Mash1 regulates neurogenesis in the ventral telencephalon

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SUMMARY

Previous studies have shown that mice mutant for the gene *Mash1* display severe neuronal losses in the olfactory epithelium and ganglia of the autonomic nervous system, demonstrating a role for *Mash1* in development of neuronal lineages in the peripheral nervous system. Here, we have begun to analyse *Mash1* function in the central nervous system, focusing our studies on the ventral telencephalon where it is expressed at high levels during neurogenesis. *Mash1* mutant mice present a severe loss of progenitors, particularly of neuronal precursors in the subventricular zone of the medial ganglionic eminence. Discrete neuronal populations of the basal ganglia and cerebral cortex are subsequently missing. An analysis of candidate effectors of *Mash1* function revealed that the Notch ligands *Dll1* and *Dll3*, and the target of Notch signaling *Hes5*, fail to be expressed in *Mash1* mutant ventral telencephalon. In the lateral ganglionic eminence, loss of Notch signaling activity correlates with premature expression of a number of subventricular zone markers by ventricular zone cells. Therefore, *Mash1* is an important regulator of neurogenesis in the ventral telencephalon, where it is required both to specify neuronal precursors and to control the timing of their production.

Key words: Mouse, Forebrain, Basal ganglia, Neural determination, Lateral inhibition, Notch signaling

INTRODUCTION

The telencephalon is the most complex region of the mammalian brain, and it contains the greatest diversity of neuronal subtypes. Recent studies have revealed important mechanisms for the regionalization of the anterior neural plate (reviewed in Rubenstein et al., 1998). Puelles and Rubenstein (1993) have proposed that the forebrain is organized into six transverse domains named prosomeres, defined by domains of expression of regulatory genes and delimited by transient constrictions in the wall of the neural tube. According to this prosomeric model, the forebrain is composed of two large subdivisions, the diencephalon and the secondary prosencephalon, with the latter itself subdivided into the hypothalamus and telencephalic vesicles. Within the telencephalic vesicles, the proliferative regions that generate the cerebral cortex dorsally, and the basal ganglia ventrally, are distinguishable at an early stage by both morphology and patterns of gene expression (Shimamura et al., 1995).

In both dorsal and ventral subdivisions of the telencephalon, neurons are produced from two populations of progenitor cells: the pseudostratified epithelium of the ventricular zone (VZ), which is lining the ventricle, and a secondary proliferative population, consisting of non-epithelial progenitor cells located in a deeper subventricular zone (SVZ; Smart, 1976; Bhide, 1996). Cell lineage experiments have shown that SVZ cells in the ventral telencephalon are derived from VZ cells (Halliday and Cepko, 1992). In the ventral telencephalon, progenitor cells are located in two bilateral elevations of the

walls of the lateral ventricle, known as the lateral and medial ganglionic eminences (LGE and MGE; Smart and Sturrock, 1979), in which the SVZ is markedly enlarged.

After neuronal precursors have left the cell cycle, they migrate out of the proliferative zones towards the periphery of the telencephalic vesicles, where they complete their differentiation. The main route of migration of telencephalic neurons is a radial route guided by the processes of radial glia. The LGE and the MGE generate principally the striatum and the pallidum, respectively (Smart and Sturrock, 1979; Deacon et al., 1994). There is evidence that a significant number of LGE-derived neurons also populate the cerebral cortex, where they reach their final positions following tangential routes of migration in the VZ, the SVZ, the intermediate zone (IZ) and the marginal zone (MZ) (reviewed in Pearlman et al., 1998). For example, a large fraction of the GABAergic interneurons of the neocortex are produced in the LGE and migrate tangentially into the cortical IZ and MZ (Anderson et al., 1997b; Tamamaki et al., 1997).

A number of regulatory genes involved in growth and early patterning of the telencephalon have recently been identified by gene targeting experiments in mouse (reviewed in Rubenstein et al., 1998). However, genes controlling neurogenesis in this forebrain territory have not yet been identified. Genes of the basic helix-loop-helix (bHLH) class have been implicated in the steps of determination and differentiation of neuronal lineages in the peripheral nervous system. *Neurogenin1* and *Neurogenin2* are required for the determination of subsets of cranial sensory neurons (Fode et al., 1998; Ma et al., 1998) and *Mash1* is required for the

generation of most autonomic ganglia, olfactory receptor neurons, and noradrenergic neurons of the hindbrain (Guillemot et al., 1993; Hirsch et al., 1998). In autonomic lineages, *Mash1* is involved in the expression of noradrenergic traits and in the terminal differentiation of neurons (Sommer et al., 1995; Hirsch et al., 1998). In contrast, *Mash1* is required in early progenitors of the olfactory epithelium to activate expression of neuronal differentiation genes and initiate neuronal differentiation (Cau et al., 1997). Consistent with a function for *Mash1* in specifying neuronal precursors, forced expression of *Mash1* renders pluripotent neural crest cells competent to respond to the neuralizing factor BMP2, and can promote neuronal differentiation of these cells (Lo et al., 1997).

A number of neural bHLH genes are expressed in the developing telencephalon. *Neurogenin1*, *Neurogenin2*, *NeuroD* and related genes are expressed in the cerebral cortex (reviewed in Lee, 1997). In contrast, *Mash1* is the only known neural bHLH gene expressed in the ventral telencephalon (Lo et al., 1991; Guillemot and Joyner, 1993), where MASH1 protein is found in a subset of VZ cells and in most SVZ cells (Porteus et al., 1994). Progenitor cells in the telencephalon are heterogenous in their proliferative and differentiation potentials (Lillien, 1998) and include pluripotent stem cells and the committed precursors that stem cells generate. *Mash1* expression in this region marks a transient population of committed neural precursors before their differentiation (Torii et al., 1998). In this article, we have investigated the function of *Mash1* in development of these telencephalic precursors. We show that *Mash1* mutant mice present multiple defects in neurogenesis in the ventral telencephalon, affecting in particular the generation of neuronal precursor cells in the SVZ of the MGE, and the timing of production of SVZ precursors in the LGE. The generation of neuronal populations in the basal ganglia and cerebral cortex is subsequently affected. This study therefore provides evidence that *Mash1* also functions as a determination gene in the brain, where it is required both to specify neuronal precursor cells and to control the timing of their generation in the ventral telencephalon.

MATERIALS AND METHODS

Wild-type and *Mash1* mutant mice

Wild-type, heterozygous and homozygous *Mash1* mutant embryos were obtained from intercrosses of *Mash1* +/− mice (Guillemot et al., 1993). For staging of embryos, midday of the vaginal plug was considered as embryonic day 0.5 (E0.5).

RNA in situ hybridization and immunohistochemistry

Embryos were fixed at 4°C in 4% paraformaldehyde for 2 hours (up to E12.5) or overnight (E15.5 and older), rinsed in phosphate-buffered saline (PBS), cryoprotected overnight in 20% sucrose in PBS and embedded in OCT (Tissue-Tek, Miles). Embedded embryos were sectioned on a cryostat at 10 μm. Section RNA in situ hybridization was performed as described in Cau et al. (1997). The cRNA probes used in this study were the following: *Dll1* (Bettenhausen et al., 1995); *Dll3* (Dunwoodie et al., 1997); *Hes5* (Akazawa et al., 1992); *GAD67* (Behar et al., 1994); *Dlx-1* (Price et al., 1991); *Dlx-5* (Simeone et al., 1994); *Lhx2* (Xu et al., 1993); *Nkx2.1* (Lazzaro et al., 1991); *SCG10* (Stein et al., 1988); *dopamine D2 receptor* (Montmayeur et al., 1991); *enkephalin* (Song and Harlan, 1994). Immunohistochemistry was
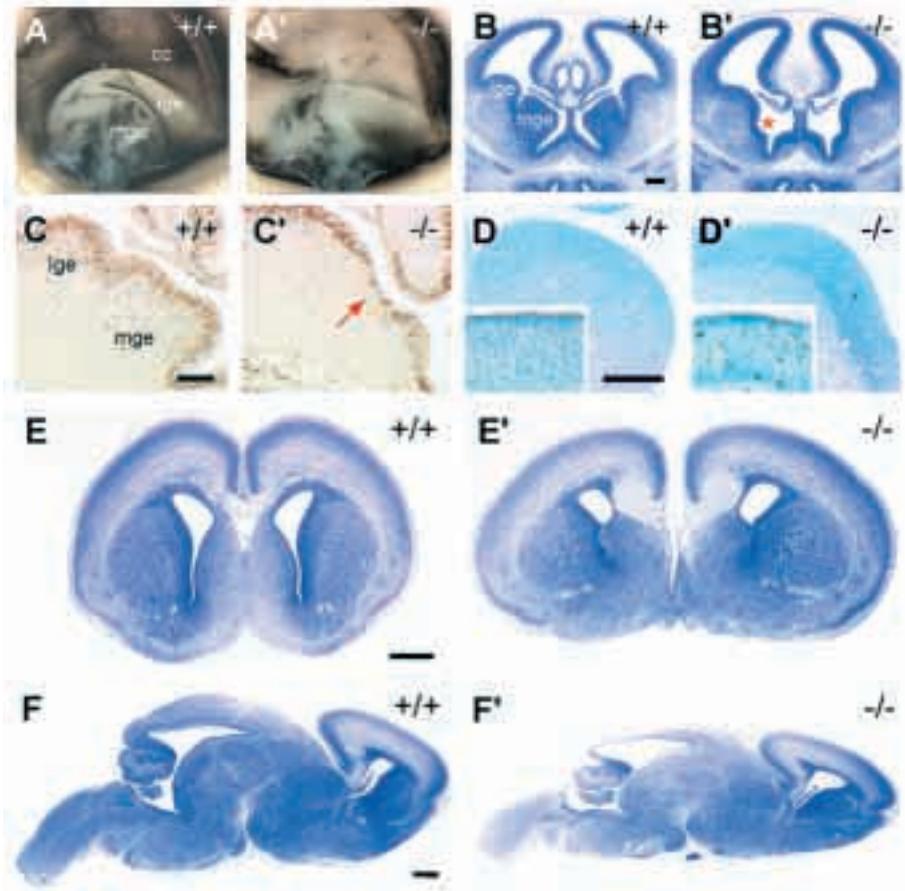

Fig. 1. Morphological defects of *Mash1* mutant brains. (A,A′) Telencephalic vesicles dissected from E12.5 wild-type (A) and mutant (A′) embryos, from which the cerebral cortex (cc) has been partially removed. In the mutant brain, the medial ganglionic eminence (mge) and to a lesser degree the lateral ganglionic eminence (lge) are reduced in size. Dorsal is up, rostral is to the right. (B,B′) Frontal sections of E12.5 wild-type (B) and mutant (B′) heads at telencephalic level, stained with Cresyl Violet. The MGE is almost completely absent at this level in the mutant telencephalon (B′, asterisk). (C,C′) BrdU incorporation by proliferating cells in the ventral telencephalon of wild-type (C) and mutant embryos (C′) at E12.5. BrdU-incorporating cells are less numerous in the VZ of the mutant MGE than in the wild-type (C′, arrow). There are also fewer dividing cells away from the ventricle in the mutant MGE, suggesting a lack of SVZ cells. (D,D′) TUNEL labelling of wild-type (D) and mutant (D′) ventral telencephalon at E12.5. There is no increase in cell death in the mutant ganglionic eminences. Olfactory epithelium from wild-type (D) and mutant (D′) E13.5 embryos are shown as controls (insets). An increase in cell death is observed in the mutant olfactory epithelium at this stage (Cau et al., 1997). (E,E′,F,F′) Sections of E18.5 wild-type (E,F) and mutant (E′,F′) brains stained with Cresyl Violet. (E,E′) Frontal sections at telencephalic levels; (F,F′) sagittal sections. The shape of the ventral forebrain and lateral ventricles is abnormal in the mutant brain, and the fibers of the corpus callosum do not cross the midline (E′). The roof of the mesencephalon is thinner in the mutant brain (F′). Bars, 200 μm (B-D); 400 μm (E,F).
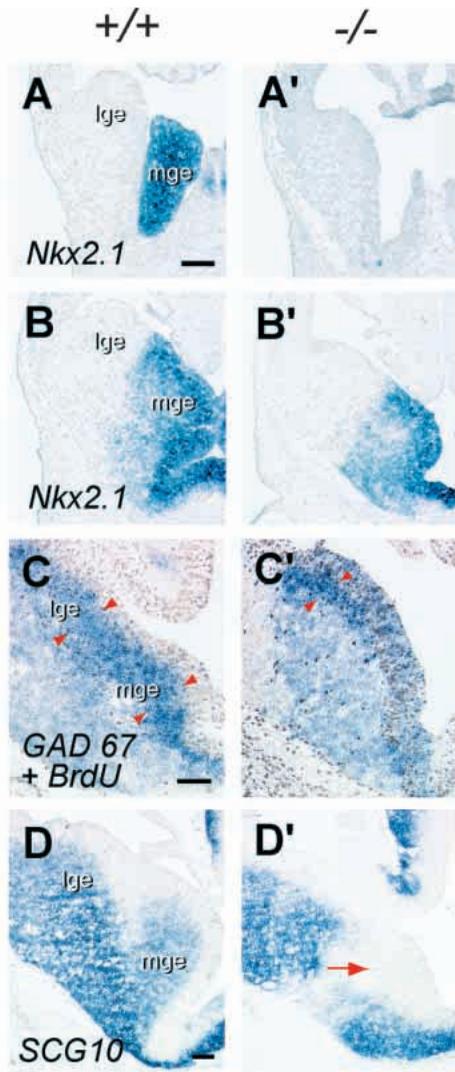
Fig. 2. Loss of neuronal precursors in *Mash1* mutant medial ganglionic eminence. Expression of *Nkx2.1* (A,A′,B,B′), *GAD67* (C,C′) and *SCG10* (D,D′) in the ventral telencephalon of E12.5 wild-type (A-D) and mutant (A′-D′) embryos. (A,A′,B,B′) The mutant MGE is reduced in size rostrally (A,A′), but is normally specified, as shown by *Nkx2.1* expression (B,B′). (C,C′) In wild-type LGE and MGE, high levels of *GAD67* expression and incorporation of BrdU (brown nuclei) mark neuronal precursors of the SVZ (arrowheads in C), whereas VZ cells are BrdU-positive but *GAD67*-negative, and neurons of the mantle zone express *GAD67* at a lower level and are BrdU-negative. The SVZ persists in the mutant LGE (arrowheads), but is missing in the MGE. (D,D′) *SCG10* expression is not affected in the mutant LGE, but is lacking in most of the mantle zone of the MGE (arrow in D′). lge, lateral ganglionic eminence; mge, medial ganglionic eminence. Bars, 200 μm (A,B); 100 μm (C,D).

Fig. 3. Loss of *Dll1*, *Dll3* and *Hes5* and ectopic *Mash1* expression in *Mash1* mutant brain. Expression of *Dll1* (A,A′,E,E′), *Dll3* (B,B′), *Hes5* (C,C′,F,F′) and *Mash1* (D,D′) in E12.5 wild-type (A-F) and mutant (A′-F′) embryos. (A-D,A′-D′) Frontal sections through the ventral telencephalon; (E,E′,F,F′) sagittal sections. The expression of *Dll1* in the VZ and SVZ of the ventral telencephalon (A,E) is absent in mutant embryos (A′, arrowhead in E′). *Dll1* expression in the tectal and tegmental areas of the mesencephalon and the spinal cord of wild-type embryos (E), is also reduced in mutant embryos (arrows in E′). The expression of *Hes5* in the VZ of the ventral telencephalon (C,F) is almost completely lost in mutant embryos (C′, arrowhead in F′). *Hes5* expression is also reduced in the spinal cord of mutant embryos (arrow in F′), whereas it is not affected in the mesencephalon. (D,D′): distribution of *Mash1* transcripts in the LGE of E12.5 wild-type (D) and *Mash1* mutant (D′) embryos. *Mash1* transcripts are present in a subset of VZ cells in wild-type telencephalon (D), and in almost all cells of the VZ in mutant LGE. Bars, 200 μm (A-C); 50 μm (D); 1 mm (E,F).

performed as described in Guillemot et al. (1993). The anti-TH (1:100 dilution) antibody was from Chemicon. Biotinylated anti-mouse and anti-rabbit immunoglobulin antibodies (1:200 dilution) and avidin-biotin complex reagents were from the Vectastain kit (Vector).

Histology, BrdU incorporation and TUNEL experiments

For histological analysis, embryos were fixed in Bouin's fixative overnight (up to E12.5) or for 3-4 days (E15.5 and older), processed for wa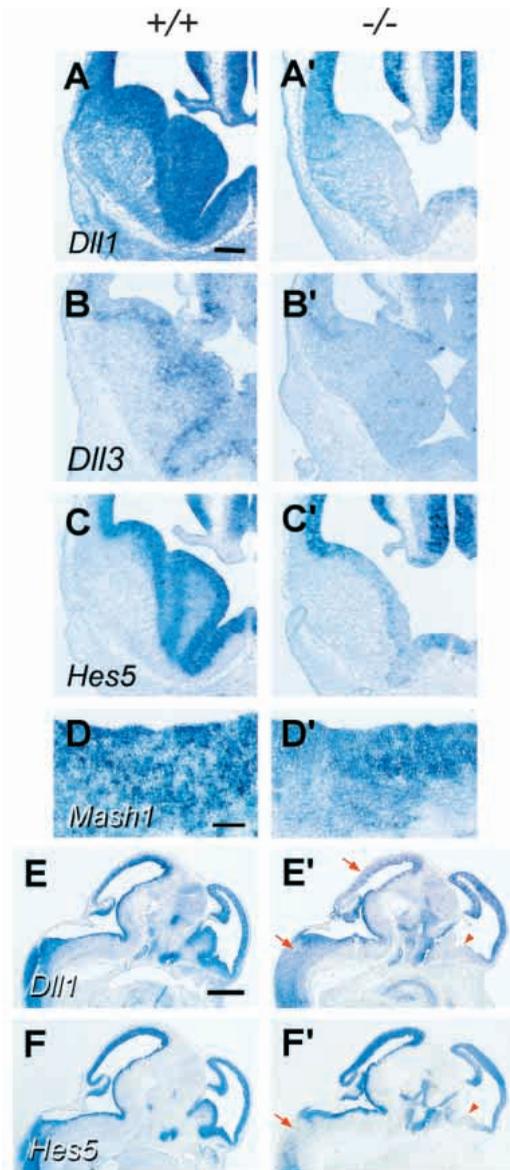x embedding, cut at 7 μm, and stained with Hematoxylin-Eosin or with Cresyl Violet. For BrdU incorporation experiments, pregnant females were injected intraperitoneally with 2 mg of BrdU (Sigma) and killed after 30 minutes (Fig. 1) or 2 hours (Figs 2 and 4), or

gestation was continued until E18.5 (Fig. 7). Embryos were processed as described above and BrdU labelling was revealed as described in Anderson et al. (1997a). The anti-BrdU monoclonal antibody (1:100 dilution) was from Boehringer Mannheim. The TUNEL procedure was performed as described in Cau et al. (1997), and sections were counterstained in Methyl Green.

RESULTS

Morphological defects in the brain of *Mash1* mutant embryos

In an effort to determine whether *Mash1* regulates neurogenesis in the CNS, we have examined the effects of a *Mash1* null mutation (Guillemot et al., 1993) on development of the ventral telencephalon, a region of the embryonic forebrain expressing *Mash1* at high levels. Telencephalic vesicles dissected from E12.5 wild-type embryos and opened dorsally clearly show the two ganglionic eminences protruding into the lateral ventricle (Fig. 1A). The medial (MGE) and lateral (LGE) ganglionic eminences contain the progenitor cells of the ventral telencephalon (Smart and Sturrock, 1979). In *Mash1* mutant embryos, the ganglionic eminences show a pronounced reduction in size, particularly the MGE, which lacks its rostral part (Fig. 1A′). The reduction of the mutant MGE is also apparent in frontal sections through the forebrain at the same stage (Fig. 1B and asterisk in 1B′).

To determine the cause of the size reduction of the MGE, we examined whether cell proliferation is affected in *Mash1* mutants at the time when the ganglionic eminences are generated, i.e. between E10.5 and E12.5 (Smart and Sturrock, 1979). Wild-type and mutant embryos were exposed between E10.5 and E12.5 to a 30-minute pulse of BrdU to label progenitor cells in S-phase of the cell cycle (Fig. 1C,C′ and data not shown). A decrease in the number of BrdU-incorporating cells was apparent in the VZ of the mutant ganglionic eminences between E10.5 and E12.5 (arrow in Fig. 1C′), and fewer labeled nuclei were detected outside the VZ in E12.5 mutant embryos, suggesting that precursor cells in the SVZ were also affected. The decrease in number of dividing cells was more conspicuous in the MGE region, which also showed more pronounced morphological defects. The reduced size of the MGE in *Mash1* mutant embryos is therefore probably due to a decrease in proliferation of progenitor cells. In agreement with this interpretation, no significant cell death was detected in the ventral telencephalon of mutant embryos between E11.5 and E13.5 using the TUNEL procedure (Fig. 1D,D′ and data not shown).

Mash1 mutant brains examined at later embryonic stages also show distinct morphological defects when compared to wild-type brains. In frontal sections through the forebrain of E18.5 embryos (Fig. 1E,E′), the lateral ventricles have an abnormal shape, the fibers of the corpus callosum do not cross the midline, and the septal area appears disorganized. Defects are also apparent in regions posterior to the telencephalon on sagittal sections (Fig. 1F,F′). Many divisions of the brain, in particular the mesencephalon, appear reduced in size. In this article, we have focused on the mutant phenotype in the telencephalon.

Loss of neuronal precursors in the medial ganglionic eminence

A molecular analysis was performed to further characterize the nature of the defects in the mutant MGE. We used the ventral forebrain marker *Nkx2.1* to study the regional specification of MGE progenitors. *Nkx2.1* is a homeobox gene whose expression in the forebrain is restricted to the MGE and, more posteriorly, to the hypothalamus (Lazzaro et al., 1991). *Nkx2.1* expression in wild-type MGE can be detected in frontal sections through the telencephalon at both rostral and caudal levels (Fig. 2A,B). In the telencephalon of *Mash1* mutants, *Nkx2.1* expression is absent at rostral levels, confirming the loss of the rostral MGE (Fig. 2A′). More caudally, however, *Nkx2.1* expression is maintained, indicating that the size reduction of the mutant MGE is not due to a defect in the regional specification of its progenitors (Fig. 2B′).

As *Mash1* has the ability to specify neuronal precursors in other lineages (see Introduction), we reasoned that loss of *Mash1* could block the production of committed neuronal precursors in the ganglionic eminences. We identified the neuronal precursors of the SVZ in the wild-type MGE and LGE by their incorporation of BrdU (Fig. 1D) and by their high level of expression of the gene encoding glutamic acid decarboxylase (*GAD67,* Behar et al., 1994; arrowheads in Fig. 2C). In contrast, the post-mitotic neurons of the mantle zone contain only low levels of *GAD67* transcripts. In mutant embryos, *GAD67* high, BrdU+ cells are present in the LGE, but are absent in the MGE, indicating that VZ cells fail to produce SVZ precursors in the remnant of MGE (Figs 1D′, 2C′). In addition, VZ progenitors ectopically express *GAD67* at lower levels throughout the mutant GE (see below for an analysis of this phenotype).

Loss of SVZ precursors should result in a reduction in neuronal production in the mutant MGE. We used the general neuronal markers SCG10 (Stein et al., 1988) to examine this possibility. SCG10 is normally expressed in the mantle zone of the MGE and LGE (Fig. 2D). This expression is absent in most of the mutant MGE (arrow in Fig. 2D′), whereas it is unaffected in the LGE. Therefore, loss of *Mash1* prevents the generation of neuronal precursors in the SVZ and the production of neurons in the mantle zone of the MGE, whereas the differentiation capacity of LGE progenitors appears unaffected. These results suggest that *Mash1* has a neuronal determination function in the MGE, where it is required for the specification of neuronal precursors.

Loss of *Dll* genes and *Hes5* expression

As neuronal precursors are present and undergo differentiation in the LGE of *Mash1* mutant mice (Fig. 2C′,D′), it was possible to study the effect of the loss of *Mash1* on later steps of neurogenesis in this region. We first examined if Notch signaling was affected in absence of *Mash1*. Proneural genes have been shown to regulate lateral inhibition in *Drosophila* and in *Xenopus*, through activation of the Notch ligand *Delta* (Künisch et al., 1994; Chitnis and Kintner, 1996). The two known mouse *Delta* homologs, *Delta-like 1* (*Dll1*) and *Delta-like 3* (*Dll 3*), are expressed in progenitor cell layers of the ventral telencephalon (Bettenhausen et al., 1995; Dunwoodie et al., 1997; Fig. 3A,B). In mutant embryos, expression of *Dll1* and *Dll3* is lost throughout the ventral telencephalon (Fig. 3A′,B′). Expression of *Dll* genes is also reduced in the mesencephalon and in the dorsal spinal cord (Fig. 3E and arrows in Fig. 3E′; data not shown), where *Mash1* is normally expressed at high levels (Guillemot and Joyner, 1993). *Mash1* is therefore necessary for the expression of two Notch ligands,

Dll1 and *Dll3*, in the ventral telencephalon and several other divisions of the CNS.

One output of Notch signaling is the activation of the genes of the *Enhancer of split [E(spl)]* family of transcriptional repressors. The murine *E(spl)*-related gene *Hes5* is expressed throughout the VZ of the embryonic CNS (Akazawa et al., 1992), and this expression is reduced or eliminated in mutant embryos in which Notch signaling is disrupted (de la Pompa et al., 1997). The expression of *Hes5* in the wild-type VZ (Fig. 3C,F) is almost completely eliminated in the mutant ganglionic eminences (Fig. 3C′) and is very reduced in the dorsal spinal cord (arrow in Fig. 3F′). Thus, using *Hes5* expression as a readout of the level of Notch signaling in the CNS, we conclude that *Mash1* is required for Notch signaling in several areas of the CNS, including the ventral telencephalon.

As a consequence of lateral inhibition, expression of proneural genes is restricted to a subset of cells in the neurectoderm of *Drosophila* and *Xenopus* embryos (reviewed in Lewis, 1996; Simpson, 1997). Similarly, *Mash1* expression is restricted to a subset of VZ cells in the ganglionic eminences (Porteus et al., 1994). To determine if this restriction of *Mash1* expression is a consequence of Delta-Notch signaling, we analysed *Mash1* transcription in wild-type and *Mash1* mutant embryos using an antisense probe containing the 3′ region of *Mash1* cDNA, which is not deleted in the knock-out allele (Guillemot et al., 1993). In contrast to their patchy distribution in VZ cells in wild-type ventral telencephalon (Fig. 3D), *Mash1* transcripts are present more uniformly in mutant VZ cells (Fig. 3D′). Therefore, lateral inhibition appears to restrict the expression of *Mash1* to a subset of progenitor cells in the VZ. Overall, these results demonstrate the very strong conservation of both regulation and function of the lateral inhibition pathway in murine neurogenesis, with activation of *Delta* expression in neuronal precursors by *achaete-scute*-related genes, and repression of these proneural genes by Notch signaling in neighbouring cells.

Mash1 mutant VZ cells prematurely express SVZ markers

Lateral inhibition controls the number of precursor cells that differentiate during primary neurogenesis and retinal development in vertebrate embryos (Chitnis et al., 1995; Henrique et al., 1997 and references therein). To determine how loss of *Mash1* and Notch signaling affects neurogenesis in the LGE of *Mash1* mutant embryos, we examined the expression of genes marking different stages in the development of neuronal lineages in the ventral telencephalon. As described above, *GAD67* is expressed in neuronal precursors of the SVZ and in neurons of the mantle zone, but is excluded from progenitors of the VZ. Members of the *Dlx* family of homeobox genes are also expressed in the ventral forebrain by cells at sequential stages of differentiation. *Dlx-1* and *Dlx-2* are expressed by subsets of VZ cells and by most cells in the SVZ (Porteus et al., 1994), while *Dlx-5* and *Dlx-6* are expressed in the SVZ and mantle zone, and not in the VZ (Anderson et al., 1997a).

In mutant embryos, *GAD67* is ectopically expressed by VZ cells of the ventral telencephalon at all developmental stages examined (Fig. 4A,A′ at E10.5; Fig. 4B,B′ at E12.5; Fig. 4D,D′ at E15.5. VZ and SVZ progenitors are marked by BrdU incorporation in Fig. 4C,C′). Similarly, *Dlx-5* is ectopically

expressed in the VZ in mutant embryo (Fig. 4E,E′ at E12.5; Fig. 4F,F′ at E15.5). *Dlx-1* transcripts are also found in virtually all cells of the mutant VZ (Fig. 4G′), an expression normally observed only in the SVZ (Fig. 4G). Therefore, SVZ markers are ectopically expressed in the VZ of the mutant ventral telencephalon.

We have also analysed the expression of the LIM homeobox gene *Lhx2*, a marker specific for the VZ cells in the forebrain (Xu et al., 1993; Fig. 4H). Expression of *Lhx2* expression is strongly reduced in the VZ of the mutant MGE, but remains at significant levels in the LGE (Fig. 4H′). This result suggests that, although mutant VZ cells have prematurely acquired SVZ characteristics, they have only partially changed phenotype in the LGE, as they maintain expression of a VZ marker.

Finally, expression of the post-mitotic neuron marker *SCG10* is restricted to the mantle zone of the LGE in mutant as in wild-type embryos (Fig. 2D,D′ and data not shown). Therefore, loss of *Mash1* and of Notch signaling affects the transition from the VZ to the SVZ but not the subsequent step in neuronal differentiation, the transition from SVZ to mantle zone, which appears to proceed normally.

Loss of specific neuronal populations in *Mash1* mutant basal ganglia and cerebral cortex

The loss of progenitors in the mutant MGE and the premature differentiation of VZ cells in the mutant LGE suggested that neuronal production could be affected by the loss of *Mash1*. To begin to examine the fate of defined neuronal populations in the telencephalon of *Mash1* mutant embryos, we studied the expression of several striatal markers, including the *dopamine receptor D2* (*D2R*), *enkephalin* and tyrosine hydroxylase (TH) (Mansour and Watson, 1995; Song and Harlan, 1994; van der Kooy, 1984). In the mutant striatum at E18.5, expression of TH is unchanged and that of *D2R* and *enkephalin* is only slightly reduced (Fig. 5A-C,A′-C′), indicating that striatal neurons are generated in the absence of *Mash1*. These observations are in agreement with the only minor reduction in size of the mutant LGE, from which these neurons derive. In the ventral telencephalon, *D2R* and *enkephalin* are also expressed by neurons of the olfactory tubercle (Fig. 5A,B). Expression of the two genes is almost absent from the olfactory tubercle in mutant brains (arrowheads in Fig. 5A′,B′). Expression of *enkephalin* in the region of the nucleus accumbens (arrow in Fig. 5B′) and that of TH in a more ventral region of the basal ganglia (arrow in Fig. 5C′) is also severely reduced. These data demonstrate that discrete neuronal populations, which remain to be identified, are missing in basal ganglia of mutant embryos.

Cell tracing experiments have shown that neurons born early in the LGE migrate near the ventrolateral surface of the telencephalon (De Carlos et al., 1996). To determine whether early born LGE neurons were affected by the loss of *Mash1*, pregnant females were injected with BrdU at E10.5 and embryos harvested at E18.5. In wild-type telencephalon, numerous neurons incorporate BrdU at E10.5 just prior to their birth and migrate ventrolaterally (arrows in Fig. 5D). The number of these neurons is drastically reduced in mutant brains (Fig. 5D′), confirming the loss of neuronal populations in the ventral telencephalon of *Mash1* mutant embryos.

It has been recently proposed that a large fraction of the GABAergic interneurons of the neocortex are born in the LGE,

from where they migrate through the cortical IZ and MZ to reach their final position (De Carlos, 1996; Anderson et al., 1997b; Tamamaki et al., 1997). We examined whether cortical interneurons were affected in *Mash1* mutant brains by studying the expression of *GAD67* and *Dlx-1*. In wild-type animals, these genes are expressed in cells located in the IZ and MZ of the cortex (Fig. 6A,B). In mutant mice, *GAD67* and *Dlx-1* expressing cells are less numerous in the IZ, and are almost completely absent in the MZ (arrows in Fig. 6A′,B′). Therefore, *Mash1* is required for the generation and/or migration of neocortical neurons with characteristics of GABAergic interneurons. The SVZ of the ventral telencephalon also generates interneurons that migrate rostrally to reach the olfactory bulb, beginning in late embryonic stages and continuing throughout adult life (reviewed in Alvarez-Buylla, 1997). Compared with wild types, the number of *GAD67*-positive cells in the olfactory bulb of mutant mice was also reduced, with some variability in both number and position of the remaining cells in the mutant bulbs (Fig. 6C,C′ at E18.5). Together, our data show that *Mash1* is required for the generation of defined neuronal populations in the basal ganglia and cerebral cortex.

DISCUSSION

Multiple neurogenesis defects in *Mash1* mutant telencephalon

Neurogenesis in the neural tube involves the production of a large variety of cell types at appropriate times and positions and in appropriate numbers. This complex process requires the regulation and coordination of cell divisions, neuronal differentiation, neuronal subtype specification and migration. Genes controlling these different processes are still poorly characterized. In this article, we have examined the function in development of the telencephalon of *Mash1*, a gene previously implicated in determination and differentiation of PNS neurons. We have observed a number of defects in the telencephalon of *Mash1* mutant mice, affecting several aspects of neurogenesis, including the proliferation and neuronal specification of progenitor cells and the timing of production of SVZ precursors. We propose that these defects correspond to two distinct functions of *Mash1*, which are both revealed in mutant ganglionic eminences. First, *Mash1* is absolutely required for the specification of neuronal precursor cells in the MGE. This specification activity is redundant in the mutant LGE where neuronal precursors are produced, thereby allowing a distinct function of *Mash1*, in the activation of Notch signaling and regulation of the

timing of production of precursor cell populations, to become apparent.

Loss of neuronal precursors in the MGE

The LGE and MGE are produced by the rapid accumulation of proliferating cells in the ventrolateral walls of the telencephalon, a process beginning around E10 in the mouse. This is soon followed by the establishment of two distinct populations of progenitor cells, in the VZ and SVZ, respectively. Cells in the embryonic SVZ have characteristics of committed neuronal precursors, as they are dividing but already express the neuronal marker *GAD67* (Behar et al., 1994; Fig. 2). In *Mash1* mutant embryos, the MGE largely fails to generate a SVZ, as revealed by a lack of high *GAD67*-expressing and BrdU-incorporating cells. There is also a deficit in *SCG10*-expressing neurons in the mantle zone (Fig. 2). These observations support the idea that *Mash1* has a neuronal
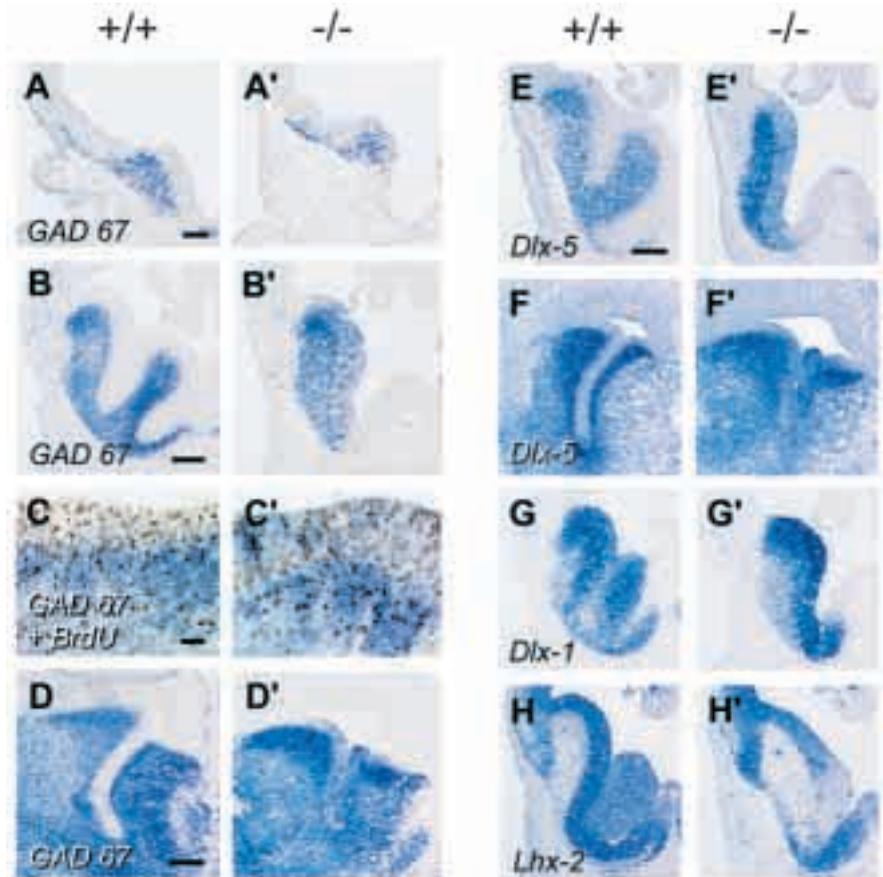

Fig. 4. *Mash1* mutant VZ cells precociously express SVZ markers. Expression of *GAD67* (A-D,A′-D′), *Dlx-5* (E,E′,F,F′), *Dlx-1* (G,G′) and *Lhx2* (H,H′) in the ventral telencephalon of wild-type (A-H) and mutant (A′-H′) embryos at E10.5 (A,A′), E12.5 (B,B′,C,C′,E,E′,G,G′,H,H′) and E15.5 (D,D′,F,F′). (A-D,A′-D′) *GAD67* is ectopically expressed in the VZ of the LGE in mutant embryos. (C,C′) In a double staining experiment with a *GAD67* RNA probe (blue) and an anti-BrdU antibody (brown), double labelled cells are found only in the SVZ in the wild-type embryo (C), but in both the VZ and SVZ in mutant embryos (C′). (E,E′,F,F′) Another marker of SVZ and mantle zone, *Dlx-5*, is ectopically expressed in the VZ of mutant embryos (E′,F′). (G,G′) *Dlx-1* is expressed in a subset of VZ cells and in all cells of the SVZ in wild-type embryos (G), while it is expressed by virtually all cells of the VZ in mutant embryos (G′). (H,H′) The expression of *Lhx2* in the VZ of the telencephalon (H), is strongly reduced in the MGE of mutant embryos (H′). Bars, 100 μm (A); 200 μm (B,D,E); 50 μm (C).

determination function in the MGE, where it is required to establish a population of neuronal precursors in the SVZ.

The mutant MGE also exhibits a more severe defect in cell proliferation, and is further reduced in size, than the LGE (Fig. 1). The increased severity of both phenotypes in the MGE suggests that a link may exist between the neuronal determination function of *Mash1* and the control of cell growth in the MGE. One possibility is that the reduction in progenitor cell proliferation could be a direct consequence of the failure to specify neuronal precursors, if neuronal precursors have a higher rate of proliferation than uncommitted neuroepithelial stem cells, a possibility which has not yet been addressed.
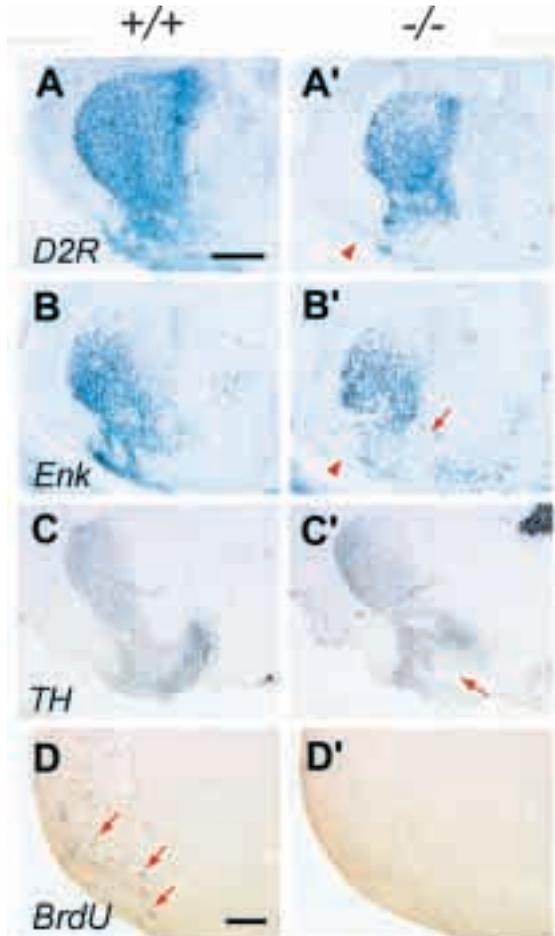
Alternatively, proliferation defects may be a more indirect consequence of the loss of *Mash1*. For example, the loss of expression of the LIM homeobox gene *Lhx2* (Fig. 4) may contribute to the decrease in cell proliferation in the mutant MGE. Expression of *Lhx2* appears to be one of the mechanisms by which telencephalic progenitors achieve a high rate of cell division, as mice mutant for *Lhx2* present a hypoplasia of the neocortex and basal ganglia and a reduction in progenitor cell proliferation (Porter et al., 1997). Further study of the molecular mechanisms underlying the neuronal determination function of *Mash1* and its interaction with the proliferation of progenitor cells will be necessary.

There is no spatial correlation in the mutant telencephalon between loss of progenitor cell populations, which is restricted to the MGE, and loss of Notch signaling, which is also observed in the mutant LGE. This suggests that the deficit in MGE progenitors is not due to a lack of lateral inhibition resulting in precocious differentiation and depletion of progenitor cells. Instead, we favor the hypothesis that *Mash1* is required to specify a neuronal fate in the progeny of neuroepithelial stem cells of the MGE, in a manner similar to that of a proneural gene in *Drosophila*, and that the failure of this specification results in a loss of progenitor cells, by an undefined mechanism.
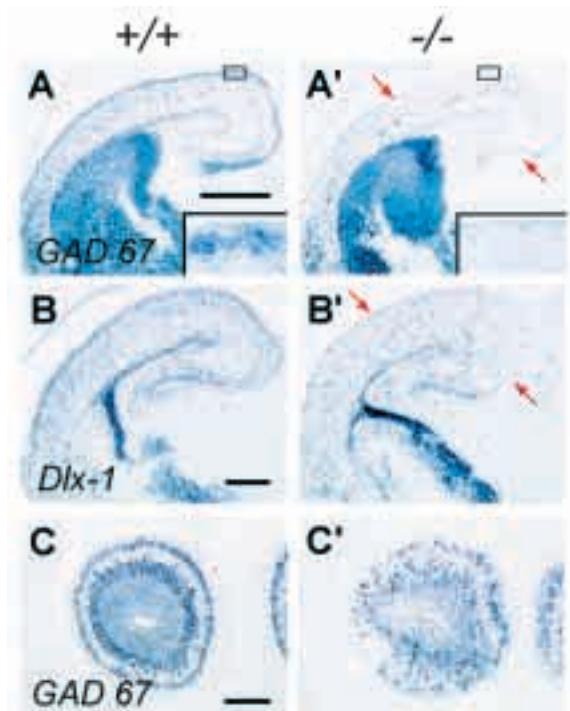

Fig. 5. Loss of specific neuronal populations in *Mash1* mutant ventral telencephalon. (A,A′,B,B′) Expression of *dopamine D2 receptor* (*D2R*) (A,A′) and *enkephalin* (B,B′) in wild-type (A,B) and mutant (A′,B′) ventral telencephalon at E18.5. *D2R*- and *enkephalin*-expressing neurons are reduced in number in the mutant striatum and are almost completely eliminated from the olfactory tubercle (arrowheads in A′ and B′). *enkephalin*-expressing neurons are also missing in the region of the nucleus accumbens (arrow in B′). (C,C′) Tyrosine hydroxylase (TH) immunohistochemistry on E18.5 wild-type (C) and mutant (C′) ventral telencephalon. TH staining in the striatum is comparable in both genotypes, but TH-expressing neurons are missing in ventral basal ganglia of the mutant brain (arrow in C′). (D,D′) Distribution in E18.5 wild-type (D) and mutant (D′) ventral telencephalon, of neurons born soon after an injection of BrdU at E10.5. Most BrdU-positive cells present ventro-laterally in the wild-type brain (arrows) are missing in the mutant brain. Bars, 400 μm (A-C); 100 μm (D).

Fig. 6. Loss of GABAergic interneurons in *Mash1* mutant neocortex and olfactory bulb. (A,A′,B,B′) Expression of *GAD67* (A,A′) and *Dlx-1* (B,B′) in the cerebral cortex of E17.5 (A,A′) and E18.5 (B,B′) wild-type (A,B) and mutant (A′,B′) embryos. Outlined areas in A and A′ are magnified in insets. A population of *GAD67, Dlx-1* expressing interneurons located in the marginal zone of the neocortex is missing in *Mash1* mutant brains (arrows in A′ and B′). (C,C′) Expression of *GAD67* in the olfactory bulb of E18.5 wild-type (C) and mutant (C′) embryos. The number of GABAergic interneurons is reduced in the mutant olfactory bulb. Bars, 400 μm (A,B); 200 μm (C).

Loss of Notch signaling

The study of primary neurogenesis in *Xenopus* embryos has shown that essential steps in the regulation of neurogenesis by the Notch pathway have been conserved between *Drosophila* and vertebrates (reviewed in Lewis, 1996). In the mouse, the neural determination genes *Ngn1* and *Ngn2* are required for expression of the *Delta* gene *Dll1* in cranial sensory neuron precursors (Ma et al., 1998; Fode et al., 1998). We have further characterized the regulation of Notch ligands during murine neurogenesis by demonstrating that *Mash1* activates *Dll1* and *Dll3* in numerous regions of the CNS. In *Drosophila*, transcription of the *Delta* gene is directly regulated by proneural proteins (Künish et al., 1994). Therefore the loss of expression of the *Delta* homologs *Dll1* and *Dll3* is probably a direct consequence of loss of *Mash1* function.

We have also used expression of the *E(spl)* related gene *Hes5* to assess the importance of *Mash1* function for Notch signaling in the CNS. *Hes5* is a structural homolog of the *E(spl)* genes of *Drosophila*, which mediate Notch function in neurogenesis (Akazawa et al., 1992; reviewed in Kageyama and Nakanishi, 1997). Mouse embryos lacking both *Hes5* and the related gene *Hes1* present a neurogenic phenotype of increased neuronal density (E. Cau, G. Gradwohl and F. G., unpublished), and fail to respond to an activated form of Notch1 (T. Ohtsukato, M. Ishibashi, G. Gradwohl, F. G., S. Nakanishi and R. Kageyama, unpublished), demonstrating that *Hes5* and *Hes1* mediate Notch signaling in neural progenitor cells. Furthermore, *Hes5* expression is down-regulated in mouse embryos mutant for *Notch1* or for the Notch co-factor *RBP-J*, demonstrating that this gene is activated in the neural tube in response to Notch signaling (de la Pompa et al., 1997). *Hes5* expression is therefore a readout of the level of Notch activity in progenitor cells, and the reduction or loss of *Hes5* expression in the ventral telencephalon and dorsal spinal cord of *Mash1* mutant embryos shows that *Mash1* is required to activate Notch signaling in these tissues.

Premature VZ cell differentiation

In contrast to the MGE, most progenitors of the LGE, including SVZ precursors, are generated in *Mash1* mutant embryos and differentiate to produce striatal neurons, demonstrating a redundancy of the neuronal determination function of *Mash1* in these cells (see below). Loss of *Mash1* however results in the precocious expression of SVZ markers by VZ cells of the LGE. This premature differentiation phenotype results from the loss of a function of *Mash1* distinct from that of neuronal precursor specification, which probably corresponds to the activation of *Delta* genes and of Notch signaling, processes which are also affected in the LGE. Delta-Notch signaling thus appears to be involved in maintaining two distinct populations of progenitors in the VZ and SVZ, respectively, by preventing the premature acquisition of SVZ characteristics by VZ progenitors. This function is reminiscent of the control by Notch signaling of the timing of progenitor cell differentiation demonstrated in *Xenopus* and chick embryos (Chitnis et al., 1995; Henrique et al., 1997 and references therein). However, blocking Notch signaling by expression of a dominant negative form of Delta-1 in the chick retina results in a premature arrest of division of neural progenitor cells and in an excess of neurons (Henrique et al., 1997). In contrast, loss of Notch signaling in *Mash1* mutants does not lead to accelerated

neuronal differentiation or to progenitor cell division arrest, suggesting that these steps are regulated in the SVZ by a pathway independent of *Mash1*, *Dll1*, *Dll3* and *Hes5*.

Loss of discrete neuronal populations in the telencephalon

Discrete neuronal populations normally produced by ventral telencephalic progenitors and located in the cerebral cortex and the basal ganglia are missing in *Mash1* mutant embryos. Some of these defects are likely to be due, at least in part, to the loss of ventral telencephalic precursor populations observed in *Mash1* mutant embryos. In addition, some neuronal losses could be an indirect consequence of the premature acquisition of a SVZ phenotype by mutant VZ cells. It has been proposed that the earliest born neurons in the LGE originate directly from the VZ, before the emergence of a SVZ (Bhide, 1996; De Carlos et al., 1996). Birth dating experiments, and *enkephalin*- and *D2R*-labelling of *Mash1* mutant brains, demonstrate the loss of neurons near the ventro-lateral surface of the telencephalon (Fig. 5), a region where early born neurons have been shown to migrate (De Carlos et al., 1996). As a result of their premature acquisition of a SVZ phenotype, progenitor cells of the mutant VZ may fail to produce these early neuronal cell types.

Genetic interactions in the telencephalon

Besides *Mash1* and *Lhx2*, mentioned above, the homeobox genes *Dlx-1* and *Dlx-2* are also important for the development of the basal ganglia. Mice mutant for both *Dlx-1* and *Dlx-2* present a striatal defect, interpreted as a block in differentiation specifically affecting late-born matrix neurons (Anderson et al., 1997a). *Dlx-1/2* expression is up-regulated in most VZ cells in *Mash1* mutant ventral telencephalon, indicating that these genes interact. This negative regulation of *Dlx-1/2* by *Mash1* is probably due to a process of lateral inhibition, rather than to a cell-intrinsic mechanism, as *Dlx-1/2* and *Mash1* have very similar expression patterns in the ventral telencephalon, in cell clusters in the VZ and almost uniform in the SVZ (Porteus et al., 1994; Liu et al., 1997), and *Mash1* and *Dlx-2* are indeed co-expressed (Porteus et al., 1994). This implies that *Dlx-1/2* may be expressed like *Mash1* in committed neuronal precursors before they migrate to the SVZ, and repressed by Delta-Notch signaling in surrounding VZ cells. The up-regulation of *Dlx-1/2* genes in *Mash1* mutant embryos would thus be a consequence of the loss of *Delta* gene expression and Notch signalling in these embryos. To determine whether this is the case and more generally whether the defects observed in *Mash1* mutant telencephalon are caused by the loss of Delta-Notch signaling or the loss of a cell-autonomous function of *Mash1*, we are generating chimeric embryos consisting of a mosaic of wild-type and *Mash1* mutant cells in order to analyse the degree of rescue of mutant defects in these chimeras.

Neuronal determination genes in the telencephalon

Mash1 has been previously shown to have determination and differentiation functions in PNS neuronal lineages. Our results show that *Mash1* is also uniquely required to generate precursor cells in the MGE. In contrast, the determination function of *Mash1* appears to be redundant in LGE progenitors, as large numbers of striatal neurons are produced from the mutant LGE. *Neurogenin* genes are unlikely to be involved in

the specification of striatal neuron precursors and to compensate for the loss of *Mash1*, as they are not detectably expressed in the ventral telencephalon of wild-type or *Mash1* mutant embryos (data not shown). Therefore, yet unidentified neuronal determination genes must exist in the developing mouse brain. New genes with neuronal determination activity may have been recruited during the course of vertebrate evolution to permit the addition of novel territories to the brain.

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