A role for neural determination genes in specifying the dorsoventral identity of telencephalic neurons

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***Neurogenin1* (*Ngn1*), *Neurogenin2* (*Ngn2*), and *Mash1* encode bHLH transcription factors with neuronal determination functions. In the telencephalon, the *Ngns* and *Mash1* are expressed at high levels in complementary dorsal and ventral domains, respectively. We found that *Ngn* function is required to maintain these two separate expression domains, as *Mash1* expression is up-regulated in the dorsal telencephalon of *Ngn* mutant embryos. We have taken advantage of the replacement of the *Ngns* by *Mash1* in dorsal progenitors to address the role of the neural determination genes in neuronal-type specification in the telencephalon. In *Ngn2* single and *Ngn1*; *Ngn2* double mutants, a population of early born cortical neurons lose expression of dorsal-specific markers and ectopically express a subset of ventral telencephalic-specific markers. Analysis of *Mash1*; *Ngn2* double mutant embryos and of embryos carrying a *Ngn2* to *Mash1* replacement mutation demonstrated that ectopic expression of *Mash1* is required and sufficient to confer these ventral characteristics to cortical neurons. Our results indicate that in addition to acting as neuronal determinants, *Mash1* and *Ngns* play a role in the specification of dorsal-ventral neuronal identity, directly linking pathways of neurogenesis and regional patterning in the forebrain.**

[*Key Words*: Cerebral cortex; thalamus; neurogenesis; mouse mutants; *Mash1*; *neurogenin*; bHLH gene]

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The molecular events controlling the generation of specific neuronal types at precise positions and in appropriate numbers in the neural tube are beginning to be deciphered. A major advance in our understanding of this process came from morphological and molecular marker analyses, which revealed that the neural tube is subdivided into transverse and longitudinal domains from early embryonic stages. In particular, it has been proposed that the forebrain neuroepithelium can be subdivided into six prosomeric units (Puelles and Rubenstein 1993). An important characteristic of the forebrain prosomeres is that progenitor cell mixing is prevented across their boundaries (Figdor and Stern 1993; Neyt et al. 1997), a restriction that is required for the subsequent generation of anatomically and functionally distinct compartments in the adult brain.

The six prosomeric units of the forebrain are transverse domains that can be further subdivided longitudinally by the restricted expression patterns of many regulatory molecules (for review, see Puelles and Rubenstein 1993). In the embryonic telencephalon, which gives rise to the cerebral cortex dorsally and the basal ganglia ventrally, homeobox genes of the empty spiracles (*Emx1*, *Emx2*) and paired homeobox (*Pax6*) families are expressed exclusively in dorsal progenitors, whereas ventral progenitors express homeobox genes of the *Nkx* (*Nkx2.1*) and *distal-less* (*Dlx1*, *Dlx2*) families (for review, see Rubenstein et al. 1998). Postmitotic neurons also have distinct molecular phenotypes in these two subdivisions of the telencephalon. For example, *GAD67*, which encodes the enzyme required for the biosynthesis of the GABA neurotransmitter, is expressed by ventral telencephalic neurons of the basal ganglia, whereas the bHLH transcription factors *NeuroD*, *Math2*, *Nscl1*, and *Nscl2* and the T-box gene *Tbr1* (Bulfone et al. 1995; Lee 1997) are expressed only in dorsally derived neurons of the cerebral cortex. Although the genetic networks leading to the generation of distinct neuronal phenotypes, cytoarchitecture, and axonal connections remain for the most part unknown, the acquisition of different molecular identities by dorsal and ventral telencephalic progeni-

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tors is likely the basis for the later diversification of neuronal types between the cerebral cortex and basal ganglia (for review, see Fishell 1997).

An important consideration regarding the specification of neuronal identities is that the pathways underlying cell-type specification must be coordinated with genetic programs that impart generic neuronal properties (for review, see Anderson and Jan 1997; Edlund and Jessell 1999). In *Drosophila*, the proneural genes, which include genes of the *achaete–scute* complex (*as-c*) and *atonal* (*ato*), confer both panneuronal properties and neuronal subtype-specific identities to neural precursors (for review, see Jan and Jan 1994). Gain-of-function analyses indicate that in addition to acting as neuronal determinants, *as-c* genes play a role in the specification of external sensory organs, whereas *ato* promotes chordotonal organ development (Chien et al. 1996; Jarman and Ahmed 1998). In addition, loss-of-function analyses and rescue experiments indicate that *as-c* genes regulate some aspects of neuronal phenotype identity in CNS precursors (Parras et al. 1996; Skeath and Doe 1996).

In vertebrates, the *ato*-related genes *Ngn1* and *Ngn2* (*Ngns*) and the *as-c*-related gene *Mash1* have neural determination functions in cranial sensory lineages (Fode et al. 1998; Ma et al. 1998), and in peripheral autonomic lineages, olfactory epithelium, and ventral forebrain (Cau et al. 1997; Lo et al. 1997; Casarosa et al. 1999; Torii et al. 1999), respectively. In addition, the spatially restricted and largely nonoverlapping patterns of expression of *Mash1* and the *Ngns* (Gradwohl et al. 1996; Ma et al. 1997) suggest that they may also play a role in the specification of distinct neuronal identities. In support of this hypothesis, it has been demonstrated that *Mash1* is required for the differentiation of a noradrenergic phenotype in hindbrain and PNS neurons, acting through the activation of, or in concert with, the *Phox2* homeodomain transcription factors (Hirsch et al. 1998; Lo et al. 1998; for review, see Goridis and Brunet 1999). Loss-of-function and gain-of-function experiments have also indicated that *Mash1* and the *ato*-related gene *NeuroD* control the ratio of interneurons of distinct classes in the retina (Tomita et al. 1996; for review, see Cepko 1999; Morrow et al. 1999). Finally, it has been demonstrated that in the chick, the *Ngns* can induce the expression of panneuronal as well as sensory-specific markers in neural crest cells, and in the dermomyotome, a mesodermal tissue (Perez et al. 1999).

In the telencephalon, *Mash1* is expressed at high levels by ventral progenitors, whereas *Ngn1* and *Ngn2* are expressed exclusively in cells of the dorsal ventricular zone (VZ) (Gradwohl et al. 1996; Sommer et al. 1996; Ma et al. 1997). Here, we have addressed the question of whether the restricted patterns of expression of *Mash1* and the *Ngns* in the telencephalon reflect a role for these genes in the specification of distinct neuronal identities in the basal ganglia and cerebral cortex, respectively. We show that *Ngn* gene function is required to prevent ectopic *Mash1* expression in the dorsal telencephalon, ensuring that the pallial and subpallial subdivisions of the telencephalon express different neural determination genes. The up-regulation of *Mash1* in the dorsal telencephalon compensates to some extent for the loss of the determination function of the *Ngns*, as neurogenesis defects in *Ngn* mutant cortices are mild, as compared with the more severe defects in the *Ngn2;Mash1* mutant cortex. However, the importance of restricting *Mash1* expression to the ventral telencephalon for correct neuronal patterning is highlighted by the observation that ectopic *Mash1* expression in *Ngn* mutants results in the misspecification of a subpopulation of early born cortical neurons, which lose expression of several dorsal markers (e.g., *Math2*, *Nscl1*, *Nscl2*, *Tbr1*) and ectopically express the ventral markers *Dlx1*, *Dlx2*, *Dlx5*, and *GAD67*. These data are supported by the results of forced expression of *Mash1* in dorsal telencephalic progenitors, which is sufficient to confer ventral characteristics to a subset of cortical neurons. These results indicate that *Mash1* and the *Ngns* are involved in coupling neural determination and the specification of neuronal phenotypes during forebrain development.

Results

Complementary expression of Ngns *and* Mash1 *in the telencephalon*

Previous expression studies have shown that *Mash1* and the *Ngns* are expressed in a complementary manner in the developing telencephalon and diencephalon, suggesting that these genes may play a role in specifying regional neuronal identity in the forebrain (Gradwohl et al. 1996; Ma et al. 1997). To begin to investigate this hypothesis, we compared the expression patterns of these genes in greater detail. To better trace *Ngn2*-expressing progenitors, a new null allele of *Ngn2* ($Ngn2^{lacZKI}$), in which coding sequences of the bHLH domain were replaced with a *lacZ* reporter, was introduced in mice by standard embryonic stem (ES) cell technology (Fig. 1A). Loss of most *Ngn2* coding sequences, including the bHLH domain, in $Ngn2^{lacZKI}$ homozygous embryos was confirmed by Southern blot (Fig. 1A) and PCR analysis (data not shown). The $Ngn2^{lacZKI}$ allele behaves as a null allele on the basis of the identical perinatal lethality and cortical phenotype observed in $Ngn2^{lacZKI}$ and $Ngn2^{neo}$ null homozygous embryos (Fode et al. 1998; this paper; data not shown).

At embryonic day (E) 12.5, transcripts for *Ngn1* (Fig. 1B) and *Ngn2* (Fig. 1C) are restricted to the VZ of the dorsal telencephalon, showing a sharp extinction of expression at the border with the ventral telencephalon. *Ngn1* is expressed in a graded manner, with higher levels in the lateral VZ, whereas *Ngn2* transcripts appear distributed more uniformly throughout the dorsal VZ. Staining of $Ngn2^{lacZKI}$ heterozygous embryos at E12.5 with X-Gal indicated that β-galactosidase activity is detected in similar restricted domains of the forebrain as *Ngn2* transcripts, including the dorsal telencephalon and dorsal thalamus (Fig. 1D). However, β-galactosidase activity is detected in a gradient in the VZ of the telencephalon, with higher levels in dorsomedial progenitors

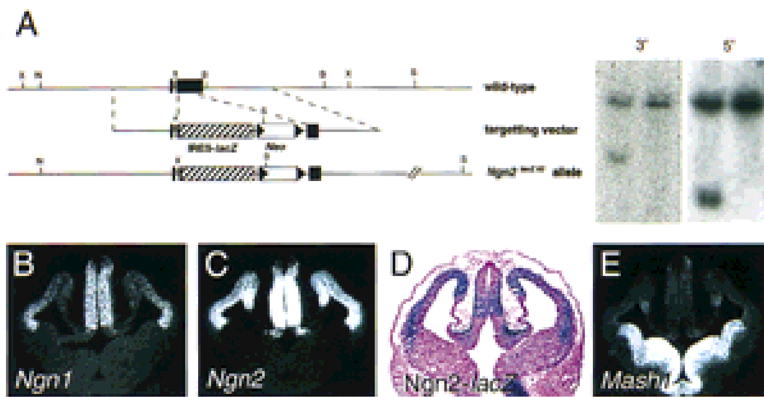

Figure 1. *Mash1* and *Ngns* are expressed in complementary patterns in the embryonic telencephalon. (*A*) A new *Ngn2* null allele was generated by replacing the sequence encoding the bHLH domain with the *lacZ* reporter (*Ngn2*^{lacZKI}). Schematic representation of the endogenous *Ngn2* locus (*top*), *Ngn2* targeting vector (*middle*), and recombined allele (*bottom*). Southern blot analysis of *Not*I–*Spe*I-digested ES cell genomic DNA with 5′ and 3′ external probes that recognize a common 20-kb wild-type band and 7.5- and 10.5-kb mutant bands, respectively. (*B*,*C*,*E*) Distribution of transcripts for *Ngn1* (*B*), *Ngn2* (*C*) and *Mash1* (*E*) on frontal sections through the forebrain of E12.5 embryos. (*D*) X-gal staining of *Ngn2*^{lacZKI} heterozygous embryos at E12.5. *Ngn1* and *Ngn2* are expressed in proliferating progenitors of the dorsal telencephalon and dorsal thalamus in an almost completely overlapping fashion. *Mash1* is expressed in a complementary manner with transcripts at high levels in the VZ of the ventral telencephalon and ventral thalamus.

(Fig. 1D) in comparison with the more uniform distribution of *Ngn2* transcripts. In addition, β-galactosidase activity is maintained in postmitotic neurons accumulating throughout the preplate, serving as a short-term lineage tracer and allowing us to conclude that *Ngn2*-derived neurons are present throughout the lateral to medial extent of the cortex.

In contrast to *Ngns*, *Mash1* is expressed at high levels in ventral progenitors located in the lateral (LGE) and medial ganglionic eminences (MGE) and only at low levels in the dorsal telencephalon (Fig. 1E). The complementary patterns of expression of these genes are also observed in the dorsal thalamus, in which *Ngn1* (Fig. 1B) and *Ngn2* (Fig. 1C) but not *Mash1* (Fig. 1E), are expressed at high levels.

Ngns *down-regulate* Mash1 *expression in dorsal telencephalon and dorsal thalamus*

Cross-regulatory interactions exist between *Ngn1* and *Ngn2* in neuronal lineages of the PNS, including the dorsal root and cranial ganglia (Fode et al. 1998; Ma et al. 1998, 1999). Thus, we examined whether the overlap in expression of *Ngn1* and *Ngn2* in the telencephalon reflects cross activation or independent means of activation, by studying *Ngn1* expression in *Ngn2* mutants and vice versa. In the dorsal telencephalon of *Ngn2* mutants, *Ngn1* expression is lost in medial and dorsal domains but is maintained in the lateral VZ (Fig. 2B). In contrast, *Ngn2* expression is detected throughout the dorsal telencephalon of *Ngn1* mutants, although the levels appear reduced (Fig. 2D). Thus, regulatory interactions between the *Ngns* exist in the forebrain, such that *Ngn2* mutant progenitors in the dorsal/medial cortex fail to express both *Ngn1* and *Ngn2*, and *Ngn1* mutant progenitors express reduced levels of *Ngn2* throughout the dorsal telencephalon.

We also investigated potential regulatory interactions between *Mash1* and the *Ngns* in the telencephalon. An up-regulation of *Mash1* expression was observed throughout the dorsomedial cortical VZ of *Ngn2* mutant embryos at E12.5 (Fig. 2G) and later stages (E13.5; Fig. 2J).

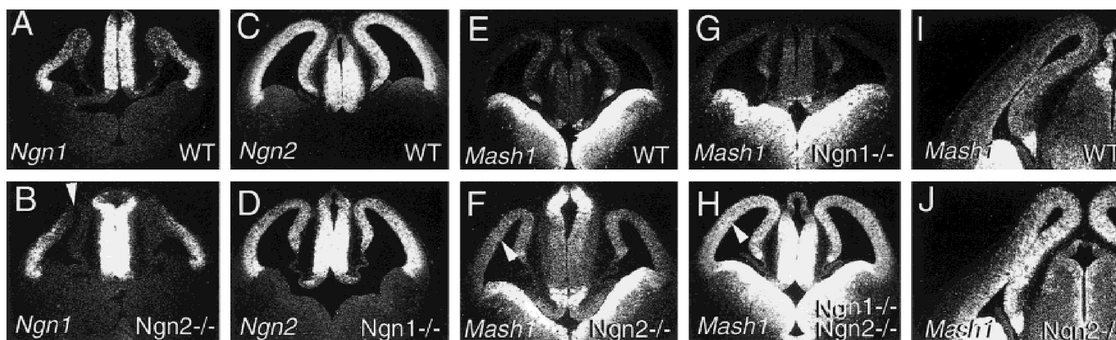

Figure 2. *Ngns* down-regulate *Mash1* transcription in the dorsal telencephalon. (*A*,*B*) Distribution of *Ngn1* transcripts on frontal sections of E12.5 wild-type (*A*) and *Ngn2* mutant (*B*) forebrain. *Ngn1* expression is dependent on *Ngn2* function in dorsal and medial cortical domains (arrowhead, *B*) and independent of *Ngn2* in a lateral cortical domain. (*C*,*D*) Distribution of *Ngn2* transcripts on frontal sections of E12.5 wild-type (*C*) and *Ngn1* mutant (*D*) forebrain. *Ngn2* is expressed at reduced levels in cortical progenitors of *Ngn1* mutants, suggesting that *Ngn2* transcription is partially dependent on *Ngn1* function throughout the dorsal telencephalon. (*E*,*J*) Distribution of *Mash1* transcripts on frontal sections of E12.5 wild-type (*E*), *Ngn1* mutant (*G*), *Ngn2* mutant (*G*), and *Ngn1; Ngn2 double mutant (H*), and of E13.5 wild-type (*I*) and *Ngn2* mutant (*J*) forebrain. *Mash1* expression is up-regulated in the VZ of the dorsal telencephalon (arrowhead in *F*and *H*) and dorsal thalamus of *Ngn2* single and more strikingly of *Ngn1;Ngn2* double mutant embryos.

This up-regulation was less apparent in the lateral VZ, in which *Ngn1* expression is maintained in *Ngn2* mutants. No changes in *Mash1* expression levels were observed in the forebrain of *Ngn1* single mutants (Fig. 2F; data not shown), but *Mash1* up-regulation was strikingly enhanced in the dorsal telencephalon and dorsal thalamus of *Ngn1;Ngn2* double mutant embryos (Fig. 2H,K). In contrast, *Ngn1* and *Ngn2* expression patterns were unaltered in the forebrain of *Mash1* mutant embryos (data not shown). Taken together, these results suggest that the *Ngns* function in a partially redundant manner to restrict *Mash1* expression to limited domains of the forebrain neuroepithelium. The nature of the regulatory interactions between *Ngns* and *Mash1* remains to be characterized.

Dorsal neurons down-regulate dorsal markers in Ngn *mutant telencephalon*

Given that the *Ngns* function as determination genes for PNS neurons (Fode et al. 1998; Ma et al. 1998), their mutation may result in neurogenesis defects in the dorsal telencephalon. To address this possibility, expression of the panneuronal marker *SCG10* was examined at E12.5 in the telencephalic preplate (PP), the layer of earliest born cortical neurons. *SCG10* expression is not sig-

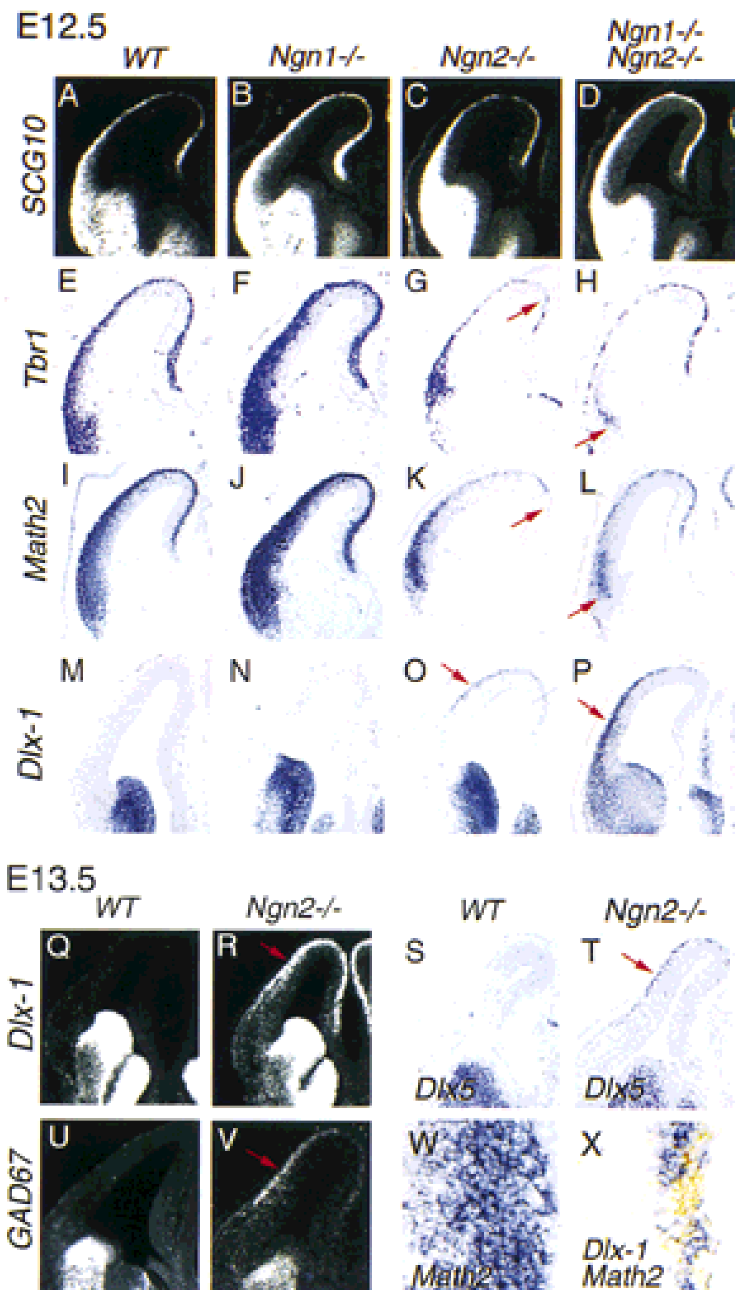

Figure 3. Cortical preplate neurons lose dorsal characteristics and acquire ventral properties in *Ngn* mutants. (*A–D*) Distribution of *SCG10* transcripts on frontal sections of E12.5 wild-type and *Ngn* mutant telencephalon. *SCG10*+ neurons are found in the telencephalic PP of wild-type embryos (*A*), *Ngn1* mutants (*B*), *Ngn2* mutants (*C*), and *Ngn1;Ngn2* double mutants (*D*). (*E–L*) Distribution of *Tbr1* transcripts (*E–H*) and *Math2* transcripts (*I–L*) on frontal sections of E12.5 wild-type and *Ngn1* mutant telencephalon, revealing a strong reduction in the expression of these dorsal-specific markers by postmitotic neurons of the dorsal and medial cortex in *Ngn2* mutants (arrows, *G* and *K*, respectively) and in lateral cortex of *Ngn1;Ngn2* double mutants (arrows, *H* and *L*, respectively). (*M–P*) Distribution of *Dlx1* transcripts on frontal sections of E12.5 wild-type and *Ngn* mutant telencephalon. This ventral marker is ectopically expressed by PP neurons in the dorsal cortex of *Ngn2* mutant embryos (arrow, *O*) and in the dorsal and lateral cortex of *Ngn1;Ngn2* double mutant embryos (arrow, *P*). (*Q*,*R*) Expression of *Dlx1* in the telencephalon of E13.5 wild-type (*Q*) and *Ngn2* mutant (*R*) embryos, demonstrating that the ectopic expression of *Dlx1* has expanded to the medial and lateral cortex in *Ngn2* mutant at this stage (arrow, *R*). (*S–V*) Expression of *Dlx5* (*S*,*T*) and *GAD67* (*U*,*V*) in the telencephalon of E13.5 wild-type and *Ngn2* mutant embryos, demonstrating that these genes are also ectopically expressed in *Ngn2* mutant cortex at this stage (arrow, *T* and *V*, respectively). High-magnification views of *Math2* (purple) and *Dlx1* (orange) expression in the dorsal cortex of E13.5 wild-type (W) and *Ngn2* mutant (X) embryos. *Math2* and *Dlx1* are not coexpressed in the *Ngn2* mutant cortex, indicating that mutant PP neurons adopt either a dorsal or a ventral fate. The same misspecification of cortical neurons was observed in $Ngn2^{neo}$ and $Ngn2^{lacZKI}$ homozygous mutants.

nificantly different in the PP of wild-type embryos and *Ngn2* single mutants (Fig. 3A,C). In *Ngn1* single (Fig. 3B) or *Ngn1;Ngn2* double (Fig. 3D) mutant embryos, there is an apparent increase in the number of *SCG10*-expressing cells, a phenotype for which we have not yet identified a cause. However, the presence of neurons in the PP of *Ngn* mutants suggested that up-regulation of *Mash1* could partially compensate for the lack of *Ngn* activity and permit cortical neurogenesis to proceed.

To examine whether the replacement of *Ngns* by *Mash1* as a determination gene in *Ngn* mutant cortical progenitors resulted in specification defects, we first analyzed the expression of several dorsal-specific neuronal markers in the telencephalon of *Ngn* mutants. Reductions in expression levels of *Tbr1* (Fig. 3G), *Math2* (Fig. 3K), *Nscl1*, and *Nscl2* (data not shown) were observed in the dorsal and medial PP of *Ngn2* mutants at E12.5. *Tbr1* and *Math2* expression levels were not reduced in the PP of *Ngn1* single mutants (Fig. 3F,J), but expression levels of these genes were further reduced in the lateral cortex of *Ngn1;Ngn2* double mutants (Fig. 3H,L).

In contrast to the dorsal postmitotic markers, no changes were observed in the level of expression of dorsal-specific progenitor markers, including *Emx1*, *Emx2*, and *Pax6*, in the *Ngn2* mutant cortex (data not shown), suggesting that with the exception of *Mash1* up-regulation, the molecular phenotype of dorsal progenitors is unaltered in *Ngn2* mutants.

Dorsal neurons ectopically express ventral markers in Ngn *mutant telencephalon*

We hypothesized that the down-regulation of dorsal-specific markers by *Ngn* mutant cortical neurons might reflect their respecification to a more ventral identity. To address this possibility, the expression patterns of several ventral telencephalic-specific genes were examined in *Ngn* mutant embryos. In wild-type E12.5 embryos, the closely related homeobox genes *Dlx1* and *Dlx2* are expressed at high levels in the VZ and SVZ of the ventral telencephalon, whereas their transcripts are not detected in dorsal domains (Figs. 3M and 6J, below, data not shown). Ectopic *Dlx1* and *Dlx2* expression was detected in the *Ngn2* mutant cortex, beginning at E12 in the rostral and dorsal cortex (E12.5; Fig. 3O; data not shown), and progressing subsequently to more medial and caudal positions (E13.5; Fig. 3R) in a manner reflecting the neurogenic gradient. Ectopic *Dlx1* expression was restricted to the neuronal layer and was not detected in the lateral cortex of *Ngn2* single mutants, in which *Ngn1* expression is maintained, nor in the cortex of *Ngn1* single mutants, which maintain *Ngn2* expression (Fig. 3N). However, an expansion of ectopic *Dlx1* expression into the lateral and caudal cortex was observed in *Ngn1;Ngn2* double mutants at E12.5 (Fig. 3P). By E13.5, additional ventral-specific genes, such as *Dlx5* (Fig. 3T) and *GAD67* (Fig. 3V), which encode a biosynthetic enzyme for the GABA neurotransmitter, were ectopically expressed by cortical neurons in *Ngn2* single mutants. Several other ventral telencephalic markers, such as *Lhx6*, *Lhx7*, *Islet1*, and *Nkx2.1*, were not ectopically expressed in *Ngn* mutant cortical neurons, suggesting that these neurons are either only partially ventralized or have activated a subtype-specific ventral differentiation program (data not shown). Consistent with the idea that the decreased expression of dorsal markers correlates with a change to a ventral-like fate by *Ngn* mutant cortical neurons, double-labeling experiments revealed that *Dlx1* and *Math2* are expressed in largely nonoverlapping and complementary populations of neurons in the *Ngn2* mutant PP (Fig. 3P).

Changes in neuronal identity in the dorsal thalamus of Ngn *mutants*

To determine whether neural *bHLH* genes are involved in specification of neuronal phenotypes throughout the forebrain, we examined neuronal patterning in the thalamus, a region in which *Mash1* and the *Ngns* are also expressed in a complementary manner. *Ngn1* and *Ngn2* are highly expressed in the dorsal thalamus (Fig. 1B,C) whereas *Mash1* expression is restricted to the ventral thalamus (Fig. 4A). Ectopic *Mash1* expression was observed in the dorsal thalamus of *Ngn2* mutants (Fig. 4B) and more extensively in *Ngn1;Ngn2* double mutant embryos, in which *Mash1* transcripts were detected at high levels throughout the different subdivisions of the dien-

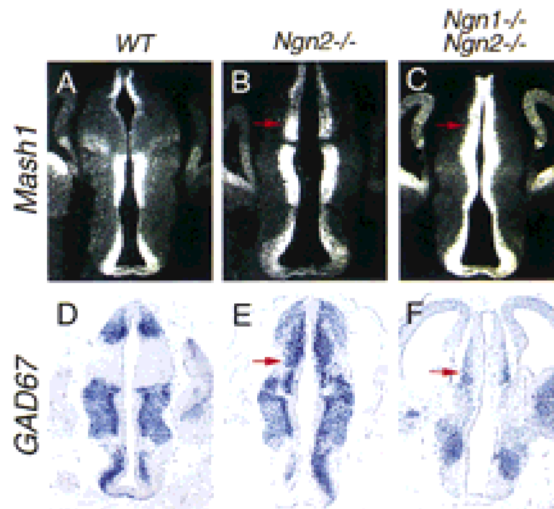

Figure 4. Mis-specification of neurons in the dorsal thalamus of *Ngn* mutants. (*A–C*) Distribution of *Mash1* transcripts on frontal sections of E12.5 wild-type and *Ngn* mutant diencephalon, showing that *Mash1* expression is up-regulated in the dorsal thalamus of *Ngn2* single mutants (arrow, *B*) and more extensively in *Ngn1;Ngn2* double mutants (arrow, *C*). Distribution of *GAD67* transcripts on frontal sections of E12.5 wild-type and *Ngn* mutant diencephalon, demonstrating an alteration in neuronal identity in the dorsal thalamus of *Ngn2* single and *Ngn1;Ngn2* double mutants revealed by the ectopic expression of *GAD67* (arrows, *E*, *F*). Morphological defects are also observed in the thalamus of *Ngn1;Ngn2* double mutant embryos (*F*), consistent with a role for the *Ngn* genes in neural determination in this region of the forebrain.

cephalon (Fig. 4C). Therefore, the *Ngns* function to restrict *Mash1* expression to limited domains throughout the forebrain neuroepithelium. In addition, consistent with the idea that altered neuronal identities arise in regions where *Mash1* expression levels are up-regulated, ectopic *GAD67* transcripts were detected in postmitotic neurons in the dorsal thalamus of *Ngn2* single mutants (Fig. 4E). The mis-specification of neurons is more severe in *Ngn1;Ngn2* double mutant embryos, because in addition to *GAD67* (Fig. 4F), *Dlx1* is also ectopically expressed in the dorsal thalamus (data not shown). Taken together with our observations in the cortex, these results indicate that the proper expression of *Ngn* and *Mash1* is required for the correct specification of neuronal identity in several subdivisions of the forebrain. In addition, the dorsal thalamus of *Ngn1;Ngn2* mutant embryos appears highly abnormal, with an enlargement of the VZ and a reduction in size of the mantle zone (Fig. 4F), suggestive of severe neurogenesis defects.

Ectopic Dlx1 *neurons are derived from dorsal progenitors*

The presence of cortical neurons with a ventral phenotype in *Ngn* mutants may arise from the mis-specification of dorsal progenitors or, alternatively, from premature and excessive tangential migration of ventrally derived neurons. In particular, a population of *Dlx1*$^+$, *GAD67*$^+$ interneurons derived from ventral precursors has been shown to migrate tangentially from the ganglionic eminences into the dorsal telencephalon beginning at E13.5 (De Carlos et al. 1996; Anderson et al. 1997). We thus used several approaches to determine whether the ectopic *Dlx1*+ neurons observed in *Ngn2* mutant cortex are dorsal or ventral in origin. As a first step to trace the origin of these neurons, we performed double-labeling experiments, using β-galactosidase activity in *Ngn2*^{lacZKI} homozygous mutant embryos to mark neurons derived from *Ngn2*-expressing mutant progenitors, and thus of dorsal origin (Fig. 1D), followed by *Dlx1* RNA in situ hybridization. Cortical neurons labeled with both X-Gal and *Dlx1* were detected (Fig. 5A; arrows in B), indicating that at least some of these neurons are derived from *Ngn2*-expressing progenitors in the dorsal telencephalon.

To provide further support for the hypothesis that ectopic *Dlx1*-expressing cells arise from cortical progenitors, explant experiments were performed in which the dorsal telencephalon was separated from ventral territories at E11, prior to the appearance of *Dlx1*+ neurons in the *Ngn2* mutant cortex. Medial, dorsal, and ventrolateral domains were dissected out and cultured separately on filters for 4 days (Fig. 5A). After culture, clusters of *Dlx1*-expressing cells were detected in large numbers in all ventrolateral explants, which include the lateral ganglionic eminence, derived from either wild-type (15/15, Fig. 5D) or *Ngn2* mutant (8/8, Fig. 5H) embryos. *Dlx1* was also detected at high levels in medial (6/6, Fig. 5J) and dorsal (7/8, Fig. 5I) explants derived from *Ngn2* mutant embryos but not in most medial (13/15, Fig. 5F) and dorsal (12/13, Fig. 5E) explants dissected from wild-type embryos after 4 days in culture. Control medial and dorsal explants dissected from the contralateral telencephalic vesicle from wild-type embryos (n = 15; Fig. 5C) and *Ngn2* mutants (n = 8; Fig. 5G), fixed immediately after harvesting, did not express *Dlx1*, confirming that ectopic *Dlx1*-expressing cells must have differentiated during the culture period. Thus, these data support the hypothesis that *Dlx1*$^+$ neurons in the *Ngn2* mutant cortex are of dorsal origin and are mis-specified.

Ngn2 *functions as a neuronal determination gene in cortical lineages*

To determine to what extent the up-regulation of *Mash1* compensates for the loss of *Ngn2* function, we examined

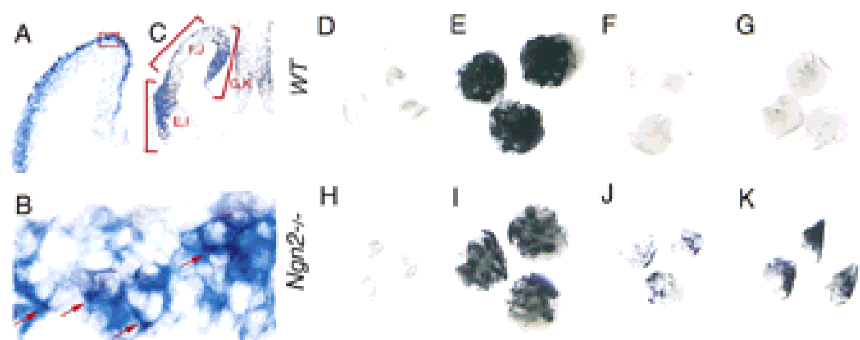

Figure 5. *Dlx1*+ cortical neurons in *Ngn2* mutants are of dorsal origin. (*A*,*B*) Double labeling of frontal sections through the telencephalon of E12.5 *Ngn2*^{lacZKI} homozygous mutant embryos with X-Gal (blue) and *Dlx1* (purple) showing that ectopic *Dlx1* neurons are X-Gal$^+$ and thus originate from *Ngn2*-expressing dorsal telencephalic progenitors. (*C*–*K*) Explants from E11 wild-type (top) and *Ngn2* mutant (*bottom*) telencephalic vesicles dissected into ventral–lateral (*E*,*I*), dorsal (*F*,*J*), and medial (*G*,*K*) domains, as defined in brackets in *C*, cultured for 4 days on floating filters and labeled for *Dlx1* transcripts. Explants of ventral–lateral domains derived from either wild-type (*E*) or *Ngn2* mutant (*I*) embryos contained a large number of *Dlx1*+neurons. Dorsal (*F*) and medial (*G*) wild-type explants did not contain a significant number of *Dlx1*$^+$ neurons; in contrast, explants from the same domains of *Ngn2* mutant embryos (*J* and *K*, respectively) contained a large number of *Dlx1*$^+$ neurons (explants were defined as positive when more than three clusters were detected, each containing more than three *Dlx1*$^+$ cells). These neurons differentiate in dorsal explants isolated from ventral telencephalon and thus originate from dorsal progenitors. A small proportion of medial (2/15) and dorsal (1/13) wild-type explants expressed *Dlx1*, possibly as a consequence of dissection errors. Confirmation that explants were dissected prior to the onset of ectopic *Dlx1* expression was obtained from the lack of *Dlx1* expression in uncultured contralateral dorsomedial explants from wild-type (*D*) and *Ngn2* mutant (*H*) embryos.

neurogenesis in the dorsal telencephalon of *Ngn2;Mash1* double-mutant embryos.

The number of neurons expressing the panneuronal marker *SCG10* is not significantly affected (Fig. 6B), and the neuronal differentiation gene *NeuroD* is normally expressed (Fig. 6E), in the cortex of *Ngn2* single mutants at E12.5. As reported previously, no defect in neurogenesis is apparent in the dorsal telencephalon of *Mash1* mutants (Casarosa et al. 1999; data not shown). In contrast, expression levels of *SCG10* and *NeuroD* are strongly reduced in *Ngn2;Mash1* mutant embryos (Fig. 6C,F), indicating that there is a reduction in number of PP neurons in double mutant embryos. *Hes5*, an *E*(*spl*)-related gene activated by Delta/Notch signaling in the mouse CNS (de la Pompa et al. 1997; Ohtsuka et al. 1999) is expressed in the VZ throughout the telencephalon, and this expression is not affected in *Ngn2* and *Mash1* single mutants (Fig. 6G,H; data not shown). However, *Hes5* expression is lost in the VZ of the dorsal/medial cortex in *Ngn2;Mash1* double mutants (Fig. 6I). *Mash1* and *Ngn2* are thus redundant for the activation of a downstream neuronal differentiation gene (*NeuroD*), the activation of Notch signaling (*Hes5*) and the generation of PP neurons in the dorsal/medial cortex (*SCG10*). These results indicate that *Ngn2* normally has a determination function for early born cortical neurons, and that the up-regulation of *Mash1* can partially compensate for the loss of this activity in *Ngn2* mutants.

Mash1 *up-regulation is required for the ventralization of* Ngn2 *mutant cortical neurons*

Although reduced in number, neurons are present in the PP of *Ngn2;Mash1* double mutant embryos, allowing us to assess whether the mis-specification of PP neurons in *Ngn2* mutants is due to the loss of *Ngn2* function or to the up-regulation of *Mash1*. We thus determined whether *Mash1* is required for the ventralization of cortical neurons by examining *Dlx1* expression in double mutant embryos. At E12.5, ectopic *Dlx1*+ neurons were detected in the PP of *Ngn2* single mutants (Fig. 6K), but not in *Ngn2;Mash1* double mutant embryos (Fig. 6L), demonstrating that *Mash1* activity is required for ectopic expression of *Dlx1* in *Ngn2* mutants. The reduction of expression of the dorsal-specific marker *Math2* in *Ngn2;Mash1* double mutants (Fig. 6O) compared with wild types (Fig. 6M) parallels that of the general neuronal marker *SCG10* (Fig. 6, cf. A and C), and double labeling with a *Math2* RNA probe and an antibody to the panneuronal marker β-tubulin showed that β-tubulin+ neurons that remain in the dorsal telencephalon of *Ngn2;Mash1* double mutant embryos maintain expression of *Math2* and are thus correctly specified (data not shown). In contrast, the increase in the number of *SCG10*+ neurons in single *Ngn2* mutants (Fig. 6B) compared with double *Ngn2;Mash1* mutants (Fig. 6C) does not lead to a significant change in *Math2* expression (Fig. 6, cf. N and O), suggesting that the additional cortical neurons resulting from *Mash1* up-regulation in *Ngn2* mutants do not acquire a dorsal identity. Taken together, these results indicate that the up-regulation of *Mash1* is

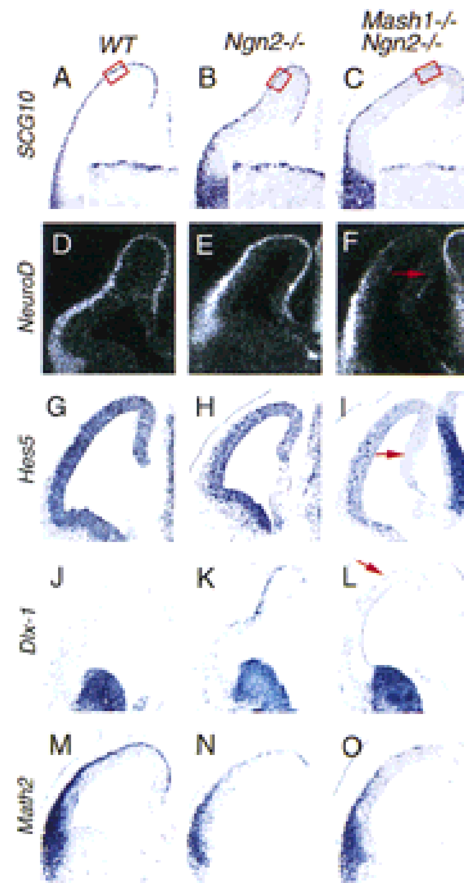

Figure 6. Up-regulation of *Mash1* is required for the mis-specification of cortical neurons in *Ngn2* mutants. (*A–C*) Distribution of *SCG10* transcripts in E12.5 wild-type, *Ngn2* mutant, and *Ngn2;Mash1* double mutant telencephalon. Neuronal loss is limited in the dorsal and medial PP of *Ngn2* single mutants (*B*) and more severe in *Ngn2;Mash1* double mutants (*C*). (*D–F*) Distribution of *NeuroD* transcripts in E12.5 wild-type, *Ngn2* mutant, and *Ngn2;Mash1* double mutant telencephalon. *NeuroD* expression is dramatically reduced in the dorsal and medial cortex of *Ngn2;Mash1* double mutants (arrow, *F*) but is not affected in *Ngn2* single mutants (*E*). (*G–I*) Distribution of *Hes5* transcripts in E12.5 wild-type, *Ngn2* mutant and *Ngn2;Mash1* double mutant telencephalon. *Hes5* expression is strongly down-regulated in the VZ of the dorsal and medial cortex of *Ngn2;Mash1* double mutants (arrow, *I*), suggesting defects in Notch signaling in these progenitors. (*J–L*) Distribution of *Dlx1* transcripts in E12.5 wild-type, *Ngn2* mutant, and *Ngn2;Mash1* double mutant telencephalon. Ectopic *Dlx1*+ neurons found in *Ngn2* mutant cortex (*K*) are absent in *Ngn2;Mash1* double mutant embryos (arrow in *L*). Up-regulation of *Mash1* is thus involved in the generation of *Dlx1*+ cortical neurons in *Ngn2* mutants. (*M–O*) Distribution of *Math2* transcripts in E12.5 wild-type, *Ngn2* mutant and, *Ngn2;Mash1* double mutant telencephalon. The reduction in *Math2* expression levels observed in the *Ngn2* mutant cortex (*N*) is also found in *Ngn2;Mash1* double mutants (*O*), thus confirming the overall reduction in number of medial and dorsal PP neurons in *Ngn2;Mash1* double mutants.

required for the mis-specification of cortical neurons in *Ngn2* mutants, including the ectopic expression of ventral markers and the down-regulation of dorsal markers.

Forced Mash1 *expression in dorsal progenitors is sufficient to induce ventral markers*

We then asked whether the up-regulation of *Mash1* in dorsal progenitors is sufficient to ventralize cortical neurons, even in the context of *Ngn2* expression. To address this question, we used homologous recombination in ES cells to generate a knock-in allele ($Ngn2^{Mash1KI}$) in which *Ngn2* coding sequences have been replaced by *Mash1* (Fig. 7A). Heterozygous $Ngn2^{Mash1KI}$ ES cells were used to generate chimeric embryos by aggregation with morula from the ROSA26 gene trap line in which *lacZ* is ubiquitously expressed (Friedrich and Soriano 1991), allowing the distinction between wild-type and $Ngn2^{Mash1KI}$ cells. By use of this approach, dorsal telencephalic progenitors heterozygous for the $Ngn2^{Mash1KI}$ allele should coexpress *Ngn2* from the wild-type allele and *Mash1* from the $Ngn2^{Mash1KI}$ allele. Chimeric embryos were sectioned and stained with X-Gal, and embryos in which mutant cells contributed nearly 100% to the dorsal telencephalon were analyzed further. As expected, a high level of *Mash1* expression was observed in dorsal telencephalic progenitors of strongly chimeric embryos at E12.5 (data not shown) and E13.5 (Fig. 7E), as compared with wild-type embryos. As a result of the forced expression of *Mash1* in *Ngn2*-expressing progenitors, ectopic expression of both *Dlx1* (Fig. 7F) and *GAD67* (Fig. 7G) was observed in cortical neurons, indicating that *Mash1* is sufficient to induce ectopic expression of ventral markers in neurons of the dorsal telencephalon, even in the context of *Ngn2* expression. This observation also supports the interpretation of the experiments in Figure 5, namely, that the presence of ventral-type neurons in the *Ngn* mutant cortex is the result of *Mash1* up-regulation in dorsal progenitors, rather than of the migration of ventral neurons. However, embryos derived from ES cells heterozygous for the $Ngn2^{Mash1KI}$ allele differ from *Ngn2* mutants in the reduced number and lateral, rather than dorsal, location of ectopic $Dlx1^{+}$, $GAD67^{+}$ cells. Therefore, the loss of *Ngn2* function appears to be also required for a more extensive ventralization of dorsal neurons.

Discussion

In this study, we have examined the role of the neural determination genes *Ngn1*, *Ngn2*, and *Mash1* in specification of neuronal phenotypes in the telencephalon. We show that the normal restriction of *Mash1* expression to ventral progenitors in the prospective basal ganglia is disrupted in *Ngn2* single and *Ngn1;Ngn2* double mutant embryos, indicating that one of the functions of the *Ngns* is to prevent *Mash1* expression in dorsal progenitors. Using both *Ngn2;Mash1* double mutant embryos and a new allele of *Ngn2* in which *Mash1* has been inserted into the locus, we demonstrate that although *Mash1* can compensate to some extent for the neuronal determination function of *Ngn2*, it respecifies a subpopulation of neurons such that they express certain ventral-specific markers and lose expression of a number of dorsal-specific genes. Thus, genetic analyses have allowed us to uncover a novel role for *Mash1* in the specification of ventral neuronal identities in the forebrain, as well as a role for the *Ngns* in the determination of cortical lineages. Taken together, these results provide new insights into how neurons with distinct phenotypes are born in different domains of the forebrain.

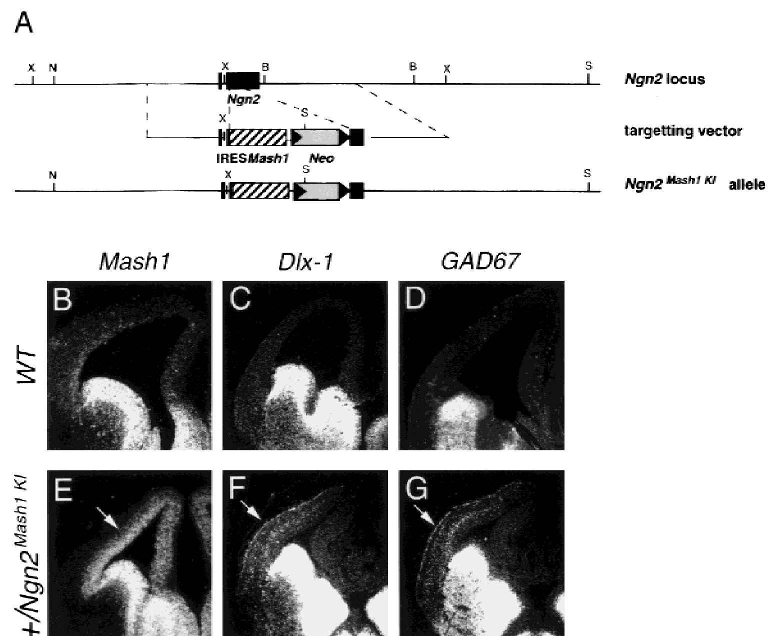

Figure 7. Forced expression of *Mash1* in dorsal progenitors is sufficient to ventralize cortical neurons. (*A*) A new allele of *Ngn2* was generated by replacing *Ngn2* coding sequences by an IRES-*Mash1* cassette ($Ngn2^{Mash1KI}$). In this allele, *Mash1* expression is controlled by *Ngn2* regulatory sequences. Schematic representation of the endogenous *Ngn2* locus (*top*), $Ngn2^{Mash1KI}$ targeting vector (*middle*), and recombined allele (*bottom*). Strongly chimeric E13.5 embryos generated with $Ngn2^{Mash1KI}$ heterozygous ES cells and a morula from the ROSA26 *lacZ* strain were analyzed. (*B*,*E*) Distribution of *Mash1* transcripts in the telencephalon of E13.5 wild-type and $Ngn2^{Mash1KI}$ chimeric embryos, demonstrating the up-regulation of *Mash1* expression in dorsal progenitors of chimeric embryos (arrow, *E*). (*C*,*D*,*F*,*G*) Distribution of *Dlx1* and *GAD67* transcripts in the telencephalon of E13.5 wild-type and $Ngn2^{Mash1KI}$ chimeric embryos, showing the presence of ectopic *Dlx1*+ cells (arrow, *F*) and ectopic *GAD67*+ cells (arrow, *G*) in the lateral and dorsal PP of chimeric embryos.

Ngns *restrict* Mash1 *expression to ventral domains in the forebrain*

Cross-regulatory interactions between neural determination genes have been reported previously in the PNS, in which *Ngn1* and *Ngn2* activate one another in complementary sets of cranial neuron precursors (Fode et al. 1998; Ma et al. 1998) and *Ngn2* activates *Ngn1* in a subset of precursors in the dorsal root ganglia (Ma et al. 1999). In a similar manner, we show that *Ngn1* expression in the dorsal/medial cortex is dependent on *Ngn2* function. In contrast, *Ngn1* is activated independently in the lateral cortex, and *Ngn2* expression is, for the most part, independent of *Ngn1* throughout the cortex. Taken together with the higher levels of *Ngn2*^{lacZKI}-driven *lacZ* activity in the dorsal/medial VZ and higher levels of *Ngn1* transcripts in the lateral VZ, these results indicate that *Ngn* genes are regulated in the cortex in a regionally restricted manner, suggesting an underlying heterogeneity of cortical progenitors. The unique identity of lateral cortical progenitors has been inferred previously from their early specification to produce neurons expressing latexin, a marker restricted to neurons of the lateral cortex (Arimatsu et al. 1999). Progenitors from other cortical regions have been shown to be similarly specified for expression of cortical area-specific markers (Cohen-Tanouji et al. 1994; Levitt et al. 1997; Tole et al. 1997). However, only a few genes are expressed in a restricted manner in cortical progenitors that would reflect such a regional specification (i.e., *Emx2*; Gusilano et al. 1996). In this respect, the differential regulation of *Ngn* gene expression provides important molecular evidence for the early regional diversification of cortical progenitors.

Mash1 and the *Ngns* are expressed for the most part in a complementary manner in the forebrain (Gradwohl et al. 1996; Ma et al. 1997), respecting well-defined boundaries described previously for other regulatory molecules (Puelles and Rubenstein 1993; Rubenstein et al. 1998). We have demonstrated that one mechanism by which these complementary patterns are established and/or maintained is through the down-regulation of *Mash1* expression in the dorsal telencephalon and dorsal thalamus by *Ngn* genes. Comparisons with other systems suggest that the establishment of the domains of neural determination gene expression is likely to be under the control of factors that pattern the early neural plate. For example, in the fly, expression of *as-c* genes in the wing imaginal disc is regulated by the homeobox proteins of the *Iroquois* complex and the GATA factor pannier, and *ato* is regulated in the eye by Shh signaling (Gómez-Skarmeta et al. 1996; Simpson 1996; Domínguez 1999), and similar interactions may be responsible for the initiation of *Mash1* and *Ngns* expression in the mammalian forebrain. The regulation of *Mash1* by the *Ngns* may represent a secondary mechanism involved in the refinement or the maintenance of these primary expression domains. In any case, our results show that cortical progenitors receive, and are competent to respond to, both *Mash1*- and *Ngn*-inducing signals. The repression of *Mash1* by *Ngns* may be essential for the correct specification of cortical progenitors, by preventing *Mash1* expression and thus ensuring that expression of *Mash1* and *Ngn* genes remains mutually exclusive.

The mechanism by which *Ngns* suppress *Mash1* expression in certain forebrain domains remains to be elucidated. One possibility is that a Notch-mediated process of lateral inhibition (Lewis 1996) or other non-cell autonomous mechanisms (Shou et al. 1999) are involved. Alternatively, the *Ngns* may function in a cell-autonomous manner to directly or indirectly repress transcription of *Mash1*. Additional experiments will be required to distinguish beween these hypotheses.

Ngn2 *functions as a neuronal determination gene in cortical lineages*

Previous studies have established that *Ngn1* (Ma et al. 1998) and *Ngn2* (Fode et al. 1998) function as neural determinants in the PNS, where they are required to activate a downstream bHLH regulatory cascade, to activate expression of Notch ligands and thereby initiate lateral inhibition, and to induce overt neuronal differentiation in subsets of sensory cranial ganglia. The idea that *Ngn2* functions in a similar manner in cortical development comes primarily from the analysis of *Mash1;Ngn2* double mutant embryos, in which the dorsal/medial cortex shows evidence of neural determination defects. In particular, this cortical region of double mutant embryos lacks expression of *NeuroD*, which is normally activated in immature CNS neurons as they leave the VZ (Lee et al. 1995), and which is induced in PNS neurons as part of a neuronal differentiation program activated by the *Ngn* genes (Fode et al. 1998; Ma et al. 1998) and by *Mash1* (Cau et al. 1997). Expression of the *Enhancer of split*-related gene *Hes5* is also down-regulated, suggesting that Delta/Notch signaling, which is required to activate expression of *Hes5* in CNS progenitors (de la Pompa et al. 1997; Ohtsuka et al. 1999), is defective in *Mash1;Ngn2* mutants. Finally, the number of neurons in the preplate of *Mash1;Ngn2* mutants is drastically reduced. The neurogenesis defects in the *Mash1;Ngn2* mutant cortex are not apparent in *Ngn2* single or *Ngn1;Ngn2* double mutants, demonstrating that *Mash1*, which is up-regulated in the dorsal VZ of these embryos, can compensate for the lack of *Ngn* gene function, activating *Notch* signaling and *NeuroD* expression and inducing cortical neuron differentiation. It is interesting to note that, in contrast to the dorsal telencephalon, the dorsal thalamus presents a severe deficit in neurons in *Ngn1;Ngn2* double mutant embryos. This demonstrates that *Ngn1* and *Ngn2* have partially redundant determination functions in this tissue, and that *Mash1* cannot compensate for the loss of *Ngns*, in spite of its up-regulation in the dorsal thalamus of *Ngn1;Ngn2* mutants, and its neural determination function in the ventral thalamus (Torii et al. 1999). The basis for this difference between dorsal thalamus and dorsal telencephalon is presently unknown.

The neurogenesis defects seen in *Mash1;Ngn2* mutants are restricted to the dorsal/medial cortex, most likely because the functions of *Mash1*, *Ngn2*, and *Ngn1*,

which is regulated by *Ngn2* in this region, are missing. In contrast, *Ngn1* is expressed independently of *Ngn2* in the lateral cortex, and is therefore likely to compensate for the lack of *Ngn2* in this domain of the *Mash1;Ngn2* mutant cortex. It is presently unknown whether *Ngn1* and *Ngn2* are involved in the development of distinct neuronal lineages in the lateral cortex, as proposed for sensory lineages in dorsal root ganglia (Ma et al. 1999), or whether the two genes function, and compensate for one another, within a unique population of progenitors in the lateral cortex.

Mash1 *ventralizes telencephalic neurons*

We have shown that up-regulation of *Mash1* in the telencephalon of *Ngn2*, *Ngn1;Ngn2*, and *Ngn2*^{Mash1KI} mutants results in ectopic dorsal expression of *Dlx1*, *Dlx2*, *Dlx5*, and *GAD67*, indicating that *Mash1* is able to specify dorsal progenitors to a more ventral fate. However, other ventral markers, including *Lhx6*, *Lhx7*, *Isl1*, and *Nkx2.1*, are not induced, which shows that *Mash1* cannot specify all types of ventral telencephalic neurons, but only a subset of them. Neurons with a similar *Dlx1/2*$^{+}$, *GAD67*$^{+}$ phenotype are normally found, albeit at a later stage (from E13.5 onward) and in smaller numbers, in the marginal and intermediate zones of the telencephalon. These neurons originate in their vast majority in the ganglionic eminences and reach the cortex by tangential migration (De Carlos et al. 1996; Anderson et al. 1997; Tamamaki et al. 1997; Lavdas et al. 1999; Zhu et al. 1999). In contrast, the ectopic *Dlx1*+ neurons in *Ngn* mutants originate from dorsal progenitors, which strongly suggests that *Mash1* is able to respecify cortical progenitors to a new fate. However, it has been proposed that some GABAergic interneurons also differentiate from cortical progenitors, on the basis of results obtained in cortical explant cultures (Götz and Bolz 1994). *Mash1*, which is expressed at low levels in the VZ of the wild-type cortex, could thus be normally involved in the specification of these neurons, and up-regulation of *Mash1* could result in a enlargement of this population of interneuron progenitors, leading to the precocious and excessive differentiation of GABAergic neurons of cortical origin. In both hypotheses, our results show that *Mash1* is able to specify a particular neuronal identity, including the GABAergic neurotransmitter phenotype, in telencephalic neurons.

Does the phenotype of *Mash1* null mutant mice support a role for *Mash1* in neuronal type specification during normal telencephalic development? In these mice, subsets of ventral telencephalic neurons are missing, but *Dlx1/2* and *GAD67* expression is maintained and there is no evidence for mis-specification among remaining ventral telencephalic neurons (Casarosa et al. 1999; Tuttle et al. 1999). One exception, however, is the population of migratory *Dlx1/2*+, *GAD67*+ cortical interneurons, which is greatly reduced in *Mash1* mutants (Casarosa et al. 1999). Although it cannot be excluded that this defect is due to the loss of progenitors in the MGE and thus corresponds to the generic determination function of *Mash1*, it may also reflect a more specific function of *Mash1* in the production of neurons with a *Dlx1/2*$^{+}$, *GAD67*$^{+}$ neuronal phenotype. The reduction in GABAergic interneurons observed in *Mash1* mutants appears to be more severe than that observed in *Nkx2.1* mutants, in which there is a complete respecification of the MGE (Casarosa et al. 1999; Sussel et al. 1999; S. Casarosa and F. Guillemot, unpubl.), suggesting that *Mash1* has additional functions in the production of these neurons beside the generation of MGE progenitors. *Mash1* may thus be both sufficient and necessary to specify this particular ventral identity, whereas it is neither sufficient nor necessary for the specification of other ventral telencephalic phenotypes (i.e. expression of *Nkx2.1*, *Isl1*, *Lhx6*, *Lhx7*).

What could be the mechanisms by which *Mash1* induces ventral-specific neuronal characteristics? Given that signaling by the secreted factor Sonic hedgehog (Shh) plays a major role in ventralization of the neural tube along its entire A/P axis (for review, see Ericson et al. 1997), including the forebrain (Barth and Wilson 1995; Ericson et al. 1995; Chiang et al. 1996; Hauptmann and Gerster 1996; Shimamura and Rubenstein 1997) it is tempting to hypothesize that *Mash1* interacts with the Shh signaling pathway in the telencephalon. One possibility is that *Mash1* specifies a ventral fate in telencephalic neurons by confering competence of telencephalic progenitors to respond to Shh. A precedent exists whereby *Mash1* confers competence to neural crest progenitors to respond to the neuralizing activity of BMP2 (Lo et al. 1997). Competence to respond to Shh appears to be regulated and to change as telencephalic development proceeds, because early exposure of telencephalic explants to Shh induces expression of MGE-specific markers (Ericson et al. 1995), later exposure induces LGE-specific markers, whereas still later, telencephalic progenitors become refractory to induction by Shh (Kohtz et al. 1998). Up-regulation of *Mash1* in dorsal progenitors could maintain the competence of these cells to respond to Shh and adopt a ventral fate, although they would have normally lost this competence at the time when Shh, which is expressed in ventral telencephalon throughout embryonic development (Miao et al. 1997), diffuses in the dorsal part of the telencephalon. However, only postmitotic neurons express ventral markers in the *Ngn* mutant cortex, and cortical progenitors remain correctly specified, which indicates that *Mash1* would only control a late step in the specification of telencephalic progenitors by Shh.

Another possibility is that *Mash1* is a downstream effector of Shh signaling in telencephalic progenitors. Expression of *Mash1* could be induced by Shh in the ventral telencephalon as part of a program of ventral-type differentiation. Analogous situations are found in the *Drosophila* eye, in which the proneural gene *atonal* is activated by hedgehog (Domínguez 1999), and in somites in which the myogenic bHLH transcription factor *MyoD* is activated by Shh signaling (Münsterberg et al. 1995). *Mash1* may thus function in a cell-autonomous manner to activate *Dlx1/2* expression and specify a GABAergic neurotransmitter phenotype in neuronal populations of

the forebrain, possibly through direct activation of *Dlx* and *GAD* genes. The close correlation between expression of *GAD67* and that of *Dlx1/2* and *Mash1* throughout the forebrain suggests a possible involvement of these transcription factors in specification of a GABAergic phenotype. In addition, the temporal sequence of their expression is consistent with the existence of a regulatory cascade between Shh, *Mash1*, and ventral-specific neuronal markers. For example, in the chick, Shh expression in the forebrain begins in the ventral diencephalon at stage 10 (Ericsson et al. 1995), whereas the chick homolog of *Mash1*, *Cash1*, is expressed in ventral telencephalon at stage 14 (Jasoni et al. 1995) and could therefore be induced by Shh acting from the ventral diencephalon in a planar manner. Expression of *Cash1* precedes that of ventral neuronal markers such as *Islet1*, which is initiated in telencephalic neurons at about stage 17, suggesting that *Cash1* could be involved in the activation of a ventral-specific program of differentiation in telencephalic neurons.

In hindbrain and autonomic neurons of the PNS, *Mash1* is required for the differentiation of a noradrenergic neurotransmitter phenotype, possibly acting through regulation of the homeobox gene *Phox2a* (Hirsch et al. 1998; Lo et al. 1998). This suggests that, although *Mash1* is likely to activate both generic and neuronal-type specific differentiation programs in various neuronal populations, it activates different programs in progenitors from different regions of the nervous system. Therefore, *Mash1* activity is context dependent, as reported previously for *Drosophila* proneural genes (Jan and Jan 1994; Brunet and Ghysen 1999).

The study of *Ngn2* and *Ngn1;Ngn2* mutants did not allow us to firmly establish a role for the *Ngns* in specification of the dorsal identity of cortical neurons. Dorsal-specific markers are down-regulated in most, but not all, cortical neurons of *Ngn* mutants, suggesting that yet additional genes may partially compensate for the loss of *Ngn* genes and specify neurons with a dorsal character. As already seen for *Mash1* in the ventral telencephalon (Casarosa et al. 1999), a loss-of-function analysis may not be sufficient to define the role of *Ngn* genes in specification of cortical neuron identities, and ectopic expression experiments, similar to those already performed in the PNS (Perez et al. 1999), may be necessary to examine this issue.

Materials and methods

Construction of Ngn2^{lacZKI} *and* Ngn2$^{Mash1/KI}$ *targeting vectors*

The structure of the *Ngn2* genomic locus was described previously (Fode et al. 1998). The *Ngn2^{lacZKI}* and *Ngn2$^{Mash1/KI}$* targeting vectors were both constructed with a 4-kb *Eag*I fragment as the 5′ arm and a 1.5-kb *Nhe*I fragment as the 3′ arm in a targeting vector containing a PGK-neo cassette flanked by *loxP* sites (Hanks et al. 1995). In both instances, the 5′ and 3′ UTR of *Ngn2* was preserved, and an internal ribosomal entry site (IRES) element (Ghattas et al. 1991) was fused upstream of the *lacZ* or *Mash1* coding sequences to ensure translational initiation.

Generation of mutant and chimeric mice

Ngn2^{lacZKI} and *Ngn2$^{Mash1/KI}$* targeting vectors were designed to replace the bHLH domain of *Ngn2* with *lacZ* and *Mash1* coding sequences, respectively, leaving 48 amino acids of amino-terminal *Ngn2* coding sequence. Targeting vectors were electroporated into R1 ES cells by described conditions of culture, transfection, and selection of clones (Wurst and Joyner 1993). Genomic DNA was examined by Southern blotting to detect homologous recombination events with *Not*I–*Spe*I digests and 5′ and 3′ external probes that were described previously (Fode et al. 1998). For the *Ngn2^{lacZKI}* allele, a recombinant ES cell clone was injected into C57BL/6J blastocysts and resulting chimeric males were bred with C57BL/6J females to obtain germ-line transmission of the mutation and the strain was maintained by crossing into a CD1 background. The loss of *Ngn2* coding sequences, including the bHLH domain, in *Ngn2^{lacZKI}* mutants was confirmed by PCR analysis with primers described in Fode et al. (1998) (data not shown). In addition, the similarity in phenotype of homozygous mutants bearing the original *Ngn2^{neo}* null allele, which removed the entire *Ngn2* coding sequence (Fode et al. 1998), and the *Ngn2^{lacZKI}* allele confirmed that the new mutation is also a null allele (data not shown). For the *Ngn2^{Mash1ki}* allele, chimeric embryos were generated by injecting 5–10 ES cells heterozygous for the mutation into morula stage (E2.5) embryos obtained from crossing males heterozygous for the ROSA26 gene trap insertion (Friedrich and Soriano 1991) with CD1 females and reimplanting embryos into pseudopregnant females. Chimeric embryos were harvested on day E12.5 or E13.5. *Ngn1* (Ma et al. 1998), *Ngn2* (Fode et al. 1998), and *Mash1* (Guillemot et al. 1993) mutant mice have been described previously. To generate *Ngn1;Ngn2* and *Ngn2;Mash1* double homozygous mutant embryos, double heterozygous mice were intercrossed.

Genotyping of Ngn1, Ngn2, *and* Ngn2^{lacZKI} *alleles*

Genotyping was performed by PCR on genomic DNA extracted from tails or embryonic yolk sacs as described previously (Fode et al. 1998). PCR genotyping of *Mash1* (Guillemot et al. 1993), *Ngn1* (Ma et al. 1998), and *Ngn2* (Fode et al. 1998) mutant and wild-type alleles was as described previously. Genotyping of the *Ngn2^{lacZKI}* allele was performed with primers situated in the upper (CCAGCTGGCGTAATAGCGAA) and lower (CGCCC-GTTGCACCACAGATG) strands of the bacterial β-galactosidase sequence. PCR conditions for the *Ngn2^{lacZKI}* allele were 30 cycles of 94°C/1 min; 60°C/1 min; 72°C/1 min.

RNA in situ hybridization and lacZ *staining*

The morning that vaginal plugs were observed is considered E0.5. Embryos were fixed at 4°C in 4% paraformaldehyde for 4 hr (E12.5) or overnight (E15.5 and older). Embryos were then rinsed in PBS, impregnated with 20% sucrose in PBS overnight at 4°C, and embedded in OCT (Tissue-Tek, Miles). Sections were cut at 10 µm on a cryostat. Nonradioactive and ^{35}S-labeled section RNA in situ hybridization was performed as described in Cau et al. (1997). Double labeling nonradioactive in situs were performed with digoxygenin and fluorescein-labeled probes. Briefly, following the development of the NBT/BCIP reaction, the antidigoxygenin antibody was destroyed by a 15 min incubation in 0.1 M glycine (pH 2.2). Sections were then washed three times for 5 min in PBS with 0.1% Tween 20 (PBT) and 10 min in MABT (100 mM maleic acid at pH 7.5, 150 mM NaCl, 0.1% Tween 20). Sections were then blocked for 1 hr in 2% Boehringer blocking reagent (BM 1096 176) made up in ma-

leic acid buffer and 20% heat-inactivated goat serum followed by an overnight incubation in blocking solution with 1/1000 dilution of anti-fluorescein antibody. Sections were then washed four to five times in MABT for 20 min and twice for 10 min in NMT (100 mM NaCl, 50 mM $MgCl_2$, 100 mM Tris-Cl at pH 9.5) and stained with 7.5 µl/ml INT in NMT buffer with 5 mM levamisole (D. Henrique, pers. comm.). β-Galactosidase staining was performed as described in Beddington et al. (1989) and double *lacZ* staining/RNA in situ hybridization was as described by Houzelstein and Tajbakhsh (1999). The probes used were described in the following publications: *Ngn1* (Cau et al. 1997), *Ngn2* (Gradwohl et al. 1996), *Mash1* (Guillemot and Joyner 1993), *Math2* (Bartholomä and Nave 1994; Shimizu et al. 1995), *NeuroD* (Lee et al. 1995); *Hes5* (Akazawa et al. 1992), *Dlx1* (Price et al. 1991), *Dlx-5* (Simeone et al. 1994); *SCG10* (Stein et al. 1988), *GAD67* (Behar et al. 1994), and *Reelin* (Ikeda and Terashima 1997).

Birthdating, immunohistochemistry, and histology

For histological analyses, embryos were fixed in Bouin's fixative from 24 hr (E12.5) up to 3 days (P0). From E15.5 to P0, brains were dissected from the skull prior to fixation. Brains were then processed for wax embedding and cut at 7 µm and stained with hematoxylin-eosin or processed for anti-BrdU immunostaining. Biotinylated secondary anti-mouse and anti-rabbit immunoglobulin antibodies (1/200 dilution) and avidin–biotin complex reagents were from the Vectastain kit (Vector). For birthdating analysis, pregnant females were injected intraperitoneally with 2 mg of BrdU (Sigma) either at E10.5, E11.5, E12.5, or E15.5 and sacrificed at E15.5 or P0. Embryos were either processed for wax sectioning for anti-BrdU immunohistochemistry or embedded in OCT for double RNA in situ/anti-BrdU immunostaining. The anti-BrdU (1/100 dilution; Boehringer Mannheim) staining was performed as described in Anderson et al. (1997).

Telencephalic explants

Telencephalic explants were dissected from E11.5 embryos and divided into dorsal, medial, and ventral-lateral domains essentially as described in Kohtz et al. (1998). Explants were cultured for 4 days on 0.02-mm Nucleopore filters floating on DMEM:F12 medium with 10% FCS. Explants were then fixed 1 hr in 4% paraformaldehyde at 4°C, rinsed two times in PBS and stored in methanol at –20°C. Wholemount RNA in situ hybridization was performed as described (Cau et al. 1997). High levels of *Dlx1* expression were defined as greater than three groups of *Dlx1*-expressing cells per explant.

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