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Delays in neuronal differentiation in *Mash1*/*Ascl1* mutants

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Abstract

The inactivation of a developmental transcription factor may lead to the complete absence of a specific cell type. More commonly, though, it only partially impairs its generation. The modalities of this partial effect have rarely been documented in any detail. Here, we report a novel function for the bHLH transcription factor Ascl1/Mash1 in the generation of the nucleus of the solitary tract (nTS). In *Mash1*$^{-/-}$ late embryos, the nTS is markedly atrophic. Tracing back the origin of this atrophy, we show that nTS precursors appear in the mutants 1 day later than in the wild type and then accumulate at a slower pace. We also show that the previously reported atrophy of the sympathetic chain in *Mash1* mutants is similarly preceded by a delay of 1 to 2 days in the appearance of differentiated ganglionic cells. Finally, we provide evidence that the acceleration imposed by *Mash1*, regardless of the production of post-mitotic cells, affects differentiation itself, both generic and type-specific.

Keywords: Mash1/Ascl1; Proneural gene; Neuronal differentiation; Transcription factor; Nucleus of the solitary tract; Sympathetic ganglia; Mouse; Knock-out

Introduction

Proneural bHLH transcription factors are key players in the process of neurogenesis. In flies, where they were first discovered, their best characterized action, paradoxically, is in non-cell autonomous inhibition of neurogenesis, by the process of lateral inhibition (Gibert and Simpson, 2003). This process is also thought to occur in the neuroepithelium of vertebrates and maintain a pool of dividing progenitors throughout the protracted period of neurogenesis (Kageyama and Nakanishi, 1997; Ross et al., 2003). The cell autonomous promotion of neuronal differentiation by proneural factors is much less understood, be it in flies or vertebrates. It is commonly construed as the "selection" of "committed" neuronal precursors from dividing progenitors, the promotion of cell cycle exit (the clearest hallmark of "commitment") and the upregulation of "generic" neuronal differentiation traits, which ensues. However, before "commitment", proneural bHLH genes pattern the neuroepithelium itself (e.g., Filippi et al., 2005; Gowan et al., 2001); during differentiation, they control subtype specific traits along with generic or pan-neural ones (e.g., Fode et al., 2000; Lee and Pfaff, 2003; Lo et al., 2002; Parras et al., 2002); and they can influence such late events as the migration of neuronal precursors (Ohsawa et al., 2005; Tiveron et al., 2003). The emerging picture is that, collectively, the actions of proneural bHLH genes span the entire course of neuronal differentiation. There is evidence that several are expressed in succession in the same lineage (e.g., Cau et al., 1997; Fode et al., 1998), each gene possibly in charge of a different phase in a multistep differentiation process. The precise definition of these steps, however, is still elusive, and awaits the identification of direct target genes, few of which are known (e.g., Lee et al., 2004).

Mash1/Ascl1 is the only murine member of the achaete/scute family of bHLH genes expressed in the nervous system. The list of neuronal types whose differentiation depend on Mash1 has steadily increased since the initial description of its null mutant phenotype (Guillemot et al., 1993). To date, it includes olfactory neurons (Cau et al., 1997; Guillemot et al., 1993) and interneurons (Parras et al., 2004), sympathetic, parasympathetic, and enteric neurons (Blaugrund et al., 1996; Guillemot et al., 1993), glomus cells of the carotid body (Kameda, 2005),

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GABAergic neurons of the cortex (Casarosa et al., 1999), central noradrenergic (Hirsch et al., 1998), and serotonergic (Pattyn et al., 2004) neurons, retinal neurons (Akagi et al., 2004; Tomita et al., 1996), V2 and dl5 interneurons of the spinal cord (Helms et al., 2005; Li et al., 2005; Parras et al., 2002) and cranial motoneurons (Ohsawa et al., 2005).

Here, we report that Mash1 is required for the proper formation of relay visceral sensory neurons of the hindbrain, i.e., of the nucleus of the solitary tract, as well as the associated primary chemoreceptors of the area postrema. We show that these two structures do form in the mutants but are atrophic, due in part to a delay in the onset of neuronal differentiation. We also show that the previously described atrophy of the sympathetic chain in *Mash1* mutants results from a similar delay in the differentiation of sympathoblasts and discuss several mechanisms.

Materials and methods

Genotyping and maintenance of mutant mice

Mash1, *Ngn2*, and *Mash1*^{KINgn2} mutant mice were produced and genotyped as previously described (Guillemot et al., 1993; Parras et al., 2002).

In situ hybridization, immunohistochemistry, and immunofluorescence

Riboprobes for *DBH*, *dHand* (gift of Y-S. Dai), *Gata3* (gift of D. Engel), *Mash1*, *Ngn2*, *Ret*, *Tlx3* (gift of Q. Ma), *Olig3* (gift of C. Birchemeier) and *Tubb3* (gift of C.W. Ragsdale) and antibodies against BrdU (Sigma), Lmx1b (gift of T. Jessell), Phox2a and Phox2b were used for in situ hybridization, immunohistochemistry, and in situ hybridization combined with immunohistochemistry as in Tiveron et al. (1996) and for immunofluorescence as in Pattyn et al. (2000). Double immunofluorescence experiments were analyzed on a TPS/SP2 Leica confocal microscope and pictures were superimposed in Photoshop (Adobe).

BrdU analysis and cell counts

BrdU was injected intraperitoneally into pregnant mice (6 mg/mouse) at E9.5, E10.5, E11.5, and E12.5, 24 or 48 h before dissecting the embryos. Embryos were then processed for double-immunofluorescence analysis using antibodies against BrdU and Phox2b, as described (Pattyn et al., 1997). For each experiment, the number of BrdU+/Phox2b+ cells was counted on transverse sections at the level of rhombomeres 7 and 8 (where nTS neurons can be identified unambiguously) of wild-type and *Mash1* mutant embryos (for each genotype, $n = 22$ sections at E10.5, $n = 14$ sections at E11.5 and E12.5 and $n = 15$ sections at E13.5, from 2 embryos). Values are means ± SEM.

Results

Normal development of the nTS

The nTS is an elongated nucleus located in the dorsal part of the hindbrain formed by first order relay sensory neurons postsynaptic to baroreceptors, chemoreceptors and osmoreceptors of the distal VIIth, IXth and Xth cranial ganglia, whose central axons form the “solitary tract” (Blessing, 1997). Neurons of the nTS are born from E9.5 to E12.5 (Taber Pierce, 1973). They emerge at rhombomeric levels 4 to 7 (Pattyn et al., 1997) from the dorsal-most part of the *Mash1*-positive domain of the neuroepithelium (Hornbruch et al., 2005; Qian et al., 2001) (Fig. 1), making this region topologically equivalent to the progenitor domain for dl3 interneurons of the spinal cord (Helms and Johnson, 2003). The post-mitotic precursors of the nTS are first detectable at E10-E10.5 by their expression of *Rnx/Tlx3* (Qian et al., 2001), *Lmx1b*, and *Phox2b* (Dauger et al., 2003) (and Fig. 1A). The onset of *Phox2b* expression slightly precedes the downregulation of *Mash1* (arrows in Figs. 1A, B). The nTS precursors migrate ventrally as soon as they are detectable and, eventually, aggregate just dorsal to the motor nucleus of the vagus nerve (Dauger et al., 2003; Qian et al., 2001) (and Figs. 1A, B).

While the nTS of *Lmx1b* knock-outs has not been examined to date, inactivation of *Tlx3* or *Phox2b* results in nTS agenesis, both genes being switched on independently of each other and *Tlx3* being required for the maintenance of *Phox2b* (Dauger et al., 2003; Qian et al., 2001). It has been argued that *Mash1*, expressed in nTS progenitors, has a specification function in nTS formation, since it can ectopically activate *Tlx3* in the chick

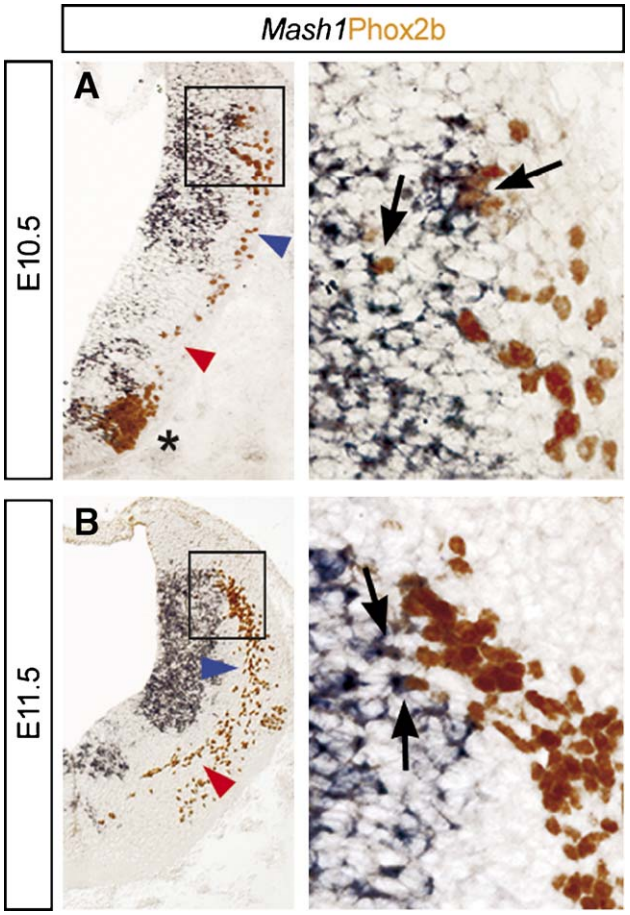

Fig. 1. Hindbrain nTS precursors derive from a *Mash1*-positive domain of the neuroepithelium. Combined in situ hybridization with *Mash1* (dark blue) and immunohistochemistry with a Phox2b antibody (orange) on transverse sections through r7 at E10.5 (A) and E11.5 (B). Only the right side of the sections is shown. Right panels are close-ups of the respective framed areas. The dorsal-most *Mash1*-positive progenitors give rise to nTS precursors, which transiently coexpress *Mash1* and the early post-mitotic marker Phox2b (arrows). Soon after they exit the ventricular zone, nTS precursors migrate ventrally (blue arrowheads) while ventrally generated Phox2b-positive motoneurons (asterisk in panel A) migrate dorsally (red arrowheads).