Divergent functions of the proneural genes *Mash1* and *Ngn2* in the specification of neuronal subtype identity

Carlos M. Parras,[1,4] Carol Schuurmans,[1,3,4] Raffaella Scardigli,[1] Jaesang Kim,[2] David J. Anderson,[2] and François Guillemot[1,5]

[1]Institut de Génétique et de Biologie Moléculaire et Cellulaire, CNRS/INSERM/Université Louis Pasteur, 67404 Illkirch cedex, C.U. de Strasbourg, France; [2]Division of Biology 216-76, Howard Hughes Medical Institute, California Institute of Technology, Pasadena, California 91125, USA

The neural bHLH genes *Mash1* and *Ngn2* are expressed in complementary populations of neural progenitors in the central and peripheral nervous systems. Here, we have systematically compared the activities of the two genes during neural development by generating replacement mutations in mice in which the coding sequences of *Mash1* and *Ngn2* were swapped. Using this approach, we demonstrate that *Mash1* has the capacity to respecify the identity of neuronal populations normally derived from *Ngn2*-expressing progenitors in the dorsal telencephalon and ventral spinal cord. In contrast, misexpression of *Ngn2* in *Mash1*-expressing progenitors does not result in any overt change in neuronal phenotype. Taken together, these results demonstrate that *Mash1* and *Ngn2* have divergent functions in specification of neuronal subtype identity, with *Mash1* having the characteristics of an instructive determinant whereas *Ngn2* functions as a permissive factor that must act in combination with other factors to specify neuronal phenotypes. Moreover, the ectopic expression of *Ngn2* can rescue the neurogenesis defects of *Mash1* null mutants in the ventral telencephalon and sympathetic ganglia but not in the ventral spinal cord and the locus coeruleus, indicating that *Mash1* contribution to the specification of neuronal fates varies greatly in different lineages, presumably depending on the presence of other determinants of neuronal identity.

The mechanisms underlying the generation by multipotent stem cells of the vast array of neuronal and glial subtypes that constitute the nervous system remain poorly characterized. Recently, evidence have been obtained that genes of the bHLH class are important regulators of several steps in neural lineage development (Brunet and Ghysen 1999; Cepko 1999; Guillemot 1999; Morrison 2001). In particular, a subset of neural bHLH genes, the proneural genes, has been shown to play a central role in the selection of neural progenitor cells. Two families of proneural genes have been identified in *Drosophila*, the *achaete-scute* genes (*ac-sc*) which control the generation of progenitors for the central nervous system (CNS) and external sense organs in the peripheral nervous system (PNS), and genes of the *atonal* family (*ato*) which control the generation of progenitors for photoreceptors, chordotonal organs, and olfactory receptors

(Modolell 1997; Campos-Ortega 1998). In vertebrates, a large number of bHLH genes are expressed in the developing nervous system, but only a fraction of them appear to have a proneural function. Loss-of-function (LOF) analyses in mouse have demonstrated that the *ac-sc* homolog *Mash1* and the *ato*-related genes *Neurogenin* (*Ngn*) *1* and *Ngn2* are required for the generation by neural stem cells of various populations of progenitor cell populations in the PNS and CNS, including progenitors in the ventral telencephalon and olfactory epithelium for *Mash1*, and in the cranial and dorsal root ganglia, the dorsal telencephalon and the ventral spinal cord for *Ngn1* and *Ngn2* (Cau et al. 1997; Fode et al. 1998, 2000; Ma et al. 1998; Casarosa et al. 1999; Horton et al. 1999; Scardigli et al. 2001). In contrast, several other bHLH genes that are also expressed by neural progenitor cells, including the *ato* orthologs *Math1* and *Math5* and *NeuroD*, do not appear to be involved in this initial step of neurogenesis (Bermingham et al. 1999; Wang et al. 2001).

Although functional studies in *Drosophila* and vertebrates initially focused on demonstrating a proneural function for bHLH proteins, the restricted expression of proneural genes in distinct populations of neural progenitors that give rise to different subtypes of neurons

raised the possibility that they may also influence neuronal fate decisions. Consistent with this idea, gain-of-function (GOF) experiments in *Drosophila* have shown that the ectopic expression of *atonal* and *achaete-scute* results in the generation of different types of sense organs (Jarman et al. 1993; Chien et al. 1996; Jarman and Ahmed 1998). In addition, rescue experiments in the embryonic CNS have demonstrated that the *achaete-scute* genes differ in their ability to specify neural precursor identities (Parras et al. 1996; Skeath and Doe 1996), and similarly, *atonal* and a closely related gene, *amos* promote the development of different types of olfactory sense organs (Goulding et al. 2000; M.L. Huang et al. 2000).

In vertebrates, there is evidence that some bHLH genes have conserved a neuronal subtype specification activity similar to that of their *Drosophila* counterparts (for review, see Brunet and Ghysen 1999; Cepko 1999; Guillemot 1999; Hassan and Bellen 2000). In the best studied case, *Mash1* has been shown by both GOF and LOF analyses to regulate the expression of the homeodomain gene *Phox2a*, which together with its relative *Phox2b* is an essential determinant of the noradrenergic neurotransmitter phenotype (Hirsch et al. 1998; Lo et al. 1998). These results have led to the proposition that *Mash1* coordinates generic and neuronal subtype specific programs of neurogenesis (Hirsch et al. 1998; Lo et al. 1998). However, both *Mash1* and *Phox2b* are required for *Phox2a* expression in neural crest-derived autonomic precursors (Hirsch et al. 1998; Lo et al. 1998; Pattyn et al. 1999), and their respective contribution to the activation of an autonomic program of differentiation during normal development has not been addressed directly. In particular, the role of *Mash1* in noradrenergic differentiation remains unclear as the loss of *Phox2a* expression in *Mash1* LOF mutants may reflect a requirement for the proneural function of *Mash1* to progress past the stage of *Phox2a* expression, as well as a more direct role in regulating *Phox2a* expression. Additional evidence for a role of *Mash1* in the specification of neuronal subtype identity is its capacity to induce the expression of GABAergic differentiation markers in dorsal telencephalic neurons (Fode et al. 2000). However, as this result is based on GOF analysis, it remains unknown whether it reflects a genuine function of *Mash1* during telencephalic development.

A role in the specification of neuronal phenotypes has also been attributed to several of the vertebrate *ato*-related genes. Ectopic expression of the *Ngn* genes in migratory neural crest and mesodermal cells has been shown to induce the expression of several sensory neuron markers as well as generic markers of neuronal differentiation, thus implicating *Ngn* genes in the specification of sensory phenotypes in the PNS (Perez et al. 1999). A role for *Ngns* in the specification of neuronal identity in the CNS has not been addressed directly, however, particularly in a situation where *Ngn* activity could be compared directly with that of *Mash1*, in a manner similar to the parallel GOF analysis of *ac-sc* and *ato* genes performed in *Drosophila* (Chien et al. 1996; Parras et al. 1996; Bray 2000). In contrast, *ato*-related

genes other than *Ngns* have been shown to have important functions in the specification of particular neuronal subtypes, including *Math1* for sensory inner ear cells and *Math5* for retinal ganglion cells (Kanekar et al. 1997; Bermingham et al. 1999; Hassan and Bellen 2000; Matter-Sadzinski et al. 2001).

Here, we have directly addressed the roles of *Mash1* and *Ngn2*, two bHLH genes with well characterized proneural functions, in the specification of neuronal subtype identity. To avoid some of the limitations inherent to traditional LOF and GOF approaches, we have used a knock-in (KI) strategy, whereby the coding sequence of one of the two genes was introduced into the locus of the second gene that was simultaneously deleted. In the resulting two strains of mice, *Mash1* precisely replaces *Ngn2* ($Ngn2^{KIMash1}$ mice) and *Ngn2* replaces *Mash1* ($Mash1^{KINgn2}$ mice) in the expression domain specific to the replaced gene. Given that these KI mutations result in the ectopic expression of a proneural gene in a distinct lineage but in a normal context, the resulting phenotypes should be relevant to the normal functions of these two genes. Moreover, the KI mutations allow for a direct comparative analysis of the activities of Mash1 and Ngn2 proneural proteins in the same cells. Finally, in this system we retain a proneural activity in neural progenitors, allowing neuronal subtype specification defects to be distinguished from proneural defects. Using these KI mutant mice, we have asked the following questions: Do both *Mash1* and *Ngn2* have an intrinsic capacity to specify neuronal subtype identity, and to what extent is their specification function influenced by the cellular context? In other words, can these proneural genes impose a new subtype specification program on progenitors, or are their activities constrained by other determinants with which they cooperate? Finally, how essential is the contribution of proneural genes to the specification of neuronal subtype identity in the different lineages where such a function has been demonstrated?

From this comparative analysis of *Mash1* and *Ngn2* functions, we conclude first that *Mash1* can respecify a variety of distinct neuronal lineages when ectopically expressed in *Ngn2*+ precursors, whereas *Ngn2* cannot respecify *Mash1*+ progenitors, indicating that *Mash1* has an instructive role in specifying the identity of various neuronal subtypes whereas the role of *Ngn2* in this process is permissive. Second, the ectopic expression of *Ngn2* can only rescue some of the neuronal populations that normally depend on *Mash1* activity, likely reflecting the existence of parallel specification pathways in a subset of *Mash1*-expressing lineages.

Results

Replacement of Mash1 *by* Ngn2 *in mice*

To systematically compare the activities of *Ngn2* and *Mash1* in contexts where proneural genes are normally active, we generated two KI mutations. An initial characterization of chimeric mice carrying a mutation in which the *Mash1* coding sequence replaces the *Ngn2* se-

quence ($Ngn2^{KIMash1}$) has been reported previously (Fode et al. 2000). The reciprocal mutation, whereby the coding sequence of *Mash1* was replaced by the sequence of *Ngn2* ($Mash1^{KINgn2}$; Fig. 1A), was generated by homologous recombination in embryonic stem cells. The mutation was transmitted to the germ line and animals heterozygous for the $Mash1^{KINgn2}$ allele were viable and fertile, whereas homozygous $Mash1^{KINgn2}$ animals died at birth in a manner similar to *Mash1* null mutant ($Mash1^{\Delta}$) animals, indicating that *Ngn2* does not have the capacity to completely rescue *Mash1* function.

We confirmed that *Ngn2* is correctly expressed in the *Mash1* expression domain of $Mash1^{KINgn2}$ mice using two RNA probes recognizing respectively the coding sequence of *Ngn2* and the 3' untranslated region (UTR) of *Mash1*, both of which hybridize to the transcript encoded by the $Mash1^{KINgn2}$ allele (Fig. 1B). In the telencephalon of wild-type mice at E12.5, *Ngn2* is expressed exclusively in the dorsal telencephalic ventricular zone (VZ) whereas *Mash1* is expressed at high levels in the ventral VZ and at low levels dorsally (Fig. 1B). In embryos homozygous for the $Mash1^{KINgn2}$ allele (referred to below as $Mash1^{KINgn2}$ embryos), chimeric transcripts coding for the Ngn2 protein were found in the VZ and subventricular zone (SVZ) of the lateral and medial ganglionic eminences at levels comparable to those of wild-type *Ngn2* transcripts in the mediodorsal telencephalon and of wild-type *Mash1* transcripts in the ventral telencephalon (Fig. 1B). Ectopic expression of *Ngn2* was also observed in other parts of the *Mash1* expression domain, including the olfactory epithelium and the autonomic nervous system (data not shown).

Ectopic Ngn2 *compensates for the loss of* Mash1 *proneural function in ventral telencephalon*

Mash1 and *Ngn2* have been shown to act as proneural genes in different regions of the nervous system (Fode et

al. 1998, 2000; Ma et al. 1998, 1999; Casarosa et al. 1999). The proneural function of *Mash1*, i.e., its role in the selection of neural progenitors and the activation of Notch signaling, has been best characterized in the olfactory epithelium (Guillemot et al. 1993; Cau et al. 1997) and ventral telencephalon (Casarosa et al. 1999; Horton et al. 1999). We first asked whether *Ngn2* has the capacity to substitute for *Mash1* proneural function and to initiate the neurogenesis program normally activated by *Mash1* by comparing the development of the ventral telencephalon and olfactory epithelium in $Mash1^{KINgn2}$ and $Mash1^{\Delta}$ mutant embryos. Strikingly, $Mash1^{KINgn2}$ embryos presented a complete rescue of the defect in ventral telencephalic progenitors observed in $Mash1^{\Delta}$ embryos at E12.5, as shown by the presence of a medial ganglionic eminence (MGE) of normal size (Fig. 2A″–F″), in comparison with the atrophic MGE in $Mash1^{\Delta}$ mutants (Fig. 2A′–F′). Moreover, MGE progenitors differentiated normally, as shown by normal expression of the homeodomain-containing gene *Prox1* in SVZ progenitors and of the panneuronal marker *SCG10* in postmitotic neurons in the mantle zone, whereas both markers are reduced in the ventral telencephalon of $Mash1^{\Delta}$ embryos, particularly in the pallidal region (Fig. 2A′, A″,B′,B″).

We then examined whether ectopic *Ngn2* in ventral telencephalic progenitors activates Notch signaling, a pathway that is defective in $Mash1^{\Delta}$ embryos (Casarosa et al. 1999; Fig. 2). Expression of the Notch ligand, *Dll1* and of the transcriptional effector of Notch signaling, *Hes5* (Ohtsuka et al. 1999) was rescued in $Mash1^{KINgn2}$ embryos (Fig. 2C″; data not shown). Moreover, transcription at the *Mash1* locus, examined with a *Mash1* 3'UTR probe recognizing transcripts from the wild-type, $Mash1^{\Delta}$ and $Mash1^{KINgn2}$ alleles of *Mash1*, was restricted to a subset of progenitor cells in the VZ of $Mash1^{KINgn2}$ embryos, as observed in wild-type embryos (Fig. 2D,D″), whereas it was more uniform in $Mash1^{\Delta}$
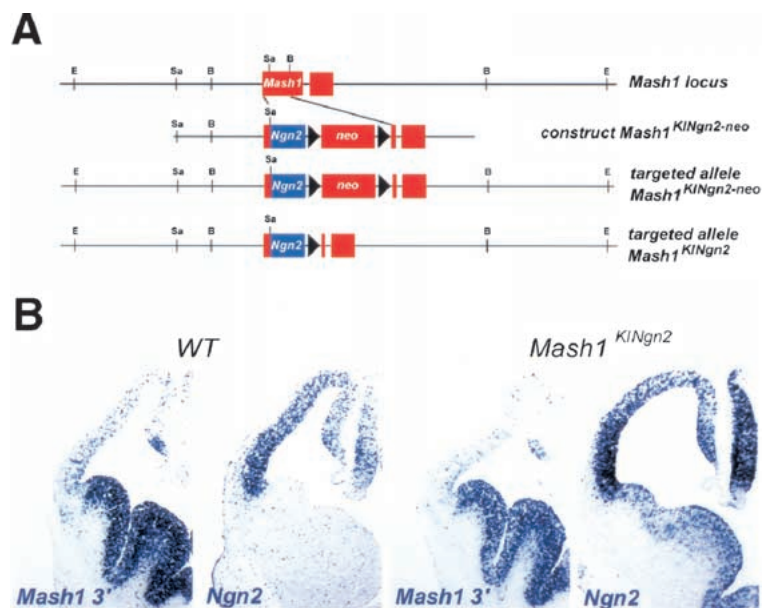

Figure 1. Targeting strategy to introduce the *Ngn2* sequence into the *Mash1* locus. (*A*) Targeting vector designed to substitute the coding sequence of *Mash1* with the coding sequence of *Ngn2*, leaving the 5' and 3' untranslated regions (UTR) of *Mash1* intact. Following transmission of the mutation to the germline, the neomycin resistance gene (Neo) was excised, giving rise to the $Mash1^{KINgn2}$ allele. (*B*) Hybridization of RNA probes for the *Mash1* 3'UTR sequence and the *Ngn2* coding sequence to frontal sections of the telencephalic region of E12.5 wild-type and $Mash1^{KINgn2}$ homozygous mutant embryos, showing that $Mash1^{KINgn2}$ transcripts are detected in the ventral telencephalon.
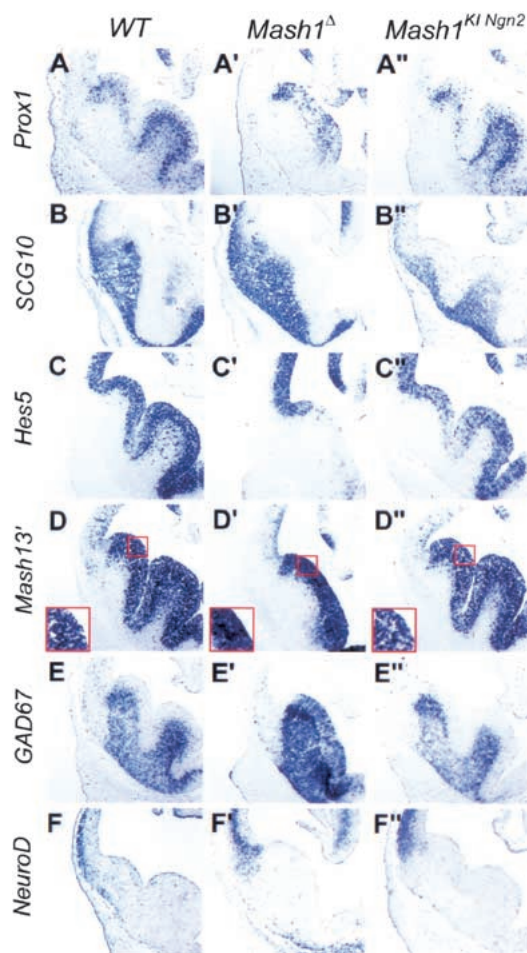
Figure 2. *Ngn2* rescues ventral telencephalic development and does not promote dorsal telencephalic fates when replacing *Mash1* in ventral telencephalic progenitors. Frontal sections at telencephalic level of E12.5 wild-type (*A–F*), homozygous *Mash1*$^\Delta$ (*A'–F'*), and homozygous *Mash1*^{KINgn2} (*A"–F"*) embryos were hybridized with RNA probes for the genes shown on the *left*. Progenitors in the medial ganglionic eminence (MGE) are missing in *Mash1* $^\Delta$ embryos (*A'–F'*), and MGE formation is rescued by *Ngn2* expression from the *Mash1* locus (*A"–F"*). Differentiation of MGE progenitors is also restored, as shown by expression of *Prox1* in subventricular zone (SVZ) progenitors (*A"*) and of *SCG10* in mantle zone neurons (*B"*). *Ngn2* expression in ventral telencephalic progenitors also restores Notch signaling, as shown by expression of the Notch effector *Hes5* in ventricular zone (VZ) progenitors (*C"*) and by the "salt and pepper" pattern of expression of *Mash1*, as detected by a *Mash1* 3'UTR probe (*D"*), whereas *Mash1* is transcribed more uniformly by VZ cells in *Mash1* $^\Delta$ embryos (*D'*). Oulined areas in *D–D"* are magnified in the insets. Neurons differentiating from *Ngn2*-expressing progenitors in the ventral telencephalon acquire a normal phenotype, as shown by their expression of ventral markers such as *GAD67* (*E"*) and by the lack of misexpression of dorsal-specific genes such as *NeuroD* (*F"*).

embryos (Fig. 2D'). This supports the conclusion that a Notch-mediated process of lateral inhibition involved in down-regulating proneural gene expression (de la Pompa et al. 1997) is restored in *Mash1*^{KINgn2} mutants. Finally,

the premature expression of the SVZ markers *Prox1*, *GAD67*, and *Dlx5* in the VZ in *Mash1*$^\Delta$ embryos was not observed in *Mash1*^{KINgn2} embryos (Fig. 2A',A",E',E"; data not shown).

Together, these data demonstrate that *Ngn2* misexpression in ventral telencephalic progenitors can fully compensate for the loss of *Mash1* in all aspects of *Mash1* proneural function in the ventral telencephalon, that is, the activation of Notch signaling in VZ progenitors, the selection of SVZ progenitors, and the differentiation of postmitotic neurons. In a similar manner, the ectopic expression of *Ngn2* resulted in normal development of the olfactory epithelium in *Mash1*^{KINgn2} embryos (data not shown).

Replacement of Mash1 *by* Ngn2 *does not change the identity of ventral telencephalic neurons*

We next addressed the possibility that *Mash1* and *Ngn2* activate distinct differentiation programs in telencephalic progenitors, as suggested by the observation that the two genes are expressed in complementary ventral and dorsal telencephalic domains that give rise to neuronal populations with different phenotypic characteristics, including different neurotransmitter phenotypes, i.e., GABAergic and glutamatergic, respectively (Wilson and Rubenstein 2000). We have shown previously that when *Mash1* is expressed from the *Ngn2*^{KIMash1} allele, it induces in a subset of dorsal telencephalic neurons the expression of the markers of GABAergic neurons, *GAD67* and *Dlx1*, suggesting that *Mash1* specifies aspects of the neurotransmitter phenotype of telencephalic neurons (Fode et al. 2000). We asked whether reciprocally, the replacement of *Mash1* by *Ngn2*, which rescues the differentiation of ventral telencephalic neurons in the pallidum (Fig. 2B"), results in the misspecification of these neurons.

We examined the phenotype of neurons differentiating from *Ngn2*-expressing MGE progenitors in *Mash1*^{KINgn2} using ventral-specific markers. Surprisingly, these neurons appeared correctly specified, as shown by the normal expression of *GAD67* and *Dlx1* at E12.5 (Fig. 2E"; data not shown). Moreover, examination of the cortex of *Mash1*^{KINgn2} embryos at E15.5 revealed normal expression patterns of *GAD67*, *Dlx1*, and *Lhx6* (Fig. 3A",B"; data not shown), indicating that interneurons migrating tangentially from the ventral telencephalon into the cortex, a neuronal population that is dramatically reduced in *Mash1*$^\Delta$ mutant embryos (Casarosa et al. 1999; Anderson et al. 2001; Fig. 3A',B'), are produced by *Ngn2*-expressing ventral telencephalic progenitors.

Finally, to ask whether *Ngn2* can impart dorsal telencephalic characteristics when misexpressed in ventral progenitors, we examined the expression of the dorsal telencephalic markers *NeuroD*, *Tbr1*, and *Math2* in *Mash1*^{KINgn2} embryos. Expression of *Tbr1* and *Math2* is dependent on *Ngn2* function in the dorsal telencephalon (Fode et al. 2000), and *NeuroD* is a direct target of *Ngn* genes in all *Ngn*-dependent lineages (Ma et al. 1996, 1998; Fode et al. 1998; H.P. Huang et al. 2000). Surpris-
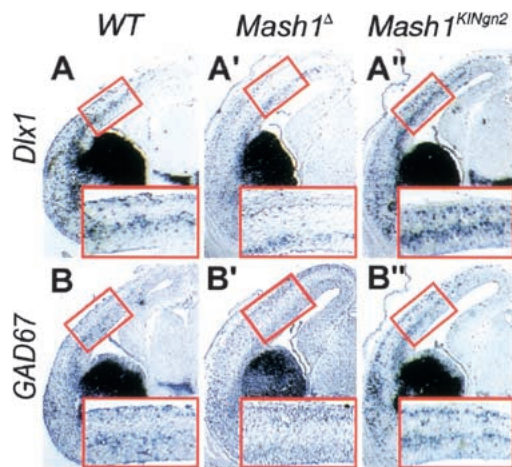
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Figure 3. *Ngn2* rescues the formation of tangentially migrating interneurons in the cerebral cortex. Frontal sections through the telencephalon of E15.5 embryos hybridized with RNA probes for markers of tangentially migrating interneurons. Interneurons expressing *Dlx1* (*A–A″*) and *GAD67* (*B–B″*) are born in the ventral telencephalon and migrate tangentially into the cortex. Many of these neurons are missing in absence of *Mash1* (*A′, B′*), and are rescued in *Mash1^{KINgn2}* embryos (*A″, B″*). Outlined areas are magnified in the insets.

ingly, none of these genes was ectopically expressed in the ventral telencephalon of *Mash1^{KINgn2}* embryos at E10.5 and E12.5 (Fig. 2F″; data not shown). Thus, in striking contrast to the previous demonstration that *Mash1* can confer ventral characteristics to dorsal telencephalic

neurons (Fode et al. 2000), *Ngn2* does not have the capacity to respecify ventral telencephalic neurons, and cannot even activate in the ventral telencephalon its direct target *NeuroD*, which is normally induced in all *Ngn*-dependent lineages.

Replacement of Ngn2 *by* Mash1 *respecifies a subset of spinal motor neurons*

The differences between the KI phenotypes of *Mash1* and *Ngn2* in the telencephalon may be caused by intrinsic differences in the activities of these two genes, or could reflect different strategies employed to specify ventral and dorsal identities in this region, with proneural genes contributing dorsally but not ventrally. To further address the possibility that *Mash1* and *Ngn2* have divergent properties in neuronal subtype specification, we examined the phenotypes of *Mash1^{KINgn2}* and *Ngn2^{KIMash1}* embryos in the ventral spinal cord, another region of the CNS where *Mash1* and *Ngn2* are expressed in distinct progenitor domains.

Ngn2 is broadly expressed in the VZ of the ventral spinal cord (Sommer et al. 1996; Scardigli et al. 2001). Loss of *Ngn2* function leads to reduced expression of a number of homeodomain proteins throughout the ventral spinal cord, including in motor neurons and V1, V2, and V3 interneurons (Scardigli et al. 2001; Fig. 4C). In contrast, *Mash1* expression in the ventral spinal cord is restricted at E10.5 to a narrow region of the VZ, coinciding with the domain that will produce V2 interneurons, characterized by expression of the homeodomain protein
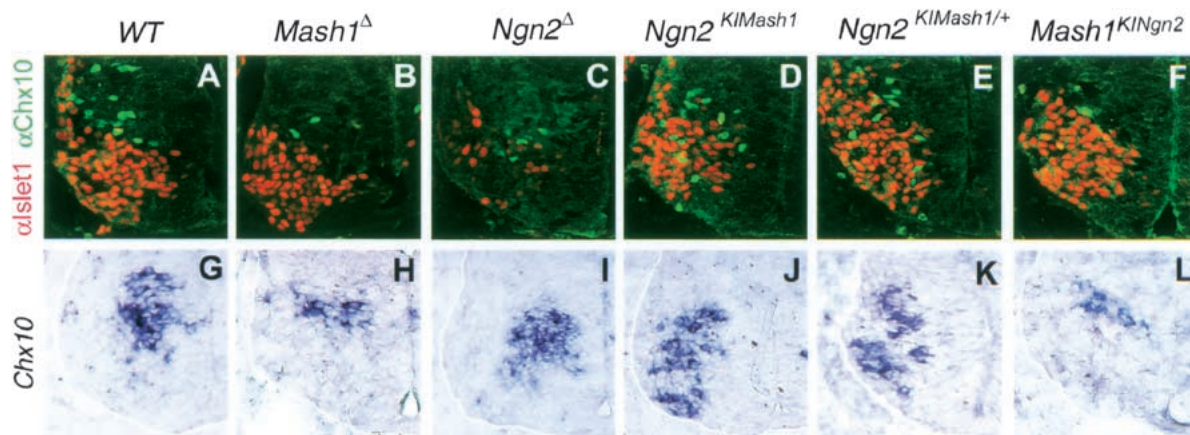

Figure 4. *Mash1* and *Ngn2* LOF and GOF phenotypes in the ventral spinal cord. (*A–F*) Double immunolabeling for the motor neuron marker Isl1 (red) and the V2 interneuron marker Chx10 (green) in transverse sections at brachial level of the ventral spinal cord of E10.5 embryos. There is a drastic reduction in number of V1 interneurons produced in the *Mash1* mutant spinal cord (*B*), and expression of subtype-specific homeodomain proteins is affected in many neuronal populations of *Ngn2* mutant embryos (Scardigli et al. 2001), including Isl1 in motor neurons and Chx10 in V1 interneurons (*C*). Expression of Isl1 is rescued by the ectopic expression of *Mash1* in most motor neurons of homozygous *Ngn2^{KIMash1}* embryos (*D*), whereas a few cells in the motor neuron population fail to express Isl1 and instead express Chx10 (*D*). Ectopic expression of Chx10 in cells ventral to the V2 interneuron population is also observed in embryos heterozygous for the *Ngn2^{KIMash1}* allele (*E*). Expression of *Ngn2* from the *Mash1* locus in *Mash1^{KINgn2}* homozygous mutant embryos does not rescue the defect in V2 interneurons resulting from the loss of *Mash1* function (*F*). (*G–L*) Sections of the brachial spinal cord of E10.5 embryos hybridized with an RNA probe for *Chx10*. Loss of *Mash1* results in a severe reduction in *Chx10* expression (*H*) which is not rescued by ectopic *Ngn2* expression in *Mash1*-expressing progenitors (*L*). In contrast, ectopic expression of *Mash1* in *Ngn2*-expressing progenitors leads to a ventral expansion of the Chx10 expression domain (*J,K*).

Chx10 (Mizuguchi et al. 2001). In $Mash1^\Delta$ embryos, expression of Chx10 was severely reduced, indicating that $Mash1$ is required for the specification and/or differentiation of V2 interneurons (Fig. 4B). In contrast, markers for motor neurons and V1 interneurons showed no overt defects (data not shown).

To determine whether the defects found in the spinal cord of $Ngn2^\Delta$ and $Mash1^\Delta$ mutants reflect a generic proneural or differentiation function of $Ngn2$ and $Mash1$ in motor neurons and V2 interneurons, or conversely, specific functions of the two genes, we examined whether these defects were rescued in $Mash1^{KINgn2}$ and $Ngn2^{KIMash1}$ mice. Labeling of the ventral spinal cord of E10.5 $Ngn2^{KIMash1}$ embryos with Chx10 and the motor neuron marker Isl1 showed that expression of these proteins was largely normal (Fig. 4D), indicating that $Mash1$ can replace $Ngn2$ for the control of the expression of neuron-specific homeodomain proteins. However, a small number of cells expressing Chx10 was intermingled with Isl1+ motor neurons (Fig. 4D), suggestive of a defect in motor neuron specification in these embryos. Ectopic Chx10+ cells, which were also observed in mice heterozygous for the $Ngn2^{KIMash1}$ allele (Fig. 4E) did not express Isl1, indicating that these neurons have not acquired a mixed phenotype. $Irx3$ expression, which marks the V2 interneuron progenitor domain and not the motor neuron progenitor domain (Briscoe et al. 2000) was in contrast unaffected in $Ngn2^{KIMash1}$ embryos (data not shown), thus excluding the possibility that the presence of Chx10+ cells ventral to their normal location is caused by abnormal mixing of progenitor populations for motor neurons and V2 interneurons. These results thus indicate that a subset of neurons in the spinal cord of $Ngn2^{KIMash1}$ embryos have been converted from a Isl1+ motor neuron phenotype to a Chx10+ V2 interneuron phenotype, demonstrating that in the ventral spinal cord as in the dorsal telencephalon, ectopic expression of $Mash1$ is capable of respecifying a subset of $Ngn2$-dependent neurons.

We asked whether, reciprocally, replacement of $Mash1$ by $Ngn2$ could rescue or respecify V2 interneurons. Examination of the spinal cord of E10.5 $Mash1^{KINgn2}$ embryos revealed that the number of Chx10+ cells was reduced to a similar extent in these embryos and in $Mash1^\Delta$ embryos (Fig. 4B). Moreover, there was no evidence for the ectopic differentiation of Isl1+ motor neurons or En1+ V1 interneurons in the V2 domain (Fig. 4F; data not shown). Therefore, ectopic expression of $Ngn2$ is not able to rescue the $Mash1^\Delta$ V2 interneuron defect or to respecify these cells into motor neurons or V1 interneurons. This data thus points to a significant difference in the properties of $Mash1$ and $Ngn2$, with $Mash1$ and not $Ngn2$ being capable of altering neuronal fates when ectopically expressed.

Ngn2 can replace Mash1 to support sympathetic neuron development

To further compare the role of $Mash1$ and $Ngn2$ in the specification of neuronal fates, we analyzed the develop-ment of noradrenergic neurons in $Mash1^{KINgn2}$ mice. $Mash1$ has an essential role in the specification of the noradrenergic neurotransmitter phenotype through regulation of the homeodomain genes $Phox2a$ and $Phox2b$, in both peripheral neurons of sympathetic ganglia and central neurons of the locus coeruleus (Hirsch et al. 1998; Lo et al. 1998). The panneuronal marker $SCG10$ and the noradrenergic markers $Phox2a$, DBH, and TH were expressed at normal levels in sympathetic precursors of $Mash1^{KINgn2}$ embryos at E10.5 (Fig. 5B″–E″; data not shown), whereas their expression was undetectable in $Mash1^\Delta$ embryos (Fig. 6B′–E′). Furthermore, and in contrast with previous experiments showing that ectopic expression of an Ngn gene can induce the expression of sensory-specific markers in nonsensory neural crest derivatives (Perez et al. 1999), no ectopic expression of the sensory markers $NSCL2$ and $Brn3$ was observed in sympathetic ganglia in $Mash1^{KINgn2}$ embryos (Fig. 5F″ data not shown). Thus, ectopic expression of $Ngn2$ in $Mash1$ mutant precursors supports sympathetic neuron differentiation, including the acquisition of a noradrenergic phenotype, and does not ectopically activate sensory neuronal traits.

Strikingly, despite the initial rescue of sympathetic neuron development, expression of $Phox2a$, DBH, and $SCG10$ was severely reduced in the sympathetic chain of $Mash1^{KINgn2}$ embryos at E13.5, to an extent similar to that seen in $Mash1^\Delta$ embryos (Fig. 5G′,H′,G″,H″). Thus, $Ngn2$ expression is not sufficient to maintain a normal differentiation program in $Mash1$ mutant sympathetic neurons. The appearance of a sympathetic mutant phenotype in $Mash1^{KINgn2}$ embryos between E10.5 and E13.5 could be attributable to a defect in proliferation or to a degeneration of $Ngn2$-expressing sympathetic precursors. TUNEL analysis revealed no increase in cell death in sympathetic ganglia of E11.5 and E12.5 $Mash1^{KINgn2}$ mutants (Fig. 5J,J′; data not shown). In contrast, a marked reduction in the number of sympathetic precursors staining with the mitotic cell-specific antibody Ki67 was observed in $Mash1^{KINgn2}$ embryos at E11.5 (Fig. 5I,I′), and 46% fewer Phox2b+ precursors incorporated BrdU in $Mash1^{KINgn2}$ mutant embryos in comparison to wild-type controls (n>3000; data not shown). These results implicate a cell proliferation defect as a major cause for the incomplete generation of the sympathetic chain in $Mash1^{KINgn2}$ embryos, and suggest that $Ngn2$ has a greater capacity than $Mash1$ to arrest proliferation of sympathetic precursors.

Ngn2 does not rescue locus coeruleus development in Mash1^{KINgn2} mutant embryos

The initial rescue of noradrenergic differentiation in $Mash1^{KINgn2}$ embryos could reflect the capacity of $Ngn2$ to directly activate the noradrenergic regulatory cascade when expressed in sympathetic precursors. Alternatively, noradrenergic differentiation in $Mash1^{KINgn2}$ embryos may be driven by the independent $Phox2b$ pathway (Pattyn et al. 1999), with $Ngn2$ providing only a

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Figure 5. *Ngn2* rescues the differentiation of noradrenergic neuronal precursors in sympathetic ganglia but does not support their proliferation. Transverse sections at forelimb level of wild-type and mutant embryos at E10.5 (*A–F*), E13.5 (*G–H*) and E12.5 (*I–J*). In *Mash1^Δ* embryos, *Phox2b* is expressed independently of *Mash1* in sympathetic ganglia (*B'*), whereas other genes associated with the noradrenergic phenotype, including *Phox2a* (*C'*) and *DBH* (*D'*), are not expressed, and postmitotic *SCG10+* neurons (*E'*) are not generated. In *Mash1^KINgn2* embryos, *Ngn2* supports the noradrenergic differentiation of sympathetic precursors, as shown by expression of *Phox2a* (*C''*) and *DBH* (*D''*), and the generation of postmitotic *SCG10+* neurons (*E''*). Hybridization on sagittal sections of E13.5 embryos with probes for *Phox2a* (*G–G''*) and *SCG10* (*H–H''*) shows that the sympathetic ganglia of *Mash1^KINgn2* embryos (*G''*, *H''*) are reduced compared with wild-type ganglia (*G*, *H*), to a level similar to that seen in *Mash1^Δ* embryos (*G'*, *H'*). Antibody staining for the mitotic marker Ki67 reveals a reduced number of dividing cells in sympathetic ganglia of *Mash1^KINgn2* embryos at E12.5 (*I'*) as compared with wild-type embryos (*I*). TUNEL analysis shows that there is no increase in cell death in sympathetic ganglia of *Mash1^KINgn2* embryos at E11.5 (*J'*). Arrowheads indicate sympathetic ganglia
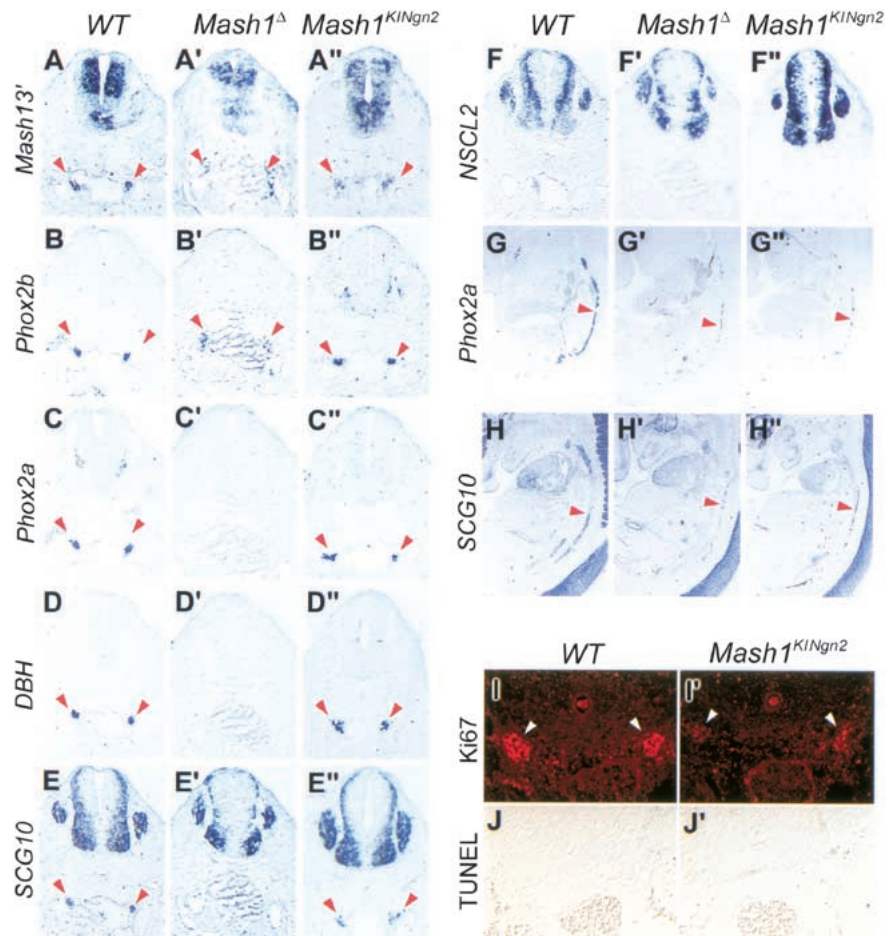
generic proneural/neuronal differentiation activity to sympathetic precursors. To further examine the potential of *Ngn2* to promote the noradrenergic phenotype, we studied locus coeruleus development in *Mash1^KINgn2* embryos. In E10.5 wild-type embryos, locus coeruleus precursors form a characteristically shaped group of cells in the rostral hindbrain that express *Mash1*, *Phox2a*, *Phox2b*, and *DBH* (Fig. 6A–C; data not shown). *Ngn2* is expressed with a similar pattern in *Mash1^KINgn2* embryos (Fig. 6A'). In E10.5 *Mash1^KINgn2* mutant embryos, expression of *Phox2a*, *Phox2b*, and *DBH* was undetectable in this region (Fig. 6B',C'; data not shown), as reported previously for *Mash1^Δ* embryos (Hirsch et al. 1998; data not shown). The locus coeruleus was also absent in *Mash1^KINgn2* mice at E13.5 (Fig. 6E'',F'') and at P0 (Fig. 6G'). Thus, *Ngn2* does not rescue noradrenergic neuron development in the locus coeruleus, suggesting that *Ngn2* does not have the potential to activate the noradrenergic differentiation pathway. Therefore, the rescue of sympathetic neuron differentiation in *Mash1^KINgn2* embryos is likely caused by the combined activities of *Ngn2*, compensating for the loss of the generic neuronal determination/differentiation function of *Mash1*, and of an independent noradrenergic specification pathway driven by *Phox2b*, which is active in sympathetic precursors and not in the locus coeruleus.

Mash1 *does not rescue dorsal root ganglia development in* Ngn1^Δ, Ngn2^KIMash1 *mutant embryos*

Ngn1 and *Ngn2* have been shown to control the generation of different populations of sensory neurons in dorsal root ganglia (DRGs), resulting in a transient defect in neurogenesis in *Ngn2* mutant mice, whereas the loss of both *Ngn1* and *Ngn2* completely blocks DRG development (Ma et al. 1999). A similar delay in onset of neuronal differentiation in DRGs was observed in *Ngn2^KIMash1* mice, using a probe for the panneuronal marker *SCG10* (Fig. 7D,E). Moreover, no differentiation of DRG neurons was observed in *Ngn1^Δ; Ngn2^KIMash1* double mutant embryos (Fig. 7F), a phenotype similar to that of *Ngn1^Δ; Ngn2^Δ* double mutants. Thus ectopic expression of *Mash1* from the *Ngn2* locus does not rescue DRG defects, indicating that *Ngn2* has a unique activity in the development of peripheral sensory ganglia that *Mash1* cannot carry out.

Discussion

Mash1 and *Ngn2*, two proneural genes belonging respectively to the *achaete-scute* and *atonal* families of bHLH factors are expressed in distinct and complementary progenitor populations throughout the nervous system (Fig.
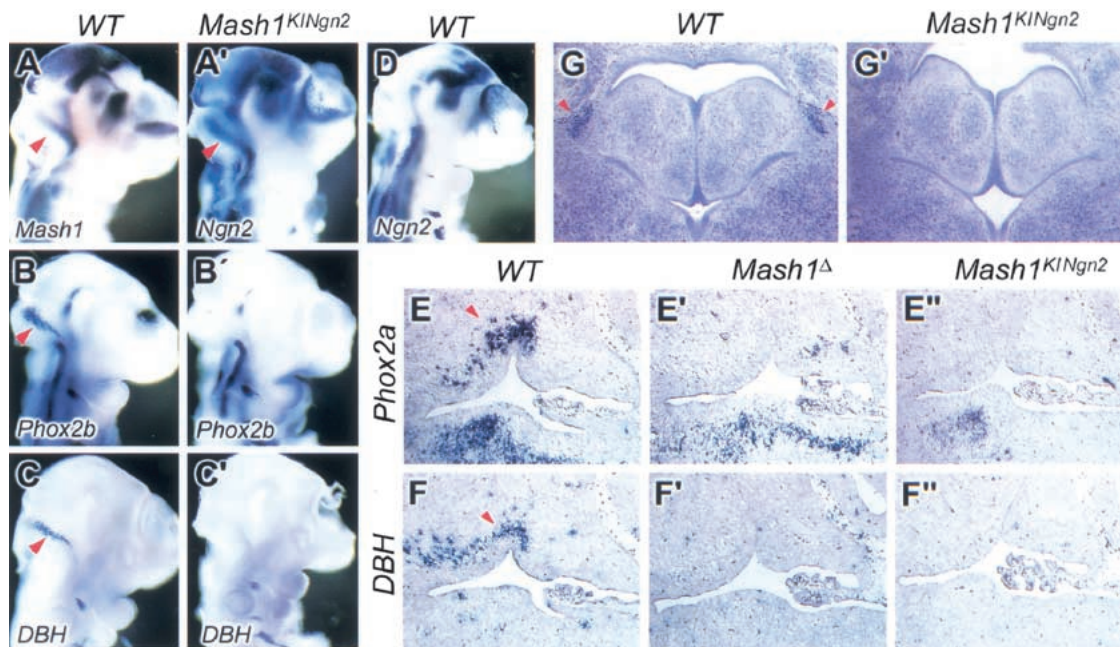
Figure 6. *Ngn2* does not rescue the development of noradrenergic neurons of the locus coeruleus. (*A–D, A′–C′*) Hybridization of E10.5 wild-type (*A–D*) and *Mash1KINgn2* embryos (*A′–C′*) embryos with probes for *Mash1* (*A*), *Phox2b* (*B*) and *DBH* (*C*), showing expression of these genes in precursors of the locus coeruleus, located in the rostral hindbrain near the rhombic lip (arrowheads). *Ngn2* is expressed in these precursors in *Mash1KINgn2* embryos (*A′*) and not in wild-type embryos (*D*). Nevertheless, noradrenergic traits, including expression of *Phox2b* (*B′*) and *DBH* (*C′*) are not induced by *Ngn2* in *Mash1KINgn2* embryos. (*E–E″, F–F″*) Sagittal sections at the level of the fourth ventricle in wild-type and mutant embryos at E13.5. *Phox2a* and *DBH* are normally expressed by the neurons of the locus coeruleus located anterior and dorsal to the fourth ventricle (arrowheads in *E* and *F*, respectively), whereas expression of the two genes is missing at the same location in *Mash1Δ* (*E′, F′*) and *Mash1KINgn2* embryos (*E″, F″*). (*G, G′*) Nissl stained coronal sections at the level of the pons at birth. The locus coeruleus is recognizable as a compact group of darkly stained neurons lateral to the fourth ventricle (arrowhead in *G*), which is missing in *Mash1KINgn2* newborns (*G′*).

8), suggesting that these genes contribute to the specification of neuronal phenotypes in the CNS and PNS. We have used a KI approach in mouse to systematically compare the activities of *Ngn2* and *Mash1* when expressed in the same cellular contexts and at stages when proneural function is normally provided. We discuss here the divergent roles of *Mash1* and *Ngn2* in neuronal subtype specification, and the variable contribution of these genes to the specification of neuronal identity in different lineages (Fig. 8).

Mash1 *is a determinant of neuronal identity*

Evidence that *Mash1* has a role in the specification of neuronal identity has been obtained in different regions of the nervous system. The strongest evidence comes from GOF experiments showing that *Mash1* can induce expression of (1) markers of GABAergic neurons, *Dlx1* and *GAD67*, in the cerebral cortex (Fode et al. 2000), (2) the noradrenergic determinant *Phox2a* and an autonomic phenotype in neural crest stem cell and neural tube cultures (Lo et al. 1998, 2002), and (3) the marker of V2 interneuron identity Chx10, when expressed in motor neuron progenitors (this paper). Induction of ectopic markers in several regions of *Ngn2KIMash1* embryos cor-

relates with a down-regulation of markers that define neuronal phenotype in these regions (e.g., Islet1 in motor neurons, *Math2* in cortical neurons), suggesting that ectopic expression of *Mash1* in *Ngn2*-expressing progenitors results in substitution of neuronal subtype-specific differentiation programs. Respecification of cortical neurons and spinal motor neurons is also observed to some extent in heterozygous *Ngn2KIMash1* embryos (Fode et al. 2000; Fig. 4E), indicating that this phenotype is indeed caused by the ectopic expression of *Mash1* and that the expression of *Ngn2* in the same cells does not significantly interfere. Moreover, it is important to notice that only a subset of neurons are respecified in both the dorsal telencephalon and motor neuron domains of homozygous *Ngn2KIMash1* embryos whereas the majority of neurons differentiate normally (Fode et al. 2000; Fig. 4D), clearly indicating that instructive cues for the specification of cortical neurons and motor neurons persist in these mutant embryos in the absence of *Ngn2* function.

From the above data, we conclude that *Mash1* is an *instructive* determinant of neuronal subtype identity, in the sense that it can override endogenous differentiation programs when ectopically expressed, thus demonstrating that it conveys neuronal subtype information and acts to some extent independently from the cellular context. A striking feature of the *Ngn2KIMash1* mutant phe-
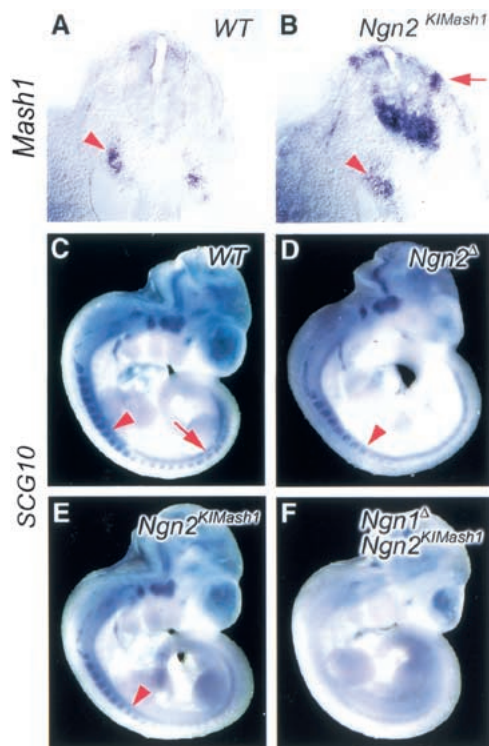
Figure 7. *Mash1* does not rescue the development of *Ngn2*-dependent sensory neurons in dorsal root ganglia. (*A*, *B*). Transverse section of E9.5 embryos sectioned following whole mount in situ hybridization with a *Mash1* probe. *Mash1* is expressed in sympathetic ganglia (arrowheads) of both wild-type and *Ngn1*$^\Delta$; *Ngn2*^{KIMash1} double mutant embryos. *Mash1* expression is also observed in migrating neural crest cells entering DRGs in a *Ngn2*^{KIMash1} embryo. (*C–F*) Mouse embryos at E10.5 are probed with the pan-neuronal marker *SCG10*. A wild-type embryo shows DRG staining well beyond the 20th somite (*C*, arrow). *SCG10* expression in *Ngn2*^{KIMash1} (*E*) is delayed in comparison to the wild-type embryo, as also observed in *Ngn2*$^\Delta$ mutants (*D*). Arrowhead points to the eighth somite (*C–E*). *Ngn1*$^\Delta$, *Ngn2*^{KIMash1} embryo (*F*) shows no *SCG10* staining in DRGs, indicating that no sensory neurons are generated by *Mash1* expression from the *Ngn2* locus.

notype is that ectopic expression of *Mash1* results in the induction of different neuronal phenotypes in different regions of the nervous system, i.e., GABAergic neurons in the telencephalon and V2 interneurons in the spinal cord. If one includes the induction of *Phox2a* in neural crest cultures, then *Mash1* is able to induce at least three different phenotypes in different progenitor populations. Within each of these regions, however, *Mash1* expression and activity are associated with only one neuronal phenotype. This suggests that the specificity of *Mash1* is modulated by regionally restricted cofactors, a hypothesis already proposed to account for the context-dependent specificity of *Drosophila* proneural proteins in sensory organ induction (Chien et al. 1996; Jarman and Ahmed 1998; Goulding et al. 2000). The identification of modulators of proneural gene activity will be critical to understand how neuronal identities are specified.

A permissive role for Ngn2 *in specification of neuronal fates*

In contrast with *Mash1*, *Ngn2* does not have an instructive role in the specification of neuronal identity, based on both GOF (this study; Mizugushi et al. 2001) and LOF studies (Scardigli et al. 2001). First, none of the tissues examined in *Mash1*^{KINgn2} mice showed evidence of re-specification of neuronal identity (Fig. 8), indicating that the replacement of *Mash1* by *Ngn2* is not sufficient to override the subtype-specific differentiation programs activated in *Mash1*-expressing progenitors. This is in clear contrast with the ectopic expression of subtype-specific markers in the telencephalon and spinal cord in *Ngn2*^{KIMash1} embryos (Fode et al. 2000; Fig. 8). Second, electroporation of *Ngn2* in the neural tube of chick embryos results in premature cell cycle exit and premature neuronal differentiation of neuroepithelial cells, as expected for a gene with proneural activity, but *Ngn2*-induced ectopic neurons do not express markers of motor neurons or ventral interneurons, consistent with the idea that *Ngn2* does not have the capacity to endow progenitor cells with neuronal subtype information in an ectopic context. In contrast, the bHLH gene *Olig2* has the capacity in the same assay to induce the expression of a number of motor neuron markers (Mizugushi et al. 2001; Novitch et al. 2001).

One interpretation of the above data could be that the function of *Ngn2* in neurogenesis is strictly that of a proneural gene being involved in the selection of progenitor cells (Fode et al. 1998) and their commitment to a "generic" neuronal fate (Nieto et al. 2001), but having no role in the specification of neuronal identity. We do not think that this interpretation is likely for several reasons. First, LOF analysis of *Ngn*s has revealed that although *Ngn1* and *Ngn2* have redundant proneural functions in the ventral spinal cord (as only *Ngn1*$^\Delta$;*Ngn2*$^\Delta$ double mutants present a marked reduction in neurogenesis; Scardigli et al. 2001), *Ngn2*$^\Delta$ single mutants present a severe reduction in expression of neuronal subtype-specific homeodomain proteins throughout the ventral spinal cord. Thus, *Ngn2* function is required in neuronal fate specification programs independantly of its proneural activity (Scardigli et al. 2001). In the dorsal spinal cord, loss of *Ngn1* and *Ngn2* functions results in the substitution of a specific population of interneurons (D3A neurons) by another (D1 neurons), but this could be attributable to repression by *Ngn*s of the subtype determinant *Math1* rather than to a direct role of *Ngn*s in specification of dorsal interneuron identity (Gowan et al. 2001).

GOF experiments also support the idea that *Ngn*s contribute to the specification of neuronal identity, in particular for peripheral sensory neurons. Forced expression of *Ngn1* in vivo by retroviral infection in the chick results in ectopic expression of several sensory neuron markers in tissues of neural and mesodermal origin (Perez et al. 1999). Expression of *Ngn1* or *Ngn2* in dissociated neural tube cell cultures induces sensory neurons more efficiently than it promotes neuronal differentia-
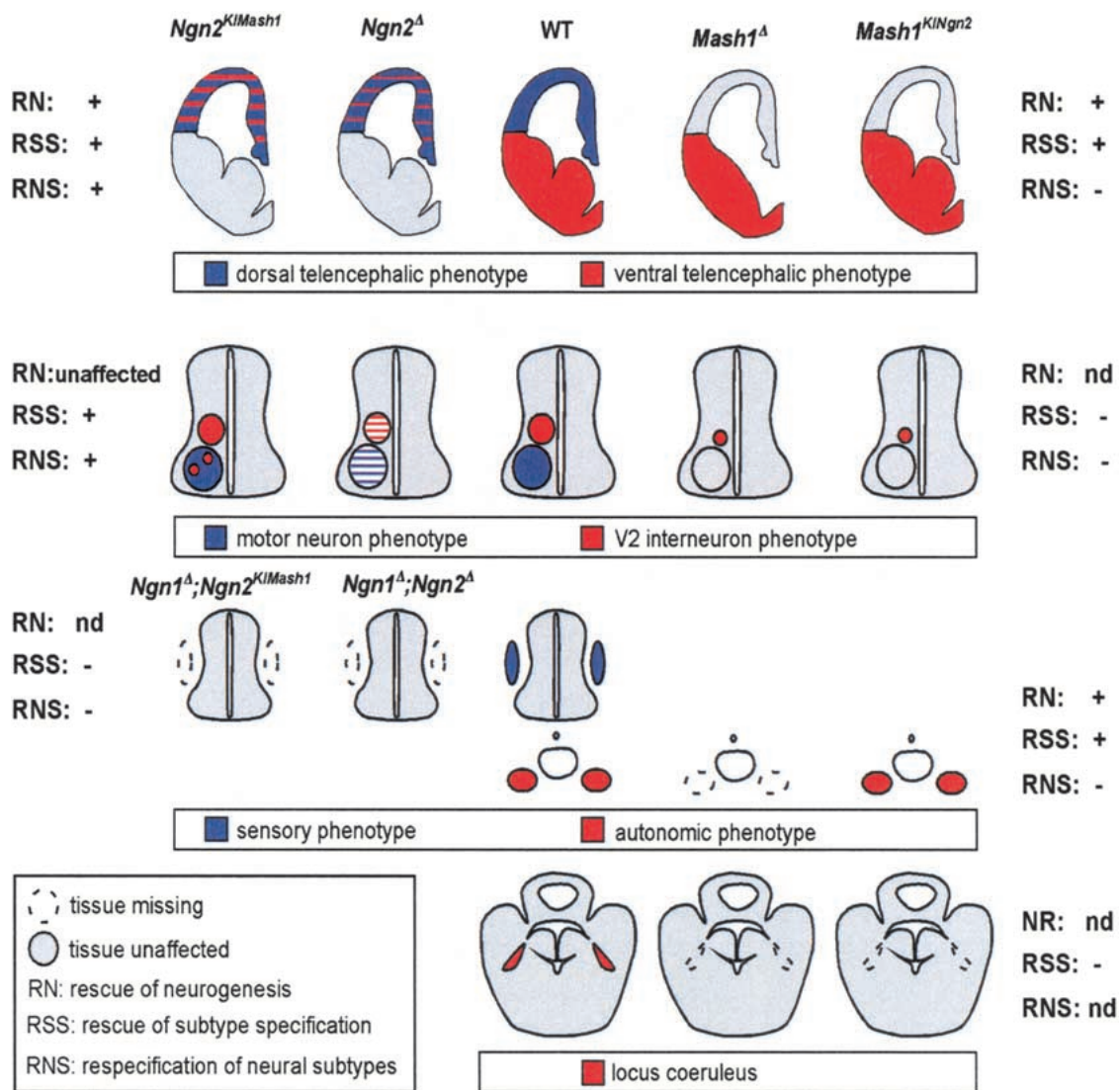
Figure 8. Summary of the phenotypic analysis of $Ngn2^{KIMash1}$ and $Mash1^{KINgn2}$ mice. The different regions of the CNS and PNS analyzed in the two KI mouse strains are schematized. Neurogenesis defects observed in null mutant mice are rescued in KI mice (indicated by RN +), except in tissues that are missing due to specification defects (indicated by RN nd). The rescue of specification defects (indicated by RSS +) varies from lineage to lineage in both KI strains, reflecting the variable contribution of proneural genes to the specification of neuronal subtype identity in different lineages. Respecification of neuronal subtypes caused by ectopic proneural gene expression (indicated by RNS +) is observed only in the CNS of $Ngn2^{KIMash1}$ mice, indicating that $Mash1$ but not $Ngn2$ has an instructive role in the specification of neuronal identity in the CNS.

tion, indicating that $Ngns$ promote the sensory identity in peripheral neurons (Lo et al. 2002). Importantly, this activity requires specific culture conditions (i.e., low BMP concentrations). In other conditions (i.e., high BMP concentrations), $Ngns$ promote instead an autonomic fate, revealing that the role of $Ngns$ in neuronal subtype specification is strongly influenced by the cellular context, which dictates the quality of the subtype that $Ngns$ promote (Lo et al. 2002). Another striking illustration of the dependence of Ngn function on the cellular context is the finding that ectopic expression of $Ngn2$ in the ventral telencephalon of $Mash1^{KINgn2}$ mice rescues the generation of $Mash1$-dependent progenitors but does not in-

duce $NeuroD$ expression, although $NeuroD$ is a direct target of Ngn genes throughout the embryo (H.P. Huang et al. 2000). This result highlights the critical role played by regionally expressed cofactors in determining the specificity of Ngn activity and the identity of the target genes that $Ngns$ activate.

The inability of $Ngn2$ to override endogenous differentiation programs when ectopically expressed may be due in some situations to its late expression with respect to the period of competence of neural progenitors for subtype specification. In the sympathetic lineage in particular, $Mash1$ is first expressed in progenitor cells that may already be restricted to an autonomic fate (Lo et al. 2002),

and this alone could explain that in *Mash1*^{KINgn2} embryos, *Ngn2* expression from the *Mash1* locus does not lead to a respecification of sympathetic neurons to a sensory fate. However, no systematic difference in temporal pattern of expression has been reported between *Mash1* and *Ngn2* in CNS progenitors, and it is therefore likely that differences in intrinsic properties rather than in timing of expression account for the different KI phenotypes of *Mash1* and *Ngn2*.

Together, results from LOF and GOF analysis support the idea that *Ngn*s are important components of the transcription machinery underlying the specification of neuronal identity. However, because *Ngn*s do not interfere with neuronal subtype-specific differentiation programs when ectopically expressed (this study), and the outcome of their activity is largely controlled by other factors (this study; Lo et al. 2002), they do not have the characteristics of instructive determinants of neuronal identity. Instead, they appear to function as *permissive* factors that are required for the normal progression of subtype specification programs, as indicated by the role of *Ngn2* in the expression of homeodomain neuronal determinant in the spinal cord (Scardigli et al. 2001), but that must act in combination with instructive factors to specify neuronal fates. In the spinal cord, the bHLH protein Olig2, which is expressed in the motor neuron progenitor domain, is a likely candidate as it can induce motor neuron differentiation when ectopically expressed in the chick neural tube (Novitch et al. 2001; Mizuguchi et al. 2001). Similarly, *Mash1* is expressed in the V2 interneuron progenitor domain (Mizuguchi et al. 2001), and we show here that it is involved in the specification of V2 interneuron identity. *Ngn2* is coexpressed with Olig2 and *Mash1* in progenitors for motor neurons and V2 neurons, respectively (Mizuguchi et al. 2001), suggesting that it may function in cooperation with these two factors, and possibly other bHLH proteins in different progenitor populations, to promote the differentiation of the different types of spinal cord neurons.

It has been proposed that the functions of progenitor selection and neuronal subtype specification, which are carried in parallel by the *ac-sc* and *ato* genes in *Drosophila*, have been uncoupled and are controlled by different bHLH genes in vertebrates (Hassan and Bellen 2000). As discussed above, the results of our study do not support this model and suggest on the contrary that vertebrate bHLH genes with proneural activity (i.e., *Mash1* and *Ngn*s) also have important and diverse functions in neuronal subtype specification, which likely involve interactions with many other determinants of neuronal identity.

Variable contribution of Mash1 to the specification of neuronal subtype identities

The rescue of the *Mash1* null mutant phenotype by ectopic expression of *Ngn2* varies in different regions of the nervous system (Fig. 8). Although development of the ventral telencephalon, olfactory epithelium and, transiently, sympathetic ganglia is normal in *Mash1*^{KINgn2}

mice, there is no rescue of V2 interneurons and locus coeruleus development. The correct differentiation of some *Mash1*-dependent noradrenergic neurons (e.g., sympathetic neurons) but not others (locus coeruleus) is striking, and can be explained by the difference in the genetic pathways specifying the noradrenergic phenotype in these two tissues (Goridis and Brunet 1999; Pattyn et al. 2000). *Mash1* and the *Phox2* genes are required for the production of noradrenergic neurons in both sympathetic ganglia and the locus coeruleus, but their interactions differ drastically in the two tissues. In the locus coeruleus, *Mash1*, *Phox2a*, and *Phox2b* act sequentially in a linear pathway, so that in absence of *Mash1*, neither *Phox2a* nor *Phox2b* is expressed (Hirsch et al. 1998; Pattyn et al. 2000). The replacement of *Mash1* by *Ngn2* in *Mash1*^{KINgn2} mice does not rescue locus coeruleus development, most likely because *Ngn2* does not share with *Mash1* the property of sufficiency to activate *Phox2a* expression and the noradrenergic differentiation pathway. This result therefore supports the conclusion that in the absence of instructive cofactors, *Ngn2* does not have the capacity to induce the ectopic expression of inappropriate neuronal-subtype specific genes or the appropriate expression of subtype-specific genes normally dependent on *Mash1*.

In contrast with the complete dependence of *Phox2a* and *Phox2b* expression on *Mash1* activity in the locus coeruleus, *Phox2b* expression is activated independently of *Mash1* in sympathetic neuron precursors, and noradrenergic differentiation in these cells requires both and independently *Mash1* and *Phox2b* activity (Hirsch et al. 1998; Pattyn et al. 2000). The rescue of *Phox2a* expression and noradrenergic differentiation that we observe in sympathetic precursors of *Mash1*^{KINgn2} mice is not caused by *Ngn2* activity alone, based on the conclusion drawn from the locus coeruleus phenotype that *Ngn2* does has the capacity to induce this phenotype (see above). More likely, *Ngn2* provides a proneural/differentiation activity that is missing in *Mash1*$^{\Delta}$ mutants, as well as a context-dependent subtype-determining function. Expression of the instructive cofactor *Phox2b* is then presumably sufficient, in conjunction with *Ngn2*, to induce noradrenergic differentiation. Thus, the rescue of the *Mash1*-null phenotype by ectopic expression of *Ngn2* in sympathetic ganglia and not in the locus coeruleus probably reflects the different strategies underlying noradrenergic development in these two tissues (Goridis and Brunet 1999).

Similar to the rescue of sympathetic neuron development, the normal development of the ventral telencephalon in *Mash1*^{KINgn2} mice presents a paradox. As already discussed, *Mash1* has been implicated in the specification of the GABAergic phenotype of ventral telencephalic neurons, based on its capacity to induce in dorsal neurons the ectopic expression of *Dlx* genes, which are essential regulators of ventral telencephalic neuron differentiation (Anderson et al. 1997) and of the GABAergic neuron marker *GAD67* (Fode et al. 1998). It was therefore unexpected that replacement of *Mash1* by *Ngn2* rescues the expression of *Dlx1* and *GAD67* and the

normal differentiation of GABAergic neurons in the cortex and ventral telencephalon. As discussed above in the context of sympathetic development, this result clearly points to the existence of a *Mash1*-independent pathway of induction of *Dlx* gene expression and GABAergic neuron specification. Whereas this putative pathway cannot alone compensate for the loss of *Mash1* function in *Mash1*$^\Delta$ mice, it is sufficient to rescue telencephalic development in *Mash1*^{KINgn2} mice in combination with the proneural activity and cofactor-dependent subtype specification function of *Ngn2*. The respective contributions of *Mash1* and other putative determinants to the specification of GABAergic neurons during normal telencephalic development remain to be established.

The role of *Mash1* in the development of V2 spinal cord interneurons appears to be more similar to that discussed above for the locus coeruleus. The ectopic expression of Chx10 observed in *Ngn2*^{KIMash1} mice indicates that *Mash1* has an instructive role in the specification of V2 interneurons, and the reduction in number of Chx10+ cells in *Mash1*$^\Delta$ mice likely reflects a requirement for this specification function, although an additional role for *Mash1* in the selection of V2 interneuron progenitors cannot be excluded. In any case, the lack of rescue of this defect in *Mash1*^{KINgn2} mice indicates that *Ngn2* alone cannot compensate for the loss of a specification function of *Mash1*, and in addition that no other instructive determinant of V2 interneuron identity can replace *Mash1* function, even when *Ngn2* activity is provided. Our analysis of *Mash1*^{KINgn2} mice therefore points to important differences in the role played by *Mash1* in subtype specification programs, with *Mash1* being the major subtype determinant in some neuronal populations (locus coeruleus, V2 interneurons) and sharing this role with other factors in other populations (ventral telencephalon, sympathetic neurons).

Variable efficiency in the rescue of Ngn-*dependent lineages by* Mash1

The capacity of *Mash1* to rescue the development of *Ngn2*-dependent lineages also varies in different regions of the nervous system (Fig. 8). The normal level of neurogenesis observed in the dorsal telencephalon of *Ngn2*^{KIMash1} demonstrates the capacity of *Mash1* to compensate for the loss of *Ngn2* proneural function (Fode et al. 2000). Similarly, *Mash1* rescues the defects in homeodomain protein expression observed in the ventral spinal cord of *Ngn2* null mutant embryos (Scardigli et al. 2001; Fig. 4), and analysis of embryos homozygous for both the *Ngn2*^{KIMash1} allele and a *Ngn1* null mutant allele (*Ngn1*$^\Delta$; *Ngn2*^{KIMash1} embryos) shows that ectopic expression of *Mash1* in *Ngn2*-expressing progenitors is sufficient to rescue the neurogenesis defect observed in the spinal cord of *Ngn1*; *Ngn2* double mutants (Scardigli et al. 2001; data not shown). More surprisingly, ectopic expression of *Mash1* does not rescue the development of dorsal root ganglia in *Ngn1*$^\Delta$; *Ngn2*^{KIMash1} embryos (Fig. 7). One possibility, supported by GOF studies in dissociated neural tube cell cultures (Lo et al. 2002), is that

*Ngn*s have a function in the specification of the sensory identity of DRG neurons that *Mash1* cannot provide, and that in the low BMP environment of DRGs, *Mash1* cannot promote an autonomic differentiation of DRG neurons.

An alternative interpretation of the lack of DRG development in *Ngn1*$^\Delta$, *Ngn2*^{KIMash1} mice is suggested by the finding that *Mash1* has a greater capacity than *Ngn2* to sustain the proliferation of sympathetic neuron precursors (Fig. 5I,I′; Lo et al. 2002), and conversely that atonal-related genes such as *Ngn*s and *NeuroD* are more efficient than *Mash1* at promoting neuronal differentiation in ectodermal cells due to a lower sensitivity to Notch-mediated lateral inhibition (Chitnis and Kintner 1996; Ma et al. 1996; Lo et al. 2002). Because of the different capacities of the two genes to promote the transition from proliferation to differentiation, the substitution of *Ngn2* by *Mash1* in DRG cells may lead to an excess of cell proliferation and a delay of differentiation that may interfere with cell survival. The idea that *Mash1* is more compatible with cell proliferation than are the *Ngn*s is supported by the observation that expression of these genes generally correlates with the proliferative status of progenitor populations. *Mash1* tends to be expressed in PNS and CNS progenitors undergoing extensive proliferation (e.g., sympathetic precursors, ganglionic eminence in the ventral telencephalon, SVZ progenitors in the dorsal telencephalon; C. Schuurmans and F. Guillemot, unpubl.) whereas *Ngn*s tend to be expressed in progenitors that differentiate rapidly (e.g., sensory precursors in dorsal root and cranial ganglia, VZ progenitors in the dorsal telencephalon). Further investigation will be necessary to elucidate the mechanisms underlying the divergent properties of *Mash1* and *Ngn2* in regulating the transition from proliferation to differentiation and in specifying neuronal subtype identity.

Materials and methods

Construction of Mash1^{KINgn2} targeting vector

The *Mash1* genomic clone (gb1.4) used to generate the *Mash1*^{KINgn2} targeting vector was described previously (Guillemot et al. 1993). A 5.5-kb *Sac*I fragment ending with the *Sac*I site located three nucleotides downstream of the start ATG was used as the 5′ arm of the targeting construct. *Ngn2* coding sequences were cloned directly downstream and in-frame with the endogenous start site of *Mash1* by introducing an *Eco*RI cloning site immediately downstream of the *Sac*I site by PCR. The 3′ arm was a 1.4-kb *Bam*H1 fragment corresponding to the 3′-most sequence of the gb1.4 clone. These fragments were cloned into pKSloxPNT, which contains a loxP-flanked PGK-neomycin cassette (Hanks et al. 1995). The targeting vector was linearized by *Xho*I and electroporated into R1 ES cells as described previously (Guillemot et al. 1993). Homologous recombination events were identified by Southern blotting using *Eco*RI to digest the genomic DNA and a 1 kb *Hpa*I fragment as a 5′- external probe, as previously described (Guillemot et al. 1993x). Positive clones were then verified with a 3′ 300-bp *Sac*I/*Nde*I external probe from pλM1B7 (kind gift of Jane Johnson, University of Texas). One recombinant ES cell clone was in-

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jected into C57BL/6J females and gave germ-line transmission of the mutation. The mutant strain was maintained by backcrossing into a CD1 outbred background.

Genotyping of Mash1^{KINgn2}, Ngn2^{KIMash1}, Ngn2$^{\Delta}$, *and* Mash1$^{\Delta}$ *mutant mice*

Ngn2^{KIMash1} and *Ngn2*$^{\Delta}$ embryos were generated by crossing heterozygous animals and genotyping was performed by PCR as described previously (Fode et al. 1998, 2000). Wild-type, heterozygous and homozygous *Mash1* mutant embryos were obtained from intercrosses of *Mash1*$^{\Delta/+}$ mice (Guillemot et al. 1993) or *Mash1*$^{KINgn2/+}$ mice. For staging of embryos, midday of the vaginal plug was considered as E0.5. Genotyping of the different *Mash1* alleles was preformed by PCR using the following primers and conditions. For the *Mash1*$^{\Delta}$ allele, upper primer CCAG GACTCAATACGCAGGG and lower primer GCAGCGCATC GCCTTCTATC, with 30 cycles of 94°C/1 min, 60°C/1 min, and 72°C/1 min. For the *Mash1*^{KINgn2} allele, upper primer ACAGTTTGGCCCGGCATGGA and lower primer AGATG TAATTGTGGGCGAAG with 30 cycles of 94°C/1 min, 60°C/1 min, and 72°C/1 min. For the *Mash1* wild-type allele, upper primer CTCCGGGAGCATGTCCCCAA and lower primer CCAGGACTCAATACGCAGGG with 30 cycles of 94°C/1 min, 64°C/1 min, and 72°C/1 min.

RNA in situ hybridization and immunohistochemistry

Embryos were fixed at 4°C in 4% paraformaldehyde for 2 h (up to E11.5) or overnight (E12.5 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, Hes5, GAD67, Dlx1, Dlx5, Lhx2,* and *SCG10,* as described in Casarosa et al. (1999); *Mash1* and *NeuroD* as described in Cau et al. (1997); *Phox2a, Phox2b,* and *DBH* as described in Pattyn et al. (1999); *Ngn2* (Gradwohl et al. 1996); *Prox1* (Torii et al. 1999); *Lhx6* (Grigoriou et al. 1998); and *Nscl2* (Perez et al. 1999). Double-label immunofluorescence was performed by simultaneous incubation with the antibodies of interest, which included monoclonal antibodies against Pax6 (Pax6), Islet-1 (40.2D6), Engrailed-1 (4G11) MNR2/HB9 (81.5C10) and Lim 1/2, all of which were obtained from the Developmental Studies Hybridoma Bank, and a Chx10 rabbit antiserum that was a gift from Johan Ericson (Karolinska Institute, Stockholm). Goat anti-mouse and goat anti-rabbit secondary antibodies conjugated to AlexA and Cy3 (Jackson Immunoresearch) were used for double labeling experiments. Confocal analysis was carried out on a Leica TCS 4D microscope.

Histology, BrdU incorporation, and TUNEL experiments

For histological analysis, embryos were fixed in Bouin's fixative for 3–4 days, processed for wax embedding, cut at 7 μm, and stained with cresyl violet. For BrdU incorporation experiments, pregnant females were injected intraperitoneally with 100 μg/g of body weight of BrdU (Sigma) and sacrificed after 30 min. Embryos were processed as described above and BrdU labeling was exposed by 40 min treatment in HCl 2N at 37°C. The anti-BrdU monoclonal antibody (1:100 dilution) was from Boehringer Mannheim. The TUNEL procedure was performed as described in Cau et al. (1997).

References

Anderson, S.A., Qiu, M., Bulfone, A., Eisenstat, D.D., Meneses, J., Pedersen, R., and Rubenstein, J.L. 1997. Mutations of the homeobox genes *Dlx-1* and *Dlx-2* disrupt the striatal subventricular zone and differentiation of late born striatal neurons. *Neuron* **19:** 27–37.

Anderson, S.A., Marin, O., Horn, C., Jennings, K., and Rubenstein, J.L. 2001. Distinct cortical migrations from the medial and lateral ganglionic eminences. *Development* **128:** 353–363.

Bermingham, N.A., Hassan, B.A., Price, S.D., Vollrath, M.A., Ben-Arie, N., Eatock, R.A., Bellen, H.J., Lysakowski, A., and Zoghbi, H.Y. 1999. *Math1*: An essential gene for the generation of inner ear hair cells. *Science* **284:** 1837–1841.

Bray, S. 2000. Specificity and promiscuity among proneural proteins. *Neuron* **25:** 1–2.

Briscoe, J., Pierani, A., Jessell, T.M., and Ericson, J. 2000. A homeodomain protein code specifies progenitor cell identity and neuronal fate in the ventral neural tube. *Cell* **101:** 435–445.

Brunet, J.F. and A. Ghysen. 1999. Deconstructing cell determination: proneural genes and neuronal identity. *Bioessays* **21:** 313–318.

Campos-Ortega, J.A. 1998. The genetics of the *Drosophila achaete-scute* gene complex: A historical appraisal. *Int. J. Dev. Biol.* **42:** 291–297.

Casarosa, S., Fode, C., and Guillemot, F. 1999. *Mash1* regulates neurogenesis in the ventral telencephalon. *Development* **126:** 525–534.

Cau, E., Gradwohl, G., Fode, C., and Guillemot, F. 1997. *Mash1* activates a cascade of bHLH regulators in olfactory neuron progenitors. *Development* **124:** 1611–1621.

Cepko, C.L. 1999. The roles of intrinsic and extrinsic cues and bHLH genes in the determination of retinal cell fates. *Curr. Opin. Neurobiol.* **9:** 37–46.

Chien, C.T., Hsiao, C.D., Jan, L.Y., and Jan, Y.N. 1996. Neuronal type information encoded in the basic-helix-loop-helix domain of proneural genes. *Proc. Natl. Acad. Sci.* **93:** 13239–13244.

Chitnis, A. and Kintner, C. 1996. Sensitivity of proneural genes to lateral inhibition affects the pattern of primary neurons in *Xenopus* embryos. *Development* **122 :** 2295–2301.

de la Pompa, J.L., Wakeham, A., Correia, K.M., Samper, E., Brown, S., Aguilera, R.J., Nakano, T., Honjo, T., Mak, T.W., Rossant, J., and Conlon, R.A. 1997. Conservation of the Notch signalling pathway in mammalian neurogenesis. *Development* **124:** 1139–1148.

Fode, C., Gradwohl, G., Morin, X., Dierich, A., LeMeur, M., Goridis, C., and Guillemot, F. 1998. The bHLH protein NEUROGENIN 2 is a determination factor for epibranchial placode-derived sensory neurons. *Neuron* **20:** 483–494.

Fode, C., Ma, Q., Casarosa, S., Ang, S.L., Anderson, D.J., and Guillemot, F. 2000. A role for neural determination genes in specifying the dorsoventral identity of telencephalic neurons. *Genes* & *Dev.* **14:** 67–80.

Goridis, C. and Brunet, J.F. 1999. Transcriptional control of neurotransmitter phenotype. *Curr. Opin. Neurobiol.* **9:** 47–53.

Goulding, S.E., zur Lage, P., and Jarman, A.P. 2000. *amos*, a proneural gene for *Drosophila* olfactory sense organs that is regulated by *lozenge. Neuron* **25:** 69–78.

Gowan, K., Helms, A.W., Hunsaker, T.L., Collisson, T., Ebert, P.J., Odom, R., and Johnson, J.E. 2001. Crossinhibitory activities of Ngn1 and Math1 allow specification of distinct dorsal interneurons. *Neuron* **31:** 219–232.

Gradwohl, G., Fode, C., and Guillemot, F. 1996. Restricted expression of a novel murine *atonal*-related bHLH protein in undifferentiated neural precursors. *Dev. Biol.* **180:** 227–241.

Grigoriou, M., Tucker, A.S., Sharpe, P.T., and Pachnis, V. 1998. Expression and regulation of *Lhx6* and *Lhx7*, a novel subfamily of LIM homeodomain encoding genes, suggests a role in mammalian head development. *Development* **125:** 2063–2074.

Guillemot, F. 1999. Vertebrate bHLH genes and the determination of neuronal fates. *Exp. Cell. Res.* **253:** 357–364.

Guillemot, F., Lo, L.C., Johnson, J.E., Auerbach, A., Anderson, D.J., and Joyner, A.L. 1993. *Mammalian achaete-scute homolog 1* is required for the early development of olfactory and autonomic neurons. *Cell* **75:** 463–476.

Hanks, M., Wurst, W., Anson-Cartwright, L., Auerbach, A.B., and Joyner, A.L. 1995. Rescue of the *En-1* mutant phenotype by replacement of *En-1* with *En-2*. *Science* **269:** 679–682.

Hassan, B.A. and Bellen, H.J. 2000. Doing the MATH: Is the mouse a good model for fly development? *Genes* & *Dev.* **14:** 1852–1865.

Hirsch, M.R., Tiveron, M.C., Guillemot, F., Brunet, J.F., and Goridis, C. 1998. Control of noradrenergic differentiation and Phox2a expression by MASH1 in the central and peripheral nervous system. *Development* **125:** 599–608.

Horton, S., Meredith, A., Richardson, J.A., and Johnson, J.E. 1999. Correct coordination of neuronal differentiation events in ventral forebrain requires the bHLH factor MASH1. *Mol. Cell. Neurosci.* **14:** 355–369.

Huang, H.P., Liu, M., El-Hodiri, H.M., Chu, K., Jamrich, M., and Tsai, M.J. 2000. Regulation of the pancreatic islet-specific gene *BETA2* (*neuroD*) by Neurogenin3. *Mol. Cell. Biol.* **20:** 3292–307.

Huang, M.L., Hsu, C.H., and Chien, C.T. 2000. The proneural gene *amos* promotes multiple dendritic neuron formation in the *Drosophila* peripheral nervous system. *Neuron* **25:** 57–67.

Jarman, A.P. and Ahmed, I. 1998. The specificity of proneural genes in determining *Drosophila* sense organ identity. *Mech. Dev.* **76:** 117–125.

Jarman, A.P., Brand, M., Jan, L.Y., and Jan, Y.N. 1993. The regulation and function of the helix-loop-helix gene, *asense*, in *Drosophila* neural precursors. *Development* **119:** 19–29.

Kanekar, S., Perron, M., Dorsky, R., Harris, W.A., Jan, L.Y., Jan, Y.N., and Vetter, M.L. 1997. *Xath5* participates in a network of bHLH genes in the developing *Xenopus* retina. *Neuron* **19:** 981–994.

Lo, L., Tiveron, M.C., and Anderson, D.J. 1998. MASH1 activates expression of the paired homeodomain transcription factor Phox2a, and couples pan-neuronal and subtype-specific components of autonomic neuronal identity. *Development* **125:** 609–620.

Lo, L, Dormand, E., Greenwood, A., and Anderson, D.J. 2002. Comparison of the generic neuronal differentiation and neuron subtype specification functions of mammalian *achaete-scute* and *atonal* homologs in cultured neural progenitor cells. *Development*. In press.

Ma, Q., Kintner, C., and Anderson, D.J. 1996. Identification of *neurogenin*, a vertebrate neuronal determination gene. *Cell* **87:** 43–52.

Ma, Q., Chen, Z., del Barco Barrantes, I., de la Pompa, J.L., and Anderson, D.J. 1998. *neurogenin1* is essential for the determination of neuronal precursors for proximal cranial sensory ganglia. *Neuron* **20:** 469–482.

Ma, Q., Fode, C., Guillemot, F., and Anderson, D.J. 1999. *neurogenin1* and *neurogenin2* control two distinct waves of neurogenesis in developing dorsal root ganglia. *Genes* & *Dev.* **13:** 1717–1728.

Matter-Sadzinski, L., Matter, J.M., Ong, M.T., Hernandez, J., and Ballivet, M. 2001. Specification of neurotransmitter receptor identity in developing retina: The chick ATH5 promoter integrates the positive and negative effects of several bHLH proteins. *Development* **128:** 217–231.

Mizuguchi, R., Sugimori, M., Takebayashi, H., Kosako, H., Nagao, M., Yoshida, S., Nabeshima, Y., Shimamura, K., and Nakafuku, M. 2001. Combinatorial roles of Olig2 and Neurogenin2 in the coordinated induction of pan-neuronal and subtype-specific properties of motoneurons. *Neuron* **31:** 757–771.

Modolell, J. 1997. Patterning of the adult peripheral nervous system of *Drosophila. Perspect. Dev. Neurobiol.* **4:** 285–296.

Morrison, S.J. 2001. Neuronal differentiation: Proneural genes inhibit gliogenesis. *Curr. Biol.* **11:** 349–351.

Nieto, M., Schuurmans, C., Britz, O., and Guillemot, F. 2001. Neural *bHLH* genes control the neuronal versus glial fate decision in cortical progenitors. *Neuron* **29:** 401–413.

Novitch, B.G., Chen, A.I., and Jessell, T.M. 2001. Coordinate regulation of motor neuron subtype identity and pan-neuronal properties by the bHLH repressor Olig2. *Neuron* **31:** 773–789.

Ohtsuka, T., Ishibashi, M., Gradwohl, G., Nakanishi, S., Guillemot, F., and Kageyama, R. 1999. *Hes1* and *Hes5* as notch effectors in mammalian neuronal differentiation. *EMBO J.* **18:** 2196–2207.

Parras, C., Garcia-Alonso, L.A., Rodriguez, I., and Jimenez, F. 1996. Control of neural precursor specification by proneural proteins in the CNS of *Drosophila. EMBO J.* **15:** 6394–6399.

Pattyn, A., Morin, X., Cremer, H., Goridis, C., and Brunet, J.F. 1999. The homeobox gene *Phox2b* is essential for the development of autonomic neural crest derivatives. *Nature* **399:** 366–370.

Pattyn, A., Goridis, C., and Brunet, J.F. 2000. Specification of the central noradrenergic phenotype by the homeobox gene *Phox2b. Mol. Cell. Neurosci.* **15:** 235–243.

Perez, S.E., Rebelo, S., and Anderson, D.J. 1999. Early specification of sensory neuron fate revealed by expression and function of neurogenins in the chick embryo. *Development* **126:** 1715–1728.

Scardigli. R., Schuurmans, C., Gradwohl, G., and Guillemot, F. 2001. Cross regulation between *Neurogenin2* and pathways specifying progenitor identity in the spinal cord. *Neuron* **31:** 203–217.

Skeath, J.B. and Doe, C.Q. 1996. The *achaete-scute* complex proneural genes contribute to neural precursor specification in the *Drosophila* CNS. *Curr. Biol.* **6:** 1146–1152.

Sommer, L., Ma, Q., and Anderson, D.J. 1996. *neurogenins*, a novel family of *atonal*-related bHLH transcription factors, are putative mammalian neuronal determination genes that reveal progenitor cell heterogeneity in the developing CNS and PNS. *Mol. Cell. Neurosci.* **8:** 221–241.

Torii, M., Matsuzaki, F., Osumi, N., Kaibuchi, K., Nakamura, S., Casarosa, S., Guillemot, F., and Nakafuku, M. 1999. Transcription factors Mash-1 and Prox-1 delineate early steps in differentiation of neural stem cells in the developing central nervous system. *Development* **126:** 443–456.

Wang, S.W., Kim, B.S., Ding, K., Wang, H., Sun, D., Johnson, R.L., Klein, W.H., and Gan, L. 2001. Requirement for *math5* in the development of retinal ganglion cells. *Genes* & *Dev.* **15:** 24–29.

Wilson, S.W. and Rubenstein, J.L. 2000. Induction and dorsoventral patterning of the telencephalon. *Neuron* **28:** 641–651.

Divergent functions of the proneural genes *Mash1* and *Ngn2* in the specification of neuronal subtype identity

Carlos M. Parras, Carol Schuurmans, Raffaella Scardigli, et al.

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