Control of noradrenergic differentiation and Phox2a expression by MASH1 in the central and peripheral nervous system

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SUMMARY

Mash1, a mammalian homologue of the Drosophila proneural genes of the achaete-scute complex, is transiently expressed throughout the developing peripheral autonomic nervous system and in subsets of cells in the neural tube. In the mouse, targeted mutation of Mash1 has revealed a role in the development of parts of the autonomic nervous system and of olfactory neurons, but no discernible phenotype in the brain has been reported. Here, we show that the adrenergic and noradrenergic centres of the brain are missing in Mash1 mutant embryos, whereas most other brainstem nuclei are preserved. Indeed, the present data together with the previous results show that, except in cranial sensory ganglia, Mash1 function is essential for the development of all central and peripheral neurons that express noradrenergic traits transiently or permanently. In particular, we show that, in the absence of MASH1, these neurons fail to initiate expression of the noradrenaline biosynthetic enzyme dopamine β-hydroxylase. We had previously shown that all these neurons normally express the homeodomain transcription factor Phox2a, a positive regulator of the dopamine β-hydroxylase gene and that a subset of them depend on it for their survival. We now report that expression of Phox2a is abolished or massively altered in the Mash1−/− mutants, both in the noradrenergic centres of the brain and in peripheral autonomic ganglia. These results suggest that MASH1 controls noradrenergic differentiation at least in part by controlling expression of Phox2a and point to fundamental homologies in the genetic circuits that determine the noradrenergic phenotype in the central and peripheral nervous system.

Key words: Noradrenergic neuron, Sympathetic ganglion, Parasympathetic ganglion, Hindbrain, Mash1, Phox2a, Mouse

INTRODUCTION

The genes that control the generation of different neuronal types from uncommitted precursor cells are still poorly characterized in vertebrates. Genetic experiments in Drosophila have uncovered a number of genes encoding transcriptional regulators, which function at successive steps in the developmental pathways leading to differentiating neurons. Among them, the proneural genes of the achaete-scute (asc) complex are basic-helix-loop-helix (bHLH) transcription factors that control the generation of neural precursors for the central (CNS) and peripheral (PNS) nervous system (reviewed in Ghysen et al., 1993; Jan and Jan, 1994). Mash1 (Johnson et al., 1990) is a mammalian homologue of asc genes, which is transiently expressed in subsets of neuronal progenitors at all axial levels of the neural tube, in the olfactory epithelium and in the retina (Lo et al., 1991; Guillemot and Joyner, 1993; Gordon et al., 1995; Jasoni and Reh, 1996; Ma et al., 1997). Its expression in the neural-crest-derived PNS progenitors is also transient and comprises the three main divisions of the peripheral autonomic nervous system: the sympatho-adrenal, parasympathetic and enteric systems (Lo et al., 1991; Guillemot and Joyner, 1993; Guillemot et al., 1993; Groves et al., 1995; Blaugrund et al., 1996; Ma et al., 1997). A null mutation of Mash1 in mice has been shown to affect the autonomic PNS, olfactory neurons and the in vitro differentiation of late-appearing retinal neurons (Guillemot et al., 1993; Tomita et al., 1996; Cau et al., 1997), but no phenotype has been reported in the brain. During development of Mash1−/− sympathetic ganglion primordia, neural crest cells arrive at the dorsal aorta, where the ganglion anlagen form, but most of them fail to express specific neuronal markers and eventually die. The status of the parasympathetic system has been studied less extensively, but the paracardiac ganglia have been reported to be eliminated by the mutation (Guillemot et al., 1993). The enteric nervous system of the stomach and intestine is present, but the mutation prevents the development of serotoninergic neurons (Blaugrund et al., 1996), and all
enteric neurons in the esophagus seem to be missing (Guillemot et al., 1993).

The downstream genes by which MASH1 controls formation and survival of specific neuronal populations have remained elusive. Good candidates are genes encoding transcriptional regulators that are selectively expressed in classes of neural cells and regulate downstream differentiation events. Phox2a and Phox2b, which encode two closely related homeodomain transcription factors with similar expression patterns, may represent such genes. Like Mash1, they are expressed early on throughout the peripheral autonomic nervous system (Valarché et al., 1993; Tiveron et al., 1996; Pattyn et al., 1997). In chick sympathetic ganglion primordia, Phox2a expression is turned on shortly after that of Cash1, the chicken Mash1 homologue, slightly before or concomitant with the noradrenaline biosynthetic enzyme tyrosine hydroxylase (TH) (Groves et al., 1995; Ernsberger et al., 1995). In the CNS, Phox2a and Phox2b are expressed in subpopulations of neurons in the brainstem and their precursors: in all adrenergic and noradrenergic neurons, in other neurons involved in autonomic reflex pathways and in a subset of cranial motor neurons (Valarché et al., 1993; Tiveron et al., 1996; Pattyn et al., 1997). In both the CNS and the PNS, there is a striking correlation between Phox2a expression and transient or permanent expression of noradrenergic features, in particular with expression of dopamine β-hydroxylase (DBH), the last enzyme of the noradrenaline synthesis pathway (Tiveron et al., 1996). Inactivation of the Phox2a gene in the mouse leads to agenesis of the major noradrenergic centre in the brain, the locus coeruleus (l.c.), and of parasympathetic ganglia, to altered expression of the noradrenergic markers TH and DBH (Fig. 1A,C,E,G,I), for instance, some sympathetic neuroblasts could have disappeared by E12.5, but the few that survive go on to express at least part of their normal differentiation program and have disappeared by E12.5, but the few that survive go on to express at least part of their normal differentiation program and have disappeared by E12.5, but the few that survive go on to express at least part of their normal differentiation program and have disappeared by E12.5, but the few that survive go on to express at least part of their normal differentiation program and have disappeared by E12.5, but the few that survive go on to express at least part of their normal differentiation program and have disappeared by E12.5, but the few that survive go on to express at least part of their normal differentiation program and have disappeared by E12.5, but the few that survive go on to express at least part of their normal differentiation program and have disappeared by E12.5, but the few that survive go on to express at least part of their normal differentiation program and have disappeared by E12.5, but the few that survive go on to express at least part of their normal differentiation program and have disappeared by E12.5, but the few that survive go on to express at least part of their normal differentiation program.

RESULTS

Initiation of Phox2a expression in sympathoblasts depends on Mash1 activity

To investigate whether Phox2a or Phox2b may be downstream targets of Mash1 in the autonomic PNS, we first examined the sympathetic ganglion primordia in Mash1<sup>−/−</sup> mice. At E10-E10.5, the earliest stage during mouse embryogenesis at which the anlagen of the sympathetic ganglia can be identified, the sympathetic neuroblasts form an almost continuous strand of cells along the dorsal aorta and express the transcription factors MASH1, Phox2a and Phox2b, the receptor tyrosine kinase Ret and the noradrenergic markers TH and DBH (Fig. 1A,C,E,G,I and see Durbec et al., 1996; Guillemot and Joyner, 1993; Guillemot et al., 1993; Tiveron et al., 1996). In Mash1<sup>−/−</sup> embryos, the neural-crest-derived sympathetic precursor cells still arrive close to the dorsal aorta where they can be identified by their expression of Ret (Guillemot et al., 1993). Accordingly, we found Ret<sup>+</sup> clusters of cells at the appropriate location of E10.5 Mash1 mutant embryos, albeit in reduced numbers (Fig. 1B). However, these cells failed to express Phox2a protein (Fig. 1B,F) and DBH (Fig. 1J), as they normally do. Remarkably, Phox2b expression was not affected by the mutation (Fig. 1D,H).

Most Mash1<sup>−/−</sup> sympathoblasts never differentiate further and have disappeared by E12.5, but the few that survive go on to express at least part of their normal differentiation program (Guillemot et al., 1993). In the region of the stellate ganglion (Fig. 1K,M), for instance, some sympathetic neuroblasts could be identified in E13.5 mutant embryos by expression of Phox2b...
protein (Fig. 1L) or DBH mRNA (not shown), and these cells now also expressed Phox2a (Fig. 1N). Hence, whereas early expression of Phox2a and DBH is abolished by the inactivation of Mash1, later expression of both markers in the surviving sympathoblasts escapes this control.

**Analysis of the Mash1−/− phenotype in parasympathetic and enteric ganglia**

We then asked whether Phox2a expression in the parasympathetic subdivision of the autonomic PNS also depends on Mash1 function. At E13.5, parasympathetic ganglia have just formed and express the Phox2a and Phox2b proteins (Fig. 2A,C,G,I). The sphenopalatine ganglion, which can be identified histologically by its characteristic shape and location, was clearly present in E13.5 Mash1−/− embryos (not shown), but did not express Phox2a (Fig. 2D). In contrast, Phox2b expression was unaffected (Fig. 2B), as was the case in sympathetic ganglion primordia. In fact, except for the ciliary ganglion (see below), all Mash1−/− parasympathetic ganglia that we examined at this stage expressed Phox2b (Fig. 2H), but had lost expression of Phox2a (Fig. 2J). Although the mutant sphenopalatine ganglion seemed of normal size and shape, the Phox2b+ cells appeared less densely packed than in wild-type embryos, suggesting additional abnormalities (Fig. 2A,B). At E16.5, the sphenopalatine ganglion was massively atrophic and a subset of the remaining ganglion cells now also expressed Phox2a, albeit very weakly (Fig. 3A-D). Other Mash1−/− parasympathetic ganglia such as the otic and submandibular ganglion had already been eliminated by then. At birth, only a few cells expressing Phox2b or detectable by histological staining were left at the site of the sphenopalatine ganglion (Fig. 3G,H). The ciliary ganglion was an exception in the sense that it was detectable neither at E13.5 (Fig. 3LJ) nor at birth using Phox2b or peripherin (not shown) as markers and perhaps never develops.

The noradrenaline-synthesizing enzymes TH and DBH have been found to be expressed in neurons of the submandibular (Grzanna and Coyle, 1978) and ciliary (Landis et al., 1987) ganglia of adult rats. In the rat sphenopalatine ganglion, TH immunoreactivity has been detected from E16.5 onwards and declined after birth (Leblanc and Landis, 1989). We found that expression of DBH in the forming mouse sphenopalatine ganglion was clearly detectable at E12.5 (Fig. 2E) and faded out thereafter. This expression was abolished in the mutants (Fig. 2F). Hence, with the possible exception of the ciliary ganglion, parasympathetic ganglia form in Mash1−/− embryos and express Phox2b, but they fail to initiate Phox2a expression on schedule, never express DBH and later degenerate.

The Mash1−/− mutation has been reported to affect the

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**Fig. 1.** Analysis of sympathetic ganglion primordia in Mash1−/− embryos. Equivalent regions of Mash1+/- embryos (left) and stage-matched Mash1−/− littermates (right) are compared. (A-D) Serial sections through the cervical region of E10.5 embryos were double-labelled by combined Ret in situ hybridization/anti-Phox2a (A,B) or anti-Phox2b (C,D) immunocytochemistry. Ret in situ hybridization signals appear as black cytoplasmic staining, Phox2 immunolabelling as orange nuclear stain. The arrows in B point to Ret+ sympathoblasts from Mash1−/− embryos, which are Phox2a−. (E-J) Sections through the thoracic region of E10.5 embryos were labelled with anti-Phox2a (E,F) or anti-Phox2b (G,H) antibodies or hybridized with a DBH probe (I,J). In E,G and F,H, immediately adjacent sections were processed for Phox2a and Phox2b immunocytochemistry. The Mash1−/− sympathetic ganglion primordia aside the dorsal aorta express Phox2b protein (H), but are Phox2a-negative (F) (arrows). The forming sympathetic ganglia express DBH in heterozygous (I), but not in homozygous (J) mutant embryos. On an adjacent section, the sympathoblasts in I and J could be visualized with a Ret probe (not shown). (K-N) Serial sections through the region of the stellate ganglion from E13.5 embryos were immunostained with anti-Phox2b (K,L) or anti-Phox2a (M,N) antibodies. The rudimentary ganglion in the Mash1−/− embryos visualized by anti-Phox2b staining (L) is now also Phox2a+ (N), da, dorsal aorta; sa, subclavian artery.
development of the enteric nervous system in several ways. On the one hand, enteric neurons in the esophagus have been shown to be eliminated by the mutation at late gestation, whereas their appearance in the stomach and intestine was delayed, but not prevented (Guillemot et al., 1993). On the other hand, the serotoninergic class of enteric neurons has been found to be missing entirely, and this has been attributed to the elimination of the early differentiating TH-expressing or ‘transiently catecholaminergic’ enteric neuronal precursors in Mash1−/− embryos (Blaugrund et al., 1996). We did not observe obvious deficits in Phox2a- or Phox2b-expressing neurons in the myenteric plexus of the stomach (Fig. 4A-D) and intestine (not shown) from E13.5 mutant embryos. In contrast, the Phox2b-expressing presumptive myenteric neurons in the wall

Fig. 2. Analysis of parasympathetic ganglion primordia in Mash1−/− embryos. Equivalent regions of Mash1+/− embryos (left) and stage-matched Mash1+/− littersmates (right) are compared. (A-F) Sagittal sections through the sphenopalatine ganglion of E13.5 (A-D) and E12.5 (E,F) embryos were labelled by anti-Phox2b (A,B) or anti-Phox2a (C,D) immunocytochemistry or by in situ hybridization with a DBH probe (E,F). In A,B and C,D, consecutive sections were processed; the Mash1−/− ganglia express Phox2b (B), but not Phox2a (D) protein. At E12.5, a subpopulation of cells expresses DBH in the Mash1+/− (E), but not in the Mash1−/− (F) ganglion. The ganglion cells in F, faintly visible through Nomarski optics, can be visualized by Phox2b or peripherin labelling on an adjacent section (not shown). (G-J) Consecutive sagittal sections through the otic ganglion from heterozygous and mutant E13.5 embryos were labelled with anti-Phox2b (G,H) or anti-Phox2a (I,J) antibodies. Note that Phox2a expression in the geniculate ganglion, a placode-derived cranial sensory ganglion that does not express Mash1 at any stage, is preserved in the homozygotes. gen, geniculate ganglion; otic, otic ganglion; ophv, ophthalmic vein; spg, sphenopalatine ganglion; V, trigeminal ganglion.

Fig. 3. Atrophy of the sphenopalatine and lack of the ciliary ganglia in Mash1−/− embryos. Equivalent regions of Mash1+/− embryos (left) and stage-matched Mash1+/− littermates (right) are compared. (A-D) Consecutive sagittal sections through the sphenopalatine ganglion from E16.5 embryos were stained with anti-Phox2b (A,B) or anti-Phox2a (C,D) antibodies. At this stage, the Mash1−/− ganglion has become atrophic (B), and the few remaining cells now also express low levels of Phox2a protein (D). (E-H) Coronal sections through the region of the sphenopalatine ganglion from newborn mice were either processed for anti-Phox2b immunocytochemistry (E,F) or for histological staining (G,H). Only a few ganglion cells (arrows) can still be detected in the homozygotes (F,H). (I,J) Coronal sections through the eye region of E13.5 embryos showing that the ciliary ganglion (asterisks in I) is missing in Mash1−/− embryos (J). The Phox2b-negative pigment epithelium of the retina is visible because of its pigmentation. nc, nasal cavity; ophv, ophthalmic vein; pe, retinal pigment epithelium; spg, sphenopalatine ganglion; Vmn, maxillary branch of the trigeminal nerve.
of the esophagus were Phox2a-negative (Fig. 4D,H). As in other parts of the enteric nervous system, transiently catecholaminergic cells are also present in the esophagus (Baetge and Gershon, 1989). Accordingly, we found that a subpopulation of the Phox2b+ cells in the E12.5 esophageal wall of wild-type embryos expressed DBH, but this expression was abrogated in the mutants (not shown).

Foregut enteric neurons thus require Mash1 function to activate the Phox2a and DBH genes, as do sympathetic and parasympathetic neurons. Together, these data show that MASH1 directly or indirectly controls Phox2a and DBH expression in the three subdivisions of the peripheral autonomic nervous system.

**CNS defects in Mash1−/− mutant mice**

In the initial description of Mash1 mutant mice, no discernible phenotype has been reported in the brain. Given that Phox2a and DBH appear to be downstream targets of Mash1 in the PNS, we examined if Phox2a expression in the CNS, particularly in the noradrenergic centres, was affected in Mash1 homozygous mutants. We first examined the l.c. at birth, when it can be visualized by histological staining. As shown in Fig. 5A,B, the l.c., easily revealed in wild-type neonates as a compact group of large, darkly stained neurons in the vicinity of the fourth ventricle, was conspicuously absent in the brainstem of Mash1 mutant mice. Lack of the l.c. at birth was confirmed by in situ hybridization for DBH and Phox2a immunocytochemistry (Fig. 5C-F).

At earlier stages of development (from E10 onward), the l.c. neuron precursors can be identified as a characteristically shaped column of cells in the isthmic region of the hindbrain, expressing DBH and Phox2a (as well as Phox2b transiently up to E11) (Tiveron et al., 1996; Morin et al., 1997; Pattyn et al., 1997 and Figs 5G,K, 6A,C). Neither Phox2a nor Phox2b proteins nor DBH mRNA could be detected at the corresponding location of Mash1−/− mutant brains, either at E13.5 on sections of the hindbrain (Fig. 5H,L; Phox2b not shown) or at E10.5 on whole-mount preparations (Fig. 6B,D; Phox2a not shown). TH mRNA, which is normally expressed by E13.5 l.c. neurons, could also not be detected in the l.c. region (Fig. 5J). Together, these data suggest that l.c. precursors never develop in the absence of MASH1.

Thorough examination of the hindbrain using either DBH or TH as markers showed that all other noradrenergic and adrenergic centres (the locus subcoeruleus and groups A1/C1, A2/C2, A4, A5 and A7) are missing in Mash1−/− embryos. At E13.5, for example, the A1/C1 group is detectable by DBH in situ hybridization in the caudal brainstem from wild-type (Fig. 5M) but not from mutant (Fig. 5N) embryos. In fact, no DBH+ cells were detectable on serial coronal sections throughout the hindbrain of E16.5 or P0 mutant mice (not shown). Since (nor)adrenergic cells outside the l.c. do not form compact nuclei and are intermingled with DBH+ cells, part of which also express Phox2a and Phox2b, we do not know whether the cells merely fail to express DBH while keeping expression of Phox2a/b, whether they have also lost Phox2a/b expression or whether they are eliminated by the mutation. Whatever the case, our results show that the proper development of the central (nor)adrenergic neurons requires Mash1 activity.

By contrast, most central Phox2a+ and Phox2b+ neurons, which are not (nor)adrenergic, appeared to be spared by the mutation, even those in close proximity to noradrenergic centres: scattered neurons of the reticular formation, the neurons in the nucleus of the solitary tract and in the motor nuclei of the IIIrd, IVth, VIIth, IXth and Xth cranial nerves (Tiveron et al., 1996). This is exemplified at birth by in situ hybridization with a peripherin probe, a convenient marker of all neurons that project into the periphery (Fig. 7A-D). These nuclei and the reticular formation appeared to contain a normal complement of Phox2a-positive cells in the mutants (see Fig. 5F,H for the reticular formation; others not shown). At E10.5, the precursors of the motor nuclei of the IIIrd, IVth and VIIth nerves already express Phox2b and are present in the mutants (Fig. 6C,D). Moreover, the peripherin probe revealed the presence of the Phox2a-negative somatic and visceral motor nuclei of the Vth, Vth and Xth cranial nerve (see Fig. 7D for the XIth nucleus, others not shown). Other histologically detectable formations such as the red nucleus (not shown) or

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**Fig. 4.** Analysis of the enteric nervous system in Mash1−/− embryos. Equivalent regions of Mash1+/− embryos (left) and stage-matched Mash1−/− littermates (right) are compared. (A-H) Consecutive sagittal sections through the stomach and lower esophagus (A-D) or the upper esophagus (E-H) of E13.5 embryos were stained with anti-Phox2b (A,B,E,F) or anti-Phox2a (C,D,G,H) antibodies. The myenteric plexus of the esophagus from the homozygotes expresses Phox2b (B,F), but not Phox2a (D,H) protein. The arrows in G point to groups of Phox2a+ neurons in the wall of the Mash1−/− esophagus. Phox2a expression in the stomach is not affected by the mutation (D). A nearby parasympathetic paracardiac ganglion (asterisk in E-G) is also Phox2b+, Phox2a− in the homozygotes (F,H), e, esophagus; oc, oral cavity; st, stomach.
the inferior olive (Fig. 7F) appeared normal as well. We also examined an important group of catecholaminergic neurons, the mesencephalic dopaminergic centres, which express TH, but neither DBH nor Phox2 genes. At E13.5, there was a normal complement of TH+ cells and fibres in the ventral tegmental area of the midbrain, where the dopaminergic precursors are located (Fig. 7G,H).

Therefore, in the CNS, MASH1 seems to control preferentially the development of (nor)adrenergic neurons. We found, however, two notable exceptions. First, the Phox2a+ cells in the embryonic spinal cord, which are not (nor)adrenergic and form a discrete column of cells in the lateral neural tube just dorsal to the sulcus limitans (Tiveron et al., 1996), could not be detected in the E13.5 mutant spinal cord, which otherwise was of normal size and shape (Fig. 7I,J). In the absence of independent markers, we cannot tell whether these cells (which are also Phox2b+) are not generated or just fail to express Phox2a. Second, the mesencephalic nucleus of the trigeminal nerve, which never expresses Phox2 genes, was absent in the mutants as assessed by peripherin labelling (data not shown). The loss or alteration of these neurons is an exception also in the sense that they are neural-crest-derived sensory neurons (Stainier and Gilbert, 1991), which are otherwise spared by the mutation (Guillemot et al., 1993).

**DISCUSSION**

**The phenotype of Mash1 mutant embryos**

Previous studies have shown that Mash1−/− embryos lack most sympathetic neurons, the paracardiac parasympathetic ganglia, the enteric neurons in the esophagus and one class of early born neurons in the enteric nervous system of the intestine and stomach (Guillemot et al., 1993; Blaugrund et al., 1996). Here we report that, in the three divisions of the autonomic PNS, precursor neurons undergo an incomplete cell-type-specific differentiation, as evidenced by their expression of Phox2b but not of Phox2a or DBH, before they degenerate. This abortive developmental sequence does not affect in the same way the entire autonomic PNS. In the enteric nervous system, it is limited to foregut neurons, which have been recently shown to derive at least partially from a separate neural crest lineage (Durbec et al., 1996). In the sympathetic nervous system, a fraction of the sympathoblasts eventually escapes MASH1 dependency and proceeds towards full differentiation, at least on the basis of Phox2a, DBH and TH expression. This could be due to Mash1 function being later provided by another bHLH protein. A schematic summary of the phenotypic changes in the different lineages of the autonomic PNS is presented in Fig. 8.

It seems paradoxical that parasympathetic ganglia, which never form in Phox2a-deficient mice (Morin et al., 1997), are present and appear grossly normal in Mash1−/− embryos up to E13.5, without expressing detectable levels of Phox2a. One may argue that levels of Phox2a that are below our detection limits are sufficient to permit the formation of parasympathetic ganglia in Mash1−/− embryos.

**Fig. 5.** Absence of the central noradrenergic neurons in Mash1−/− mutant mice. Equivalent regions of Mash1+/− embryos (left) and stage-matched Mash1−/− littermates (right) are compared. (A-F) Coronal sections through the pons of newborn mice were Nissl-stained (A,B), labelled by in situ hybridization with a DBH probe (C,D) or by anti-Phox2a immunocytochemistry (E,F). In the heterozygotes, the lc can be visualized lateral to the fourth ventricle by histological staining (A) as well as by expression of DBH (C) and Phox2a protein (E). It is absent in the homozygotes by these criteria (B,D,F). Note that Phox2a+ neurons of the reticular formation (asterisk), which are DBH-negative, are preserved in the Mash1−/− embryos. (G-L) In sagittal sections of E13.5 heterozygous embryos, the lc anlage can be identified by its characteristic shape and location and by expression of Phox2a (G), TH (I) and DBH (K). It is lacking in the homozygous mutants (H,J,L). The Phox2a+, DBH+ cells in the locus subcoeruleus are also missing, whereas the DBH−, Phox2a+ neurons of the reticular formation (asterisks) are present in the Mash1−/− embryos. (M,N) In sagittal sections through the caudal hindbrain of Mash1+/− E13.5 embryos, hybridization with a DBH probe reveals the (nor)adrenergic cells in areas A1 and C1; there are no DBH+ neurons in Mash1−/− embryos in this region. Rostral is to the right. 4v, fourth ventricle; A1/C1 (nor)adrenergic cell groups A1 and C1; lc, locus coeruleus; lsc, locus subcoeruleus.
limit suffice to allow for ganglion formation in \(Mash1^{-/-}\) embryos. The other possibility is that formation of these ganglia requires Phox2a activity only in the presence, but not in the absence of MASH1. One explanation would be that in the absence of MASH1 (and Phox2a) a compensatory up-regulation of another bHLH gene may allow for expression of Phox2b and ganglion formation. Alternatively, an early function of Phox2a could be to counteract MASH1 activity, which may prevent overt differentiation. Clearly, further work is required to determine the epistatic relationship between the \(Mash1\) and \(Phox2a\) mutations.

A further difference between the \(Mash1\) and \(Phox2a\) mutant phenotypes that demands explanation is that, in \(Phox2a\) knock-out mice, \(DBH\) expression in the sympathetic ganglion primordia is not affected, possibly because of the presence of Phox2b (Morin et al., 1997; Pattyn et al., 1997). In \(Mash1^{-/-}\) embryos, the \(DBH\) gene fails to be activated despite the presence of Phox2b. These data can be reconciled by assuming that, in this lineage, Phox2b and Phox2a are functionally equivalent, but need to cooperate with MASH1 or a different, as yet unidentified MASH1-dependent pathway to promote \(DBH\) expression.

In the initial description of the \(Mash1^{-/-}\) phenotype, no deficiencies had been found in the brain and spinal cord using a variety of different markers (Guillemot et al., 1993). Here, we demonstrate that, in fact, all central adrenergic and noradrenergic neurons are lacking in \(Mash1^{-/-}\) embryos, from the earliest stage when they can be detected. On the contrary, and with one exception, the other nuclei that we examined in the midbrain and hindbrain were present, in line with the previous analysis using a different set of markers (Guillemot et al., 1993) and the normal macroscopic appearance of the brain. However, the \(Mash1^{-/-}\) phenotype is not specific for \(Phox2a^+\), \(DBH^+\) neurons. The olfactory neuron lineage, in which neither gene is expressed, is another major lineage affected by the mutation (Guillemot et al., 1993; Cau et al., 1997). In the spinal cord, we find that a group of Phox2a+ interneurons, which are \(DBH^+\), is affected, and in the mesencephalon and metencephalon, we cannot detect the

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**Fig. 6.** Phenotype of the \(Mash1^{-/-}\) mutation in the mid-hindbrain region at E10.5. Whole-mount in situ hybridizations of wild-type and homozygous \(Mash1\) mutants with \(DBH\) (A,B) and Phox2b (C,D) probes show that the l.c. precursors (arrows), clearly visible in wild-type embryos (A,C), are absent in the mutants (B,D). The Phox2b+ primordia of the nuclei of the IIIrd (oculomotor) and IVth (trochlear) cranial nerve are not affected by the mutation, nor is the strong Phox2b signal in the fourth rhombomere (asterisk), where the precursors of the motor nucleus of the VIIth (facial) cranial nerve arise.

**Fig. 7.** Lack of Phox2a+ neurons in the spinal cord and preservation of non-noradrenergic nuclei in the mid-hindbrain of \(Mash1^{-/-}\) mice. Equivalent regions of \(Mash1^{+/+}\) embryos (left) and stage-matched \(Mash1^{-/-}\) littermates (right) are compared. (A-F) Coronal sections through the pons (A,B) and medulla (C-F) of newborn mice were labelled by in situ hybridization with a peripherin probe (A-D) or by Nissl stain (E,F). The motor nucleus of the facial (VIIth) nerve in the pons (A,B), the dorsal motor nucleus of the vagal (Xth) nerve and the nucleus of the hypoglossal (XIIth) nerve (C,D) as well as the inferior olive in the medulla, easily recognized by its typical morphology (E,F), are preserved in the homozygous mutants. (G,H) Sagittal sections through the midbrain of E13.5 embryos were labelled by anti-TH immunocytochemistry. The dopaminergic neurons of the substantia nigra and of the ventral tegmental area of the midbrain are preserved in \(Mash1^{-/-}\) mutants. (I,J) In transverse sections of the E13.5 spinal cord, anti-Phox2a labelling reveals a column of cells in the alar plate of heterozygous (I), but not of homozygous (J) embryos. Note that the overall size and shape of the spinal cord is not altered in the homozygous mutants. sn, substantia nigra; VII, motor nucleus of the VIIth cranial nerve, X, dorsal motor nucleus of the Xth cranial nerve; XII, nucleus of the XIIth cranial nerve; io, inferior olive.
Mash1 is a transcription factor of the same class as Phox2a, and expression is largely confined to proliferating neuroblasts. Apart from the PHD1/neurogenin1 and NeuroD in the olfactory receptor neuron lineage (Cau et al., 1997) and eHAND in sympathoblasts (Ma et al., 1997). Cross-regulations among neuronal-fate-determining bHLH genes are common, and have been observed between Drosophila achaete and scute (Jan and Jan, 1993) as well as for Xenopus neurogenin1 and neuroD (Ma et al., 1996).

A candidate for mediating the action of Mash1 in the olfactory neuron lineage is PHD1/Unccx-4.1, which encodes a transcription factor of the same class as Phox2a (Rovescalli et al., 1996; Saito et al., 1996) and whose expression is largely eliminated in the Mash1 mutant epithelium (Saito et al., 1996). However, because the olfactory epithelium of Mash1 mutants is already severely atrophic when PHD1 is normally turned on, it cannot be ruled out that the loss of PHD1 expression simply reflects the loss of PHD1-expressing cells.

Phox2a, like Mash1, is expressed throughout the three divisions of the autonomic PNS and, where this has been studied (Groves et al., 1995; Ernsberger at al., 1995), its expression is initiated shortly after that of Mash1, making it a possible candidate for mediating MASH1 function in the PNS. Our results strongly suggest that Phox2a is indeed a downstream target of MASH1 in the autonomic PNS. In
sympathetic and parasympathetic ganglia and in the myenteric plexus of the foregut, Mash1 was found to control Phox2a expression, although it remains to be determined how direct this regulation is. As shown in the accompanying paper (Lo et al., 1998), forced expression of Mash1 in neural crest cells promotes expression of Phox2a. In the CNS, we are unable to score Phox2a expression in the affected neurons for want of independent markers. It is, however, striking that the I.c., whose precursors normally express Phox2a as soon as they are generated and before they express bona fide noradrenergic markers (Tiveron et al., 1996; Pattyn et al., 1997), is missing in both the Mash1 and Phox2a knock-out mice (Morin et al., 1997). Clearly, development of this lineage depends on both factors, suggesting that they function in the same genetic cascade.

Phox2b, a recently identified close relative of Phox2a, is expressed in the autonomic PNS in a pattern that largely overlaps that of Phox2a and Mash1 (Pattyn et al., 1997) and could thus also be a potential target of Mash1. However, Phox2b expression was spared by the Mash1 mutation at all sites of the PNS where Phox2a was affected, in line with previous suggestions that its promoter has largely diverged from that of Phox2a (Pattyn et al., 1997).

Implications for Mash1 function

Classically, the proneural genes of the Drosophila asc complex have been thought to endow cells with the potential to adopt a general neural fate. It has become clear, however, that they already impose constraints on the type of precursor to be generated, probably by controlling the expression of neuronal-type selector genes. In the Drosophila PNS, for example, achaete and scute are required for the formation of external sensory organs, but not of chordotonal organs (Dambly-Chaudière and Ghysen, 1987; Jarman et al., 1993, 1995). In the CNS of the fly, correct specification of a well-characterized precursor cell requires expression of achaete or scute, which cannot be replaced by other family members (Skheath and Doe, 1996; Parras et al., 1996).

The question to what extent Mash1 functions as a proneural gene and to what extent as a neuronal-type-specific determinant has not been resolved. The fact that the mutation affects classes of neurons completely unrelated by lineage or function has been taken to suggest that Mash1 is required for some basic mechanism of neurogenesis, rather than for the specification of particular neuronal phenotypes (Guillemot et al., 1993). Similarly, forced expression of Mash1 has been shown to confer neurogenic competence to neural-crest-derived cells from the fetal gut (Lo et al., 1997) and to promote expression of pan-neural markers in neural crest cells (Lo et al., 1998), which are proneural functions. However, neural crest cells expressing early neuronal markers, but unable to differentiate further, have been isolated from Mash1−/− mice (Sommer et al., 1995). This argues that MASH1 controls differentiation of cells already committed to a neuronal fate. Possibly, the null mutation uncovers only the neuronal-type-specific function of MASH1 because of functional redundancy with other bHLH genes for its proneural activity, which is revealed by gain-of-function experiments.

The results presented here strongly support the idea that MASH1 is required not only for conferring pan-neuronal properties, but also for the implementation of specific aspects of neuronal differentiation and, in particular, for expression of the noradrenergic phenotype. Given the widespread expression of Mash1 in the rhombencephalic neuroepithelium (Guillemot and Joyner, 1993), its specific requirement in the (nor)adrenergic brainstem neurons is striking. In the autonomic PNS precursors, we show that Mash1 activity is required for activation of the DBH gene. Promotion of expression of Phox2a, which has been shown to be a positive regulator of the DBH gene in vivo (Morin et al., 1997) and in vitro (Zellmer et al., 1995; Yang, C., Kim, H.-S., Seo, H., Kim, C.-H., Brunet, J.-F. and Kim, K.-S., unpublished data), appears to be a key step controlled by MASH1 in these cells. This may also be the case for central noradrenergic neurons, of which all normally express Phox2a and a fraction depend on it for their survival.

Therefore, we suggest that MASH1 promotes the expression of noradrenergic traits in part by controlling expression of Phox2a. Once induced by MASH1, Phox2a, in conjunction with extracellular signals (Groves et al., 1995; Groves and Anderson, 1996; Reissmann et al., 1996), may activate DBH and other differentiation genes of autonomic and noradrenergic neurons. The need for additional factors is underscored by the observation that forced expression of Phox2a in undifferentiated neural crest cells is not sufficient to drive DBH expression (Lo et al., 1998).

These data place Mash1 somewhere at the top of a hierarchy of genes controlling differentiation and survival of autonomic and noradrenergic neurons. Finally, the regulation by MASH1 of a specific neuronal differentiation trait (the (nor)adrenergic phenotype) in both the central and peripheral nervous system, hints at a fundamental unity of the transcriptional control of neuronal-type specification in neural tube and neural crest derivatives.

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