventricular zone. Scale bar: ~100 μm.

not complete as the VZ maintains some neuroepithelial characteristics, such as positioning of the M-phase nuclei near the ventricle (Fig. 2H,H′).

Previous analysis of the *Mash1* mutant at E12.5 showed that it has reduced expression of *Dll1* and *Hes5* (Casarosa et al., 1999). To determine whether the abnormalities that we observe at E10.5 are associated with misregulated Notch signaling, we studied expression of *Dll1*, *Jag1*, *Hes1*, *Hes5*, *Notch1*, *Notch2* and *Notch3* at E10.5 and E11.5 (Fig. 3; data not shown). At E10.5, while the expression of the *Notch* genes appears normal (Fig. 3A,A′; data not shown), expression of *Dll1* was at background levels in the MGE, and was greatly reduced in the LGE (Fig. 3B,B′). At this age, *Jag1* expression, which is concentrated between the MGE and LGE, appears relatively normal (Fig. 3D,D′).

To assess whether the reduction in *Dll1* expression resulted in reduced levels of Notch signaling, we studied the expression of *Hes1* and *Hes5*; these bHLH genes are effectors of Notch signaling (Ohtsuka et al., 1999). *Hes5* expression was reduced in the mutant subcortical telencephalon at E10.5 and E11.5, indicating that Notch signaling was indeed reduced by the lack of *Mash1* (Fig. 3C,C′,I,I′). The low levels of Hes1 expression made it difficult to assess whether its expression was altered in *Mash1* mutants (Fig. 3F,F′).

These findings suggest that an early function of *Mash1* in the subcortical telencephalon is to regulate the rate at which early progenitors differentiate into late progenitors. In *Mash1* mutants, early progenitors (P1 and P2) precociously take on the molecular features of the late progenitors (P3). One possibility is that MASH1-dependent expression of *Dll1* in P2 and P3 progenitors mediates lateral inhibition that prevents precocious maturation of neighboring progenitors. To test this hypothesis, we studied the effect of losing *Dll1*-mediated Notch signaling in *Dll1* mutants.

Dll1 mutants have reduced Notch signaling yet maintain N1 neurogenesis

Dll1 mutants die around E11.5 (Hrabe de Angelis et al., 1997). At earlier ages, forebrain development appears to be relatively normal (Fig. 4), lacking increased levels of apoptosis (data not shown), allowing us to analyze subcortical development between E10.5-E11. Like the *Mash1* mutants, *Dll1* mutants have reduced *Hes5* expression in the subcortical telencephalon (Fig. 4A,A′), suggesting decreased Notch signaling. In addition, they have reduced expression of RC2 (Fig. 4F,F′), implying accelerated differentiation of the progenitor cells.

Unlike *Mash1* mutants, *Dll1* mutants have N1 neurogenesis based on several criteria. At E10.5, the distribution of MASH1 and DLX2 expressing cells in the VZ is similar to wild-type littermates, although the thickness of this zone is reduced (Fig. 4B,B′), whereas the mantle zone of both the LGE and MGE have an abundance of MASH1–/DLX2+ cells (Fig. 4B,B′). In

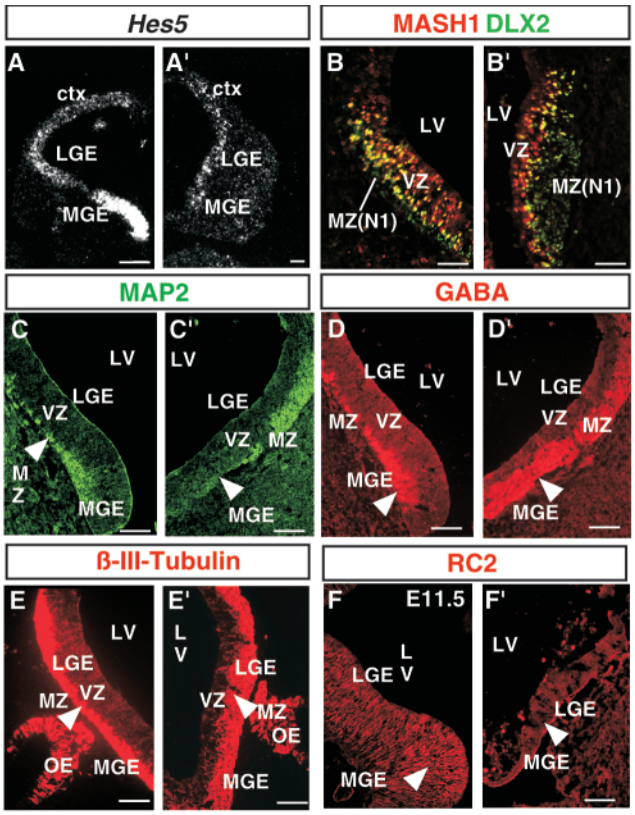

Fig. 4. *Dll1* mutants show increased early neurogenesis and reduced Notch signaling. Dark-field and fluorescence photomicroscopic images of coronal hemi-sections of control (left side) and *Dll1* mutant (right side) telencephalons at E10.5 (A-E′) and E11.5 (F,F′) studied using ^{35}S-labeled radioactive in situ hybridization (A,A′) and immunofluorescence (B-F′). *Dll1* mutants show reduced *Hes5* expression (A,A′), which is indicative of decreased Notch signaling, thinning of the VZ [with reduced RC2 expression (F,F′)], and an expansion of the mantle zone that expresses DLX2 (B,B′), β-III-tubulin (C,C′) and GABA (D,D′). These neurons may not have differentiated completely based on the low level of MAP2 expression particularly in the presumptive MGE (arrowhead in E,E′). Similar to the *Mash1* mutants, GABA expression appears in maturing neurons before MAP2 expression (compare D,D′,E,E′). ctx, cortex; LGE, lateral ganglionic eminence; LV, lateral ventricle; MGE, medial ganglionic eminence; MZ, mantle zone; OE, olfactory epithelium; VZ, ventricular zone. Scale bars: ~100 μm.

addition, there are mantle zone cells with robust expression of GABA and β-III- tubulin (Fig. 4D,D′,E,E′). However, early differentiation is not equal in the LGE and MGE; the LGE shows much more expression of MAP2 than does the MGE (Fig. 4C,C′). A similar difference in LGE/MGE development is seen in the *Mash1* mutant at E11.5 (Fig. 2D,D′). Casarosa et al. (Casarosa et al., 1999) also observed MGE hypoplasia at E12.5. We are uncertain why the MGE is more severely affected than the LGE in both of *Mash1* and *Dll1* mutants.

From these observations we hypothesize that in the absence of *Dll1* function, early VZ progenitors (P1 and P2) precociously differentiate. *Mash1* mutants share this phenotype. However, unlike *Mash1* mutants, *Dll1* mutants produce early-born neurons (N1). These observations suggest that *Mash1* is required cell autonomously for the generation of N1 cells. *Dll1* and *Mash1* mutants also differ in that DLX2 is more widely expressed in the VZ of *Mash1* mutants (compare Fig. 2C,C′ with Fig. 4B,B′; see Discussion).

Acquisition of the late progenitor properties in *Mash1* mutants is correlated with precocious DLX2 expression in the VZ. A previous study showed that *Dlx1* and *Dlx2* are required for the generation of late-born neurons (Anderson et al., 1997a). Thus, *Dlx1*, *Dlx2* and *Mash1* appear to have complementary roles in the temporal control of neuronal specification and differentiation. Towards understanding the mechanisms underlying these observations, we examined whether *Dlx1* and *Dlx2* regulated *Mash1* expression and/or Notch signaling in the subcortical telencephalon.

Loss of *Dlx1* and *Dlx2* function leads to an expanded domain of Notch signaling in the SVZ of the subcortical telencephalon

Previously, we have demonstrated that *Dlx1/2* mutants have defects in the differentiation of subcortical neurons produced after ~E12.5 (Anderson et al., 1997a; Marin et al., 2000). However, when E15.5 mutant SVZ cells were dissociated and cultured in vitro, they were able to proceed in their differentiation program (Anderson et al., 1997a), suggesting that cell-cell contact may have blocked their differentiation. Thus, we examined whether this phenotype was related to a defect in Notch signaling.

Unlike in the *Mash1* mutant, the production of early-born neurons (N1) appears normal in the *Dlx1/2* mutants at E10.5, based on expression of GABA and MAP2 (Fig. 5A,A′,B,B′, and data not shown). The VZ of the *Dlx1/2* mutant also shows normal molecular properties at E11.5 based on the expression of RC2 and genes in the Notch signaling pathway (*Notch1*, *Notch3*, *Dll1*, *Hes1*, *Hes5* and *Mash1*) (Fig. 5C,C′,D,D′,E,E′,F,F′ and data not shown).

While early progenitor specification (P1 and P2) and early neurogenesis (N1) appear to be normal in *Dlx1/2* mutants, molecular defects in late progenitors (P3) become apparent with the onset of SVZ formation. Subtle increases in the expression of *Dll1*, *Hes5* and *Mash1* are detected as early as E11.5 (Fig. 5D,D′,E,E′,F,F′). The molecular defects in the late progenitors become more obvious at later stages, such that the SVZ shows an expanded domain of *Mash1*, *Dll1*, *Hes5* and *Notch1* expression at E15.5 (Fig. 6A,A′,D,D′,E,E′,F,F′). These phenotypes persist through P0, when these animals die.

The ectopic MASH1+ cells in the *Dlx1/2* mutants are mitotically active, based on their expression of PCNA (Fig. 6A,A′). As shown by the increased expression of *Hes5* (Fig. 6E,E′), the expanded *Notch1* and *Dll1* expression results in elevated Notch signaling. These observations together suggest that one of the key functions of *Dlx1/2* in promoting late neurogenesis is through downregulating Notch signaling, perhaps by repressing *Mash1* expression. Note that expression of *Notch3* and *Hes1* is not appreciably changed in the mutant (Fig. 6B-C′), suggesting that unique combinations of receptor/ligand interactions may lead to expression of different effector molecules (*Hes1* versus *Hes5*, for example) (see Discussion).

Consistent with the expanded Notch signaling domain, *Dlx1/2* mutant SVZ cells express high levels of transcription factors that are normally restricted primarily to the VZ, such as *COUP-TF1*, *Gsh1*, *Gsh2* and *Lhx2* (Fig. 6G-H′, data not shown) (Anderson et al., 1997a). The persistent expression of VZ

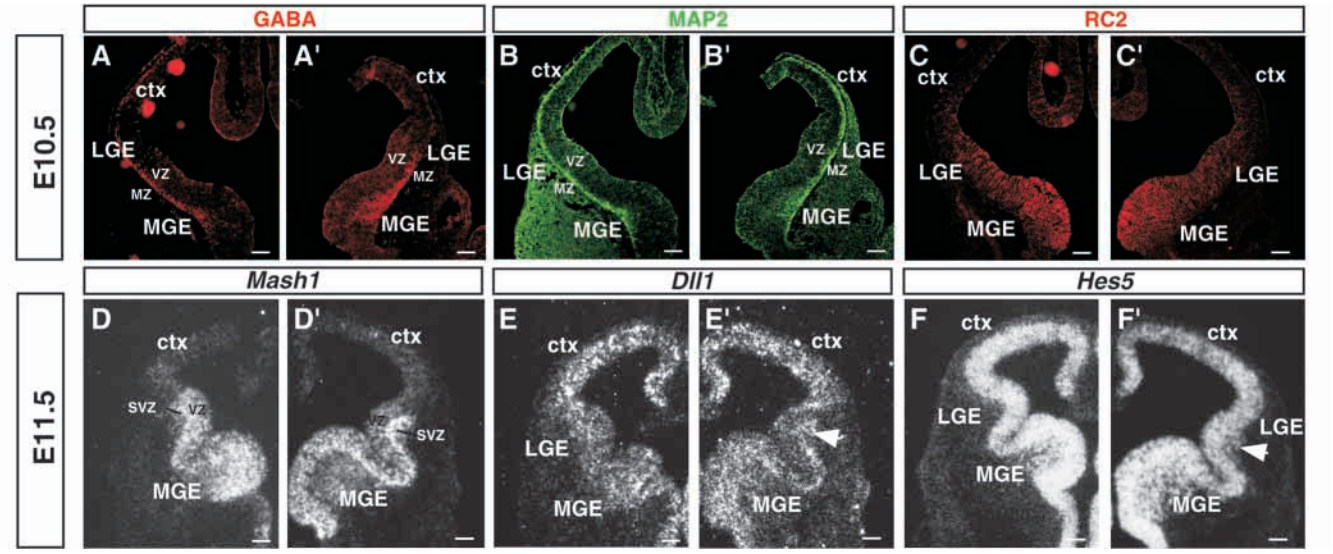

Fig. 5. *Dlx1/2* mutants show normal neurogenesis at E10.5, and begin to show elevations in *Mash1*, *Hes5* and *Dll1* expression by E11.5 in the SVZ. Coronal hemi-sections of control (left side) and *Dlx1/2* mutant (right side) telencephalons at E10.5 (A-C′) studied with immunofluorescence, and at E11.5 (D-F′), studied with radioactive in situ RNA hybridization. At E10.5 early neurons, assessed by expression of GABA and MAP2 (A,A′,B,B′) and radial glia, assessed by expression of RC2 (C,C′), appear normal in the *Dlx1/2* mutants. At E11.5 expression of *Mash1*, *Dll1* and *Hes5* (D-F′) increase in the SVZ (arrowheads). Scale bars: 100 μm.

markers is associated with reduced expression of SVZ markers such as *Dlx5*, *Dlx6*, *SCIP/Oct6* and *Six3* (Anderson et al., 1997a; Zerucha et al., 2000) (data not shown). While the expression of general neuronal markers (MAP2 and β-III-tubulin) is maintained in the *Dlx1/2* mutants (Fig. 6I,I′,K,K′, and not shown), the block in differentiation affects expression of neuronal sub-type genes, such as the dopamine receptor 2 (*Drd2)* and *Darrp* (Fig. 6J,J′) (Anderson et al., 1997a). Thus, we suggest that *Dlx1/2* function is required to specify and differentiate P3 progenitors by repressing the genes that are normally expressed in VZ progenitor cells (e.g. *Mash1*, *Gsh1/2*, *Lhx2*, *COUP-TF1*) and by activating genes expressed in the SVZ (e.g. *Dlx5*, *Dlx6* and *SCIP/Oct6*) and MZ (e.g. *Drd2*).

Recent studies show that ectopic Notch signaling, either through constitutively active Notch1 (Notch-IC) expression or through treatment with soluble *Dll1*, predisposed neuronal progenitors to take on a glial fate (Morrison et al., 2000; Tanigaki et al., 2001). In addition, ectopic expression of Notch-IC leads to the generation of radial glia in the mammalian telencephalon (Gaiano et al., 2000). As *Dlx1/2* mutants show ectopic Notch signaling, we studied whether radial glia are also affected in these mutants. Although the early expression of RC2 is normal in the *Dlx1/2* mutants (Fig. 5C,C′), we find persistent expression of RC2 and nestin at E18.5 (Fig. 6L,L′, and data not shown), when the radial glial scaffolding appears to have mostly collapsed in the wild-type animals. This phenotype is consistent with the ectopic Notch signaling observed in these animals (Fig. 6D-F′), and it suggests that there is an accumulation of radial glia cells in the *Dlx1/2* mutants.

DISCUSSION

We present genetic evidence that temporally distinct waves of neurogenesis in the subcortical telencephalon differentially depend on the *Mash1* bHLH genes and *Dlx1* and *Dlx2* homeobox genes. Generation of the earliest neurons (N1) requires *Mash1*, whereas generation of later-born neurons (N2 and others) depends on *Dlx1* and *Dlx2*. Below, we discuss evidence that *Mash1* and *Dlx1/2* perform these functions through having opposite effects on Notch signaling in early (P1 and P2) and late (P3) progenitor cells (Fig. 7).

MASH1 and DLX2 expression define subsets of subcortical progenitors

Previous studies have identified primary and secondary progenitor populations in the LGE (Halliday and Cepko, 1992; Bhide, 1996; Sheth and Bhide, 1997). We show that MASH1 and DLX2 expression can be used to define subsets of LGE progenitors. Combinatorial expression of these transcription factors, in conjunction with the expression of proliferation and differentiation markers, provides evidence for at least three types of progenitors in the ventricular zone (P1, MASH1–/DLX2–; P2, MASH1+/DLX2–; P3, MASH1+/DLX2+) (Figs 1, 7).

Prior to E9.5, there are only MASH1-/DLX2- cells (P1 progenitors) in the subcortical telencephalon. Then, MASH1 expression is induced in the VZ, which is rapidly followed by DLX2 expression; their expression marks the appearance of P2 (MASH1+/DLX2–) and P3 (MASH1+/DLX2+) progenitors. From E10.5, P3 cells (MASH+/DLX2+) accumulate as a layer intercalated between the VZ and MZ; these cells are postulated to be the incipient SVZ. By E12.5, an additional population of MASH–/DLX2+ cells accumulates between the SVZ and the MZ (not shown); these might represent another type of progenitor cells (P4).

Prior to E10.5, DLX2+ neurons are generated that have migrated to the MZ, where they express GABA, GAD65, GAD67, MAP2 and β-III-tubulin (Fig. 1A-D and not shown). These cells are the early born neurons (N1) and we propose

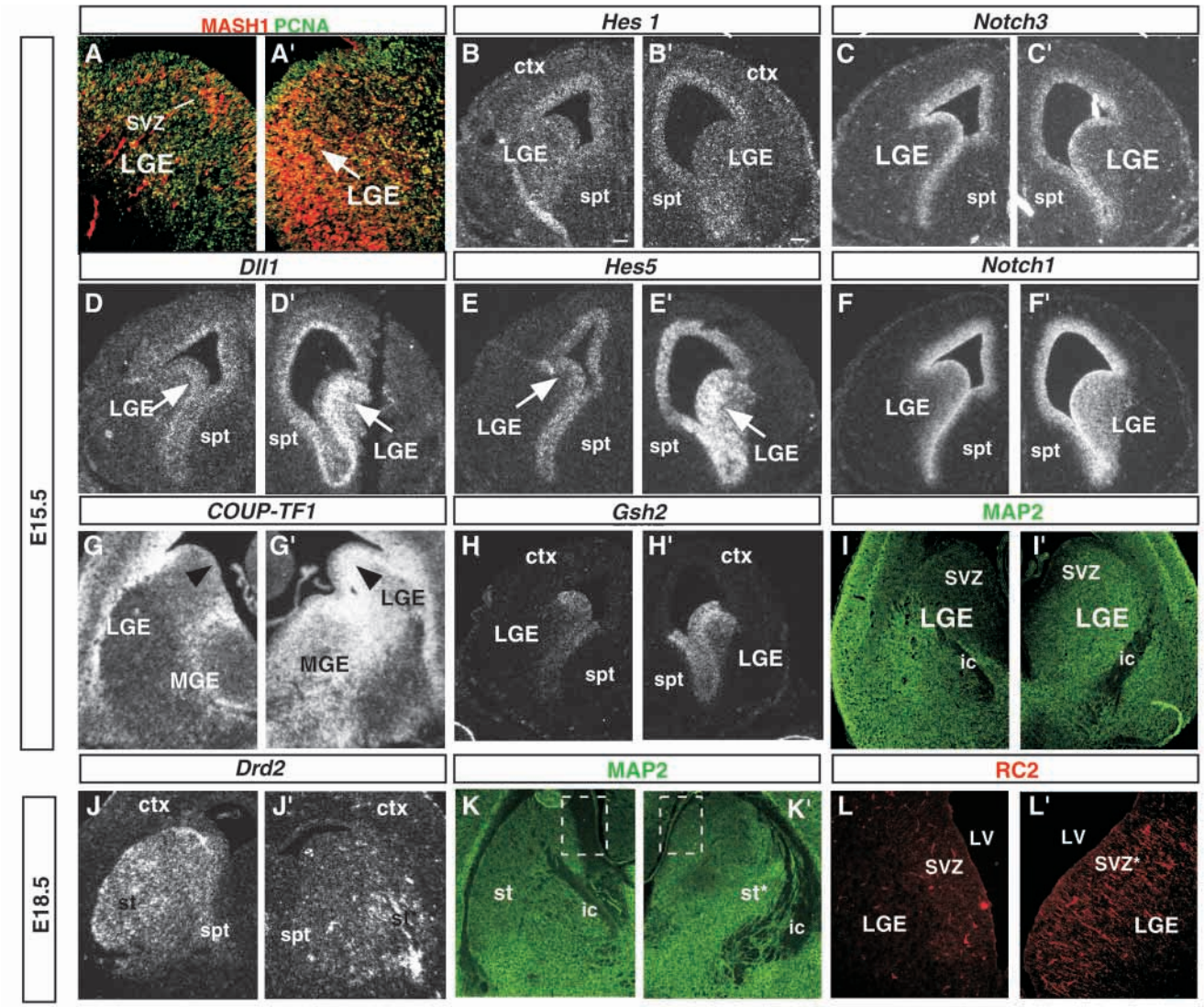

Fig. 6. *Dlx1/2* mutants show expanded Notch signaling domains and a block in differentiation of SVZ cells. Dark-field and fluorescence photomicroscopic images of coronal hemi-sections of control (left side) and *Dlx1/2* mutant (right side) telencephalons at E15.5 (A-I′) and E18.5 (J-L′) studied using radioactive in situ hybridization (B-E′,G-H′,J,J′) and immunofluorescence (A,A′,I,I′,K,K′,L,L′). (G-H′)The *Dlx1/2* mutant SVZ shows elevated expression of VZ markers (*COUP-TF1*; *Gsh2*).These defects are correlated with increases in MASH1, *Dll1* and *Hes5* expression (A,A′,D-E′), particularly in the SVZ. Note that while the *Hes5* and *Notch1* (E-F′) expression domain expands, *Hes1* and *Notch3* (B-C′) expression remains normal in the *Dlx1/2* mutants. The persistence of progenitor molecular properties as late as E18.5 (not shown) is linked with persistent expression of a radial glial marker (RC2; L,L′). The defects in SVZ differentiation are correlated with reduced expression of striatal markers, such as dopamine receptor 2 (*Drd2*) (J,J′), while a general marker of neurogenesis (MAP2) appears normal in the mantle zone (I-K′). Note, however, that MAP2 expression extends much closer to the ventricle in the mutant at E18.5 (boxed areas in K,K′). ctx, cortex; ic, internal capsule; LV, lateral ventricle; spt, septum; st, striatum. Scale bars: ~100 μm.

that they are derived from the VZ progenitors (either P1 or P2). From E11.5, the next wave of neurons (N2) are generated, and their appearance coincides with increased RC2 expression in the VZ and the emergence of the SVZ progenitors. We suggest that N2 neurons are mainly generated from P3 progenitors. Formal lineage analysis is needed to test this model.

The presence of primary (VZ) and secondary (SVZ) progenitor zones is reminiscent of the developing CNS of invertebrates. In *Drosophila*, neuroblasts are the primary neuroepithelial cells. Neuroblasts produce ganglion mother cells, which (like SVZ cells) have a reduced mitotic potential (Doe et al., 1998).

Evidence for cell autonomous and non-autonomous functions of *Mash1*

Previous reports demonstrated that *Mash1* is required for early neurogenesis (Casarosa et al., 1999; Horton et al., 1999; Marin et al., 2000) and for Notch signaling via regulating *Dll1* and *Dll3* expression (Casarosa et al., 1999). The previous evidence that *Mash1* mutants have reduced Notch signaling in progenitor cells was based on analysis at E12.5 and later ages (Casarosa et al., 1999). This is several days after the initiation of *Mash1* expression in neural progenitors (E9.75), and after the formation of the postulated P1, P2 and P3 progenitors. We found that by E10.5, *Mash1*

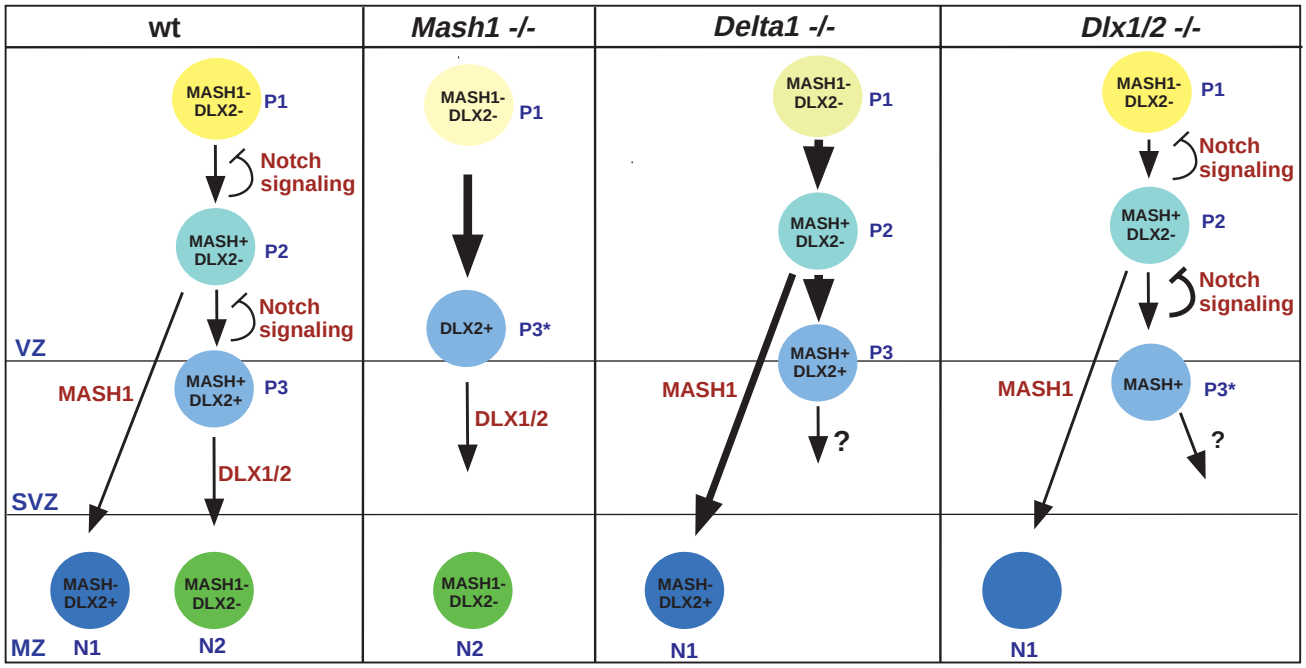

Fig. 7. Models showing progenitor cell (P) and neuronal (N) maturation in the LGE of wild-type, *Mash1*$^{-/-}$, *Dll1*$^{-/-}$ and *Dlx1/2*$^{-/-}$ embryos. MASH1–/DLX2– neuroepithelial progenitors (P1) give rise to MASH1+/DLX2– (P2) and MASH1+/DLX2+ (P3) progenitors around E10. An alternate model would have P1 cells separately generate P2 and P3 states. Lineage analysis will be needed to determine the relationships between the postulated progenitor cells and their derivatives. We propose that P2 cells mature into early neurons (N1), while P3 cells proliferate to form the SVZ progenitor zone and produce later-born neurons (N2). *Mash1* is required cell-autonomously to specify P2 progenitors and hence N1 neurons. *Mash1* is also required non cell-autonomously: increasing *Dll1* expression to prevent neighboring cells from acquiring the P3 fate prematurely via Notch-dependent lateral inhibition. In *Dll1* mutants, P1, P2 and P3 progenitors are present, but their maturation is accelerated due to decreased Notch signaling. By contrast, early neurogenesis is normal in the *Dlx1/2* mutants but P3 progenitors are blocked in their ability to differentiate. This phenotype is attributed to the failure to downregulate Notch signaling during specification and/or differentiation steps of P3 maturation. The arrows between cell states do not necessarily imply that mitosis is required. The enlarged arrows represent hypothesized increases in the rate of the indicated process.

mutants have reduced expression of *Dll1* and *Hes5* (Fig. 3B,B′,C,C′). The decreased *Hes5* expression is likely to be due to reduced Notch signaling caused by reduced *Dll1* expression.

To determine directly the contribution that the reduced *Dll1* expression plays in the *Mash1*$^{-/-}$ phenotype, we compared subcortical development in *Mash1* and *Dll1* mutants at E10-E11 (Figs 2, 3, 4). This comparison provided evidence that *Mash1* has at least two functions between E10-E11. The first is a *Dll1*-independent role in promoting the production of P2 progenitors and N1 neurons. The second is a *Dll1*- (and perhaps *Delta3*-) dependent role in preventing adjacent progenitors from differentiating.

Mash1 mutants produce very few MAP2+, GABA+ N1 neurons at E10.5 (Fig. 2A,A′,B,B′). This is consistent with the BrdU birth dating results showing a reduction in the number of subcortical neurons leaving the mitotic cycle at E10.5 (Casarosa et al., 1999). However, *Dll1* mutants produce N1 neurons (Fig. 4B,B′,D,D′,E,E′). Thus, we propose that *Mash1* expression is autonomously required in P2 progenitors to generate N1 neurons in the LGE (Fig. 7).

Dll1 and *Mash1* mutants also differ in that DLX2 is more widely expressed in the VZ of *Mash1* mutants (compare Fig. 2C,C′ with Fig. 4B,B′). This finding could be due to the persistent expression of another Notch ligand (such as *Delta3*), in the *Dll1* mutants and not the *Mash1* mutants. It could also be explained if MASH1 has an autonomous function in preventing premature maturation of VZ cells.

We suggest that the reduction in Delta-mediated lateral inhibition increases the rate at which progenitor cells differentiate in both the *Dll1* and *Mash1* mutants. In *Dll1* mutants, thinning of the VZ (which contains P1, P2 and P3 cells) is accompanied by increased mantle zone, consistent with the precocious differentiation of the neural progenitors. In *Mash1* mutants, early progenitors appear to become depleted based on the nearly ubiquitous expression of DLX2 in the VZ at E10.5 and E11.5 (Fig. 2C,C′). In addition, at E10.5 and E11.5, cells expressing SVZ markers (such as *Gad67* and *Dlx5)* are now present in the VZ (Fig. 3E,E′,G,G′,H,H′ and data not shown); expression of these markers increases at later stages (Casarosa et al., 1999; Horton et al., 1999). Therefore, the reduction in lateral inhibition accelerates the generation of later progenitors, similar to what has been observed in vertebrate retinal development (Dorsky et al., 1997; Henrique et al., 1997). While early progenitors differentiate into more mature progenitor cells (based on the expression of DLX2, *Dlx5* and *Gad67*), they appear to maintain a neuroepithelial structure, because the VZ has periventricular M-phase nuclei at E11.5 (Fig. 2H,H′).

Dlx1 and *Dlx2* are required to downregulate Notch signaling

Previous analysis of *Dlx1/2* mutants showed that early LGE and MGE neurogenesis was largely unperturbed, whereas the differentiation of neurons born after ~E12.5 was reduced (Anderson et al., 1997a; Marin et al., 2000). The abnormal differentiation led to a block in radial and tangential migration that results in the accumulation of partially differentiated neurons in periventricular ectopia (Anderson et al., 1997a; Anderson et al., 1997b; Marin et al., 2000). However, dissociation of mutant SVZ cells facilitated further differentiation in vitro, suggesting that cell-contact mediated inhibition may have contributed to the phenotype (Anderson et al., 1997a).

Consistent with these characteristics, we show that *Dlx1/2* mutants exhibit increased levels of *Hes5* expression, implying that differentiation may be blocked due to increased levels of Notch signaling (Fig. 5E,E′). At E11.5 *Dll1* and *Mash1* expression are elevated in the SVZ (Fig. 5D,D′,E,E′); these abnormalities become more severe at later stages (Fig. 6D,D′,H,H′). As MASH1 and DLX2 are co-expressed in some progenitors (P3), a potential mechanism underlying this phenotype would be that *Dlx1* and *Dlx2* repress *Mash1* expression (directly or indirectly) as P3 cells mature. In *Dlx1/2* mutants, failure to downregulate *Mash1* expression would lead to elevated levels of *Dll1* expression; this, in turn, would increase Notch signaling and *Hes5* expression in adjacent cells.

The elevations in Notch signaling are correlated with the persistent expression of some VZ markers in the SVZ (e.g. *Notch1*, *Hes5 Gsh2*, *COUP-TF1* and *Lhx2*) (Fig. 6) (Anderson et al., 1997a). In addition, the radial glial marker, RC2, is more highly expressed in the mutant LGE at E18.5 (Fig. 6L,L′). This is consistent with the observation that increases in Notch signaling results in the generation/maintenance of radial glia (Gaiano et al., 2000; Tanigaki et al., 2001).

Evidence that activation of different Notch receptors may be differentially coupled to *Hes1* and *Hes5* expression and function

DLX1 and DLX2 are the first homeobox transcription factors reported to downregulate Notch signaling. However, although loss of *Dlx1/2* function leads to an upregulation of *Hes5* expression (Fig. 5F,F′, Fig. 6E,E′), *Hes1* expression is not appreciably altered (Fig. 6B,B′). Furthermore, although there is increased *Notch1* expression throughout the mutant SVZ (Fig. 6F,F′), *Notch3* expression remains restricted to the VZ (Fig. 6C,C′). The following models could explain this differential effect on the expression of *Notch3* and *Hes1* versus *Notch1* and *Hes5*. *Notch3* receptor activation may specifically lead to *Hes1* expression in early VZ progenitors (P1 cells). When P2 and P3 cells form, they upregulate *Dll1* expression, which signals through *Notch1* to induce *Hes5*. Alternatively, DELTA1 binds to either NOTCH1 or NOTCH3, but activation of *Hes5* is mediated only through the *Notch1* receptor. Thus, in the *Dlx1/2* mutants, where early P1 and P2 fates appear normal, *Notch3* and *Hes1* expression are normal. However, specification and differentiation of P3 cells are abnormal in *Dlx1/2* mutants, leading to ectopic *Notch1* and *Hes5* expression.

The distinct roles of *Notch3/Hes1* and *Notch1/Hes5* are supported by more restricted expression of *Notch3* (Irvin et al., 2001) (Fig. 6), and differences between *Notch1* and *Notch3* functions (Beatus et al., 1999). Furthermore, *Hes1* and *Hes5* have been shown to play differential roles in the development of the inner ear (Zine et al., 2001) and olfactory epithelium (Cau et al., 2000). Additional studies are needed to determine whether there are different ligand specificities for *Notch1* and *Notch3* receptors and/or different signal-transduction cascades downstream of *Notch1* and *Notch3* in the developing telencephalon.

bHLH and homeobox genes function together in the temporal regulation of cell fate specification and differentiation

We present evidence that *Mash1* and *Dlx1and2* regulate development of temporally distinct sets of LGE-derived neurons (see model in Fig. 7). We suggest that *Mash1* has a cell-autonomous function in the formation of the N1 neurons. In addition, *Mash1*, through controlling *Delta* expression, has a non cell-autonomous function in regulating the rate at which adjacent progenitors mature. Thus, in the absence of *Mash1* or *Dll1*, VZ progenitors receive reduced Notch signaling, and therefore precociously acquire later progenitor fate or become postmitotic (Figs 2, 3, 4, 7). On the contrary, *Dlx1/2* function is required to reduce Notch signaling for differentiation of SVZ progenitors. Thus, in the absence of *Dlx1/2* function, Notch signaling persists and differentiation is impeded (Figs 5, 6, 7). The model presented in Fig. 7 suggests that P1, P2 and P3 are sequential cell states. An alternate model would have P1 cells separately generate P2 and P3 states. Lineage analysis will be needed to determine the relationships between the postulated progenitor cells.

Although *Mash1* and *Dlx1/2* mutants affect distinct populations of LGE neurons, we suggest that bHLH and homeobox genes function coordinately to regulate differentiation at all stages of LGE development. Thus, although DLX1 and DLX2 are expressed in early neurons, their function does not appear to be essential in the early lineage. *Dlx5* and *Dlx6* are candidate genes for compensating for *Dlx1* and *Dlx2* function at early stages, as expression of these genes is preserved in N1 cells in *Dlx1/2* mutants (data not shown) (Anderson et al., 1997a). Furthermore, although *Mash1* function is essential for early LGE lineages, it is expressed throughout the period of LGE neurogenesis. While at this point little is known about the later functions of *Mash1*, there is evidence that it does regulate the properties of LGE progenitor cells at E15.5 and E18.5, as these show elevated expression of GABA (Fig. 2K,K′,L,L′). In addition, perhaps other bHLH genes, such as *Olig2*, may compensate for *Mash1* function at later stages (Takebayashi et al., 2000). Thus, future studies should focus on testing the function of other homeobox and bHLH transcription factors in the temporal control of differentiation in the subcortical telencephalon.

This work was supported by the research grants to K. Y. from Tourette's Syndrome Foundation, and to JLRR from Nina Ireland, NARSAD, and NIMH RO1 MH49428-01, RO1 MH51561-01A1 and K02 MH01046-01. We thank Song Cha Yun and Fiona Zhong for their excellent technical assistance, Susan Yu for her administrative assistance, and all members of the Rubenstein laboratory for their

helpful discussions and encouragement. In particular, we thank Jason Long, Sonia Garel, Oscar Marin, Youlin Ruan, Inma Cobos and Cathy Dye for critical reading of the manuscript.

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