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

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SUMMARY

Notch signaling has a central role in cell fate specification and differentiation. We provide evidence that the \textit{Mash1} (bHLH) and \textit{Dlx1} and \textit{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 \textit{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 \textit{Mash1} mutants, suggests that \textit{Mash1} regulates early neurogenesis through Notch- and Delta-dependent and -independent mechanisms. While \textit{Mash1} is required for early neurogenesis (E10.5), \textit{Dlx1} and \textit{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: \textit{Mash1}, \textit{Dlx2}, \textit{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 cell 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 (\textit{Delta}, \textit{Serrate}, \textit{Lag2}). In mammals, there are four Notch genes (\textit{Notch1-Notch4}) and five DSL ligand genes (\textit{Dll1, Dll3, Dll4, Jag1} and \textit{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 [\textit{CBF1} (\textit{Rbpsuh} – Mouse Genome Informatics) \textit{Suppressor of Hairless} (\textit{SuH}), \textit{Lag1}]. During neurogenesis, Notch activation of CSL reduces the expression of WRPW-bHLH transcription factors that inhibit neuronal differentiation (e.g. HES genes or \textit{Drosophila Enhancer of Split}) and represses the expression of proneural bHLH transcription factors (e.g. \textit{Mash1} or \textit{Achaete-Scute} in
There are several classes of proneural bHLH genes expressed in the mammalian telencephalon, including an Achaete-Scute homolog Mash1 (Asc1l – 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 (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 demonstrated 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 negatively 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 C57Bl/6), Mash1 mutant (Guillemot et al., 1993) and Dlx1/2 mutant (Qu 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 (Mash1, 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, Drl2 – 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 35S-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 #586604). 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 double-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 C57Bl6) 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 x 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.
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) 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) is more coherently arranged, and can be distinguished as a SVZ (Fig. 1H), which persists as development proceeds.

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 MASH1 and 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 N1 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...
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 ofDll1 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,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). Note that although MAP2 expression is low in the mutant MGE at E11.5, GABA expression is now present (Fig. 2E). 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). These ectopic DLX2+ cells have properties of proliferating 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+ cells in the LGE 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.
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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 ofDll1 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 ofDll1, 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 telecephaIon 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. 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 35S-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.
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 these observations, we examined whether Dll1 and Dlx2 regulated Mash1 expression and/or Notch signaling in the subcortical telencephalon.

**Loss of Dll1 and Dlx2 function leads to an expanded domain of Notch signaling in the SVZ of the subcortical telencephalon**

Previously, we have demonstrated that Dll1/2 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

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**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 35S-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.
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. 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.](image-url)
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. 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.
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 Hes1 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 result 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.

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