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Comparison of the generic neuronal differentiation and neuron subtype specification functions of mammalian *achaete-scute* and *atonal* homologs in cultured neural progenitor cells

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SUMMARY

In the vertebrate peripheral nervous system, the proneural genes neurogenin 1 and neurogenin 2 (*Ngn1* and *Ngn2*), and *Mash1* are required for sensory and autonomic neurogenesis, respectively. In cultures of neural tube-derived, primitive PNS progenitors NGNs promote expression of sensory markers and MASH1 that of autonomic markers. These effects do not simply reflect enhanced neuronal differentiation, suggesting that both bHLH factors also specify neuronal identity like their *Drosophila* counterparts. At high concentrations of BMP2 or in neural crest stem cells (NCSCs), however, NGNs like MASH1 promote only autonomic marker expression. These data suggest that that the identity specification function of NGNs is more sensitive to context than is that of MASH1. In NCSCs, MASH1 is more sensitive to Notch-mediated inhibition of neurogenesis and cell cycle arrest, than are the NGNs. Thus, the two proneural genes differ in other functional properties besides the neuron subtype identities they can promote. These properties may explain cellular differences between MASH1- and NGN-dependent lineages in the timing of neuronal differentiation and cell cycle exit.

Key words: Rat, MASH1, Atonal, Neurogenins, Neurogenesis, bHLH

INTRODUCTION

Proneural genes encode basic helix-loop-helix (bHLH) transcription factors that are essential for early steps in neurogenesis, in both *Drosophila* (Campuzano and Modolell, 1992) and vertebrates (reviewed by Guillemot, 1999). In *Drosophila*, proneural genes such as *achaete-scute* (*ac-sc*) and *atonal* (*ato*) play at least two roles. They select neural or neuronal precursors from a field of equivalent neuroepithelial cells, a function sometimes equated with promoting generic neuronal differentiation (Jarman and Ahmed, 1998), and they also specify neuronal subtype identity (Anderson and Jan, 1997; Hassan and Bellen, 2000). Thus, for example, in the fly peripheral nervous system (PNS), *ac-sc* specifies external sensory (ES) organ identity, while *ato* primarily specifies chordotonal (CD) organ identity (Jarman et al., 1993; Chien et al., 1996). However ATO can specify ES as well as CD identities, depending on context, while AC-SC exclusively promotes ES identity (Jarman et al., 1993; Jarman and Ahmed, 1998).

Vertebrate homologs of proneural genes, such as the *ato*-related neurogenins (NGNs) (Gradwohl et al., 1996; Ma et al., 1996; Sommer et al., 1996) and the *ac-sc* related *Mash1* (*Ascl1* – Mouse Genome Informatics) (Johnson et al., 1990; Guillemot and Joyner, 1993), are required for neurogenesis in distinct lineages (Guillemot et al., 1993; Fode et al., 1998; Ma et al., 1998) and are sufficient to promote generic neuronal differentiation both in vivo (Zimmerman et al., 1993; Turner and Weintraub, 1994; Ma et al., 1996; Blader et al., 1997; Perez et al., 1999) and in vitro (Lo et al., 1998; Farah et al., 2000; Sun et al., 2001). Whether these bHLH factors are sufficient to promote distinct neuronal subtype identities, however, is less clear.

In *Drosophila*, establishment of the identity-specification functions of the proneural genes has crucially depended on comparative gain-of-function (GOF) studies (Jarman et al., 1993; Jarman and Ahmed, 1998). By contrast, there is no reported GOF study in which the specific neuronal subtypes promoted by mis-expression of *Mash1* and NGNs have been quantitatively compared side-by-side in the same vertebrate system. Although retroviral mis-expression of these bHLH factors has been performed in rodent cortex (Cai et al., 2000), no detailed information was reported on the relative ratios of different neuronal subtypes generated by the *ac-sc* and *ato* homologs. The ability of *Xenopus ac-sc* and *ato* homologs to promote pan-neuronal marker expression has been directly compared (Chitnis and Kintner, 1996), but specific neuronal subtypes have not been examined.

Many non-comparative studies of the neuronal subtype specification function of vertebrate proneural genes have been

performed. There is evidence that constitutive or ectopic expression of NGNs, and other *ato* homologs, yields specific neuronal subtypes in vivo (Blader et al., 1997; Kanekar et al., 1997; Olson et al., 1998; Perez et al., 1999; Gowan et al., 2001). There is also evidence that MASH1 contributes to the specification of neuronal subtype identity in the CNS (Fode et al., 2000). However, in the absence of comparative data, it remains unclear whether *ato* and *ac-sc* homologs would both promote the same neuronal subtype(s) in a given cellular context. Moreover, because it is difficult to control the extracellular environment in such in vivo experiments, it is not clear whether these proneural genes autonomously determine specific neuronal subtypes, or rather simply provide a permissive context that allows identity specification by local cell-extrinsic signals (Scardigli et al., 2001). This issue can be addressed by performing comparative GOF experiments with NGNs and *Mash1* in vitro, where the extracellular environment can be manipulated independently of the expression of these bHLH factors.

In the vertebrate PNS, NGNs are required for sensory neuron development (Fode et al., 1998; Ma et al., 1998; Ma et al., 1999), while *Mash1* is, conversely, required for autonomic neurons (Guillemot et al., 1993). We have compared the subtype(s) of PNS neurons promoted by NGNs and *Mash1* when these genes are misexpressed in primitive PNS progenitors derived from the neural tube, or in neural crest stem cells (NCSCs) (Stemple and Anderson, 1992). In neural tube progenitors dorsalized by low concentrations of BMP2, NGNs promote exclusively sensory neurogenesis, while MASH1 promotes only autonomic neurogenesis. However at higher concentrations of BMP2, or in NCSCs, NGNs promote only autonomic neurogenesis. These results parallel those obtained for *ac-sc* and *ato* in the *Drosophila* PNS (Jarman et al., 1993; Jarman et al., 1995; Jarman and Ahmed, 1998), and suggest that MASH1 and the NGNs, like their *Drosophila* counterparts, both have precursor-selection and subtype-specification functions, despite recent arguments to the contrary (Brunet and Ghysen, 1999; Hassan and Bellen, 2000). However, the subtype-specification function of NGNs is more sensitive to context, like that of ATO (Jarman et al., 1993; Jarman and Ahmed, 1998). Our experiments also reveal differences in the relative sensitivity of MASH1 and NGNs to Notch-mediated inhibition of neurogenesis and cell cycle exit. These differences may explain cellular differences between MASH1- and NGN-dependent lineages in the timing of neuronal differentiation and cell cycle exit, in both the PNS and CNS.

MATERIALS AND METHODS

Cell culture and retroviral infection

Rat E10.5 neural tubes were dissected as described (Lo et al., 1998). About 40-60 pooled neural tubes were trypsinized, washed thoroughly and replated at 15,000 cells per 4 mm diameter cloning ring (an area of 0.126 cm^2), in a 35 mm fibronectin-coated dish. Cells were allowed to settle down for 1 hour before the rings were removed. Cells were cultured in a defined medium as described elsewhere (Greenwood et al., 1999), except that basic fibroblast growth factor (bFGF, 12 ng/ml; UBI) was included at all times. Where indicated, the culture medium was supplemented with BMP2 (gift from Genetics Institute). The neural tube cells reaggregated after 24 hours and, depending on the concentration of BMP2, were surrounded by many dispersed cells after 48 hours. Retroviral infection was performed on 24 hour cultures, and viruses were added at an m.o.i. (multiplicity of infection) of one viral particle per cell for 3-4 hours at 37°C. Infected cells were washed once and cultured for an additional 3 days before fixation and staining.

The full-length coding sequences of MASH1, NGN1 (NEUROD3 – Mouse Genome Informatics) and NGN2 (ATOH4 – Mouse Genome Informatics) were amplified from plasmids (Perez et al., 1999) using PFU-polymerase (Stratagene) as described (Lo et al., 1998). These genes were cloned into a pLZRS vector (Kinsella and Nolan, 1996) which was modified to contain a cassette encoding IRES-nuclear super GFP with five Myc epitope tags (super GFP was a gift from Barbara Wold). The pLZRS retroviral vector used the nuclear replication and retention functions of the human Epstein-Barr virus (EBV) to maintain the retroviral constructs as episomally replicated plasmids in 293-T-based retroviral packing cells (Ory et al., 1996). Production of high titer virus was modified slightly from elsewhere (Burns et al., 1993; Okada et al., 1999); details can be provided upon request.

Monoclonal antibody production

DNA sequences encoding the N terminus of the bHLH domain of NGN2 and the full-length coding sequence of SOX10 were cloned into pGEX-4T3 (Pharmacia). After IPTG induction, the GST fusion proteins were extracted from bacteria and purified by GST column. The immunization protocol was as described elsewhere (Lo et al., 1991). Initial screening of hybridoma supernatants was performed in pLZRS-NGN2 or pLZRS-SOX10 retroviral infected chicken embryonic fibroblast (CEF) cells, followed by screening on rat E12 to E13 sections. For anti-NGN2, out of 545 clones, two mouse IgG1-(5C6 and 8B1) and one mouse IgG2a-(7G4) – secreting hybridomas were selected for their specificity to NGN2. The specific staining of all three antibodies was blocked by the NGN2-GST fusion, but not by an NGN1-fusion protein. For anti-SOX10, out of more than 800 clones, three mouse IgG1-secreting hybridomas, 20B7,20A7 and 13C9, were selected. Epitope-mapping experiments indicated that 20B7 recognizes a determinant in the first 65 amino acids, which contains sequences unique to SOX10. Anti-NGN2 and anti-SOX10 hybridoma supernatants were used at a 1:20 dilution.

Immunocytochemistry

Rat embryos or cells were fixed in freshly prepared 4% paraformaldehyde in PBS. After sucrose sinking, the embryos were embedded and stored at –80°C. Only freshly sectioned embryos were used for staining. Antibodies were applied for 1-2 hours at room temperature for cell culture and overnight at 4°C for sections. Monoclonal antibodies were obtained and used as follows: BRN3A (POU4F1 – Mouse Genome Informatics) (1:50 hybridoma supernatants, mouse IgG1, Chemicon), GFP (1:200, mouse IgG2a, clone 3E6, Molecular Probe), TUJ1 (1:1000, mouse IgG2a, Covance), Myc tag (1:4 hybridoma supernatant, mouse IgG1, clone 9E10,ATCC), NF160 (1:200, mouse IgG1, clone NN18, Sigma), RET [1:4 hybridoma supernatant (Lo and Anderson, 1995)] and NeuN (1:200, Chemicon), and anti-BrdU (1:100, Caltag). Rabbit polyclonal antibodies were obtained and used as follows: BRN3A (1:1000, gift from Eric Turner), PHOX2B (PMX2B – Mouse Genome Informatics) (1:1000, gift from Goridis and Brunet), Myc tag (1:500, Santa Cruz), GFP (1:500, Molecular Probe), anti-NF-M (1:500, Chemicon) and peripherin (1:1000, Chemicon). Donkey anti-mouse or rabbit secondary antibodies conjugated to FITC, PE, Cy3 and Cy5 were obtained from Jackson ImmunoResearch). Goat anti-mouse IgG1 or IgG2a conjugated with PE or FITC were obtained from Southern Biotechnology. Goat anti-mouse IgG1 or IgG2a conjugated with Alexa 488 or Alexa 568 were obtained from Molecular Probes. Secondary antibodies were used at 1:200 to 1:300 dilution and applied for 1 hour at room temperature. For double- or triple labeling, a cocktail containing the required antibodies was mixed, filtered, and centrifuged to remove aggregates before use.

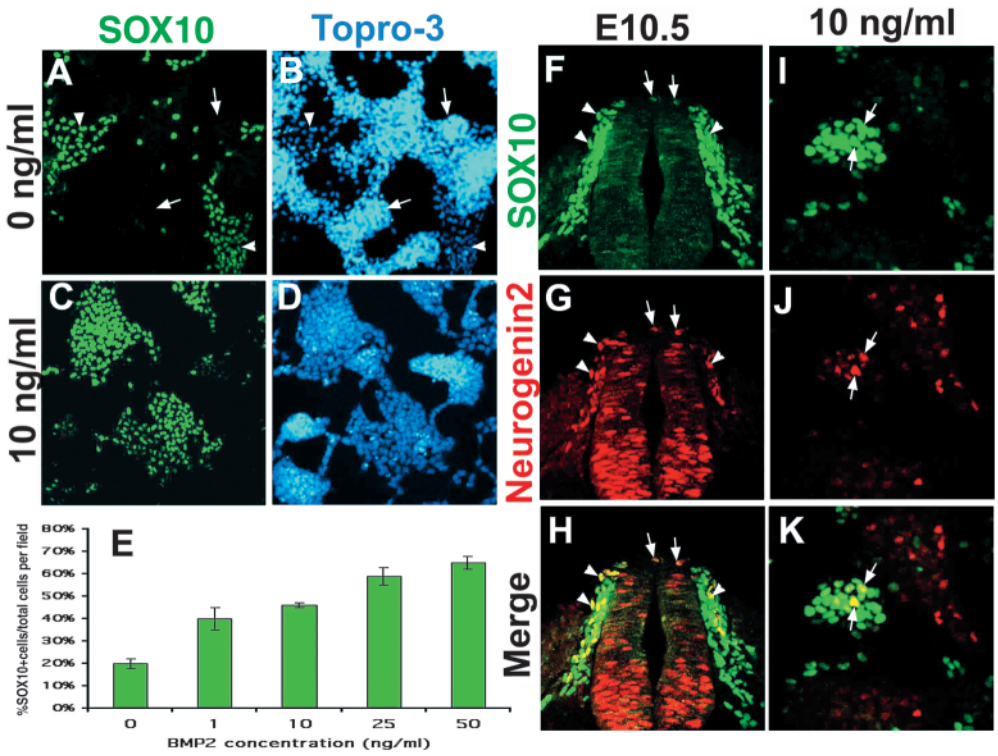


Fig. 1. SOX10$^+$ and NGN2$^+$ cells develop in dNT cultures. (A-E), cultures grown for 48 hours in the indicated concentrations of BMP2 were stained with anti-SOX10 and counter-stained with the nuclear dye Topro-3. (E) The percentage of SOX10$^+$ cells was scored. The data are derived from two independent experiments. Arrows indicate dense reaggregates of SOX10$^-$ neural tube cells, arrowheads indicate relatively more dispersed SOX10$^+$ neural crest cells. (F-H) Double-labeling of a section of E10.5 rat spinal cord with anti-SOX10 and anti-NGN2. Arrows indicate NGN2$^+$ cells at dorsal margin of the spinal cord, arrowheads indicate double-positive cells in the neural crest migration pathway. (I-K) dNT culture grown for 48 hours in 10 ng/ml BMP2 double-labeled for SOX10 and NGN2. Arrows indicate double-positive cells.

For BrdU analysis, GFP or NF-M positive cells were detected using mouse anti-GFP or rabbit anti-NF-M, followed by Alexa 488-conjugated goat anti-mouse IgG2a or Cy5-conjugated donkey anti-rabbit secondary antibodies. BrdU incorporation was detected as described previously (Novitch et al., 1996) using a mouse anti-BrdU antibody and Alexa 568-conjugated goat anti-mouse IgG1 secondary antibody (Molecular Probes)

Positive cells were visualized using a Olympus microscope or Leica TCS SP confocal Microscope. Randomly selected 10× or 20× fields were sequentially scanned with different laser beams for FITC, Cy3

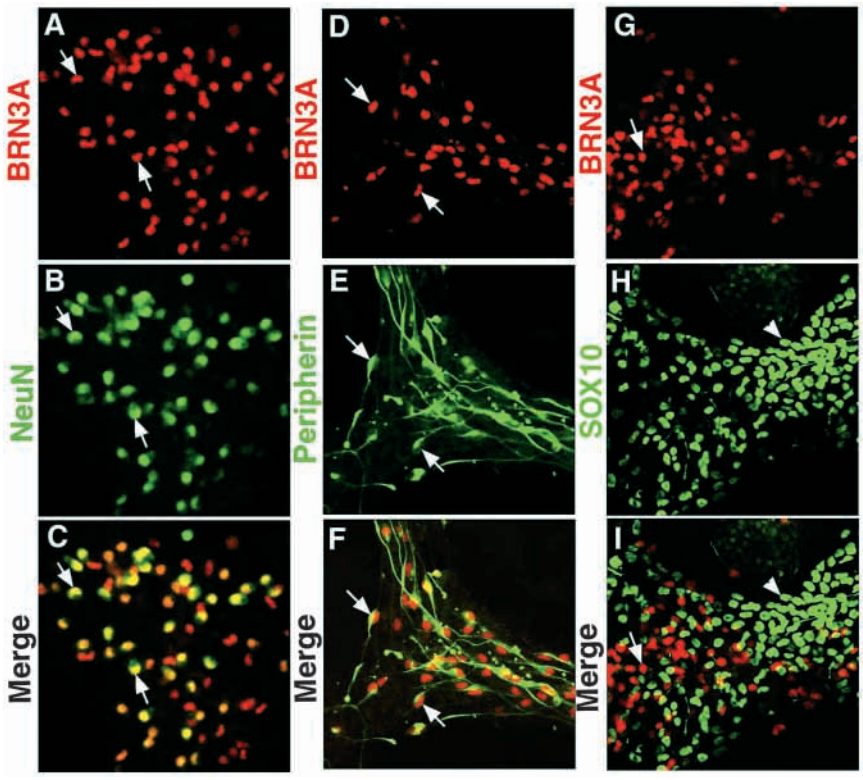


Fig. 2. BRN3A$^+$ cells in dNT cultures are peripheral sensory neurons. Cultures grown for 3 days in 10 ng/ml BMP2 were double-labeled for BRN3A (A,D,G) and the indicated markers (B,E,H). NeuN is a pan-neuronal marker; peripherin is a peripheral neuron-enriched marker. Some BRN3A$^+$ cells also co-express RET (not shown). (G-I) The BRN3A$^+$ cells co-expressing NeuN and peripherin were often found in close association with clusters of SOX10$^+$ cells. However the BRN3A$^+$ cells are SOX10$^-$ as the latter marker is rapidly downregulated during neuronal differentiation (not shown; L. L., J. Kim and D. J. A., unpublished).

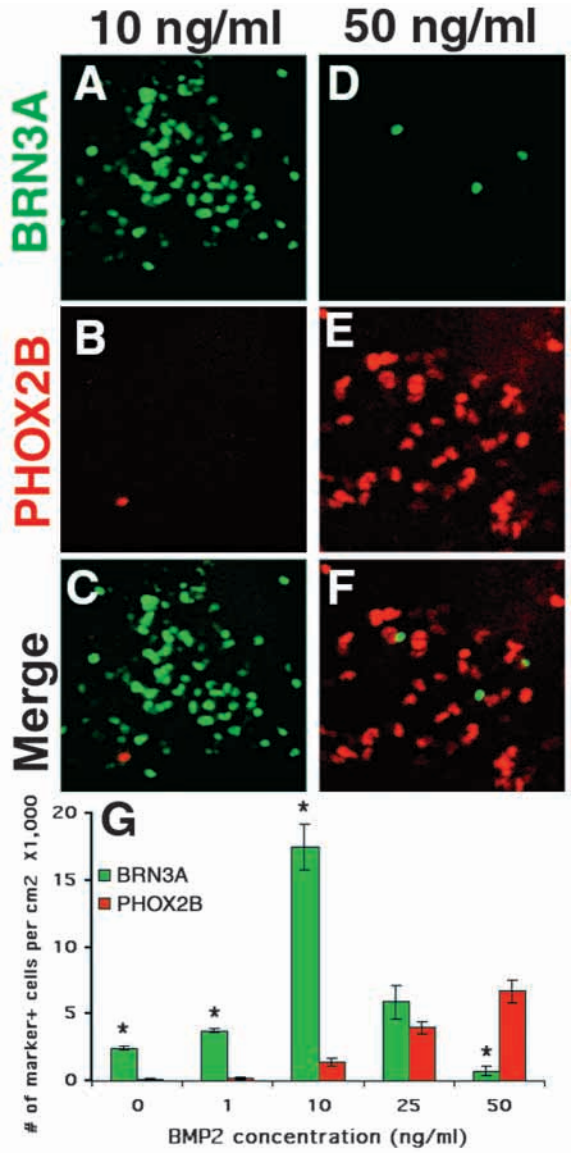


Fig. 3. Effect of BMP2 concentration on differentiation of BRN3A$^+$ and PHOX2B$^+$ cells in dNT cultures. Cultures grown for 4 days in the indicated concentrations of BMP2 were fixed and double-labeled with anti-BRN3A (A,D) and anti-PHOX2B (B,E). (G) The number of BRN3A$^+$ or PHOX2B$^+$ cells/cm^2 was measured in a standard cloning ring. Numbers indicate the mean±s.e.m. of two independent experiments with quadruplicate dishes in each experiment. *The number of BRN3A$^+$ cells is significantly different (P<0.01) from the number of PHOX2B$^+$ cells at the indicated dose of BMP2.

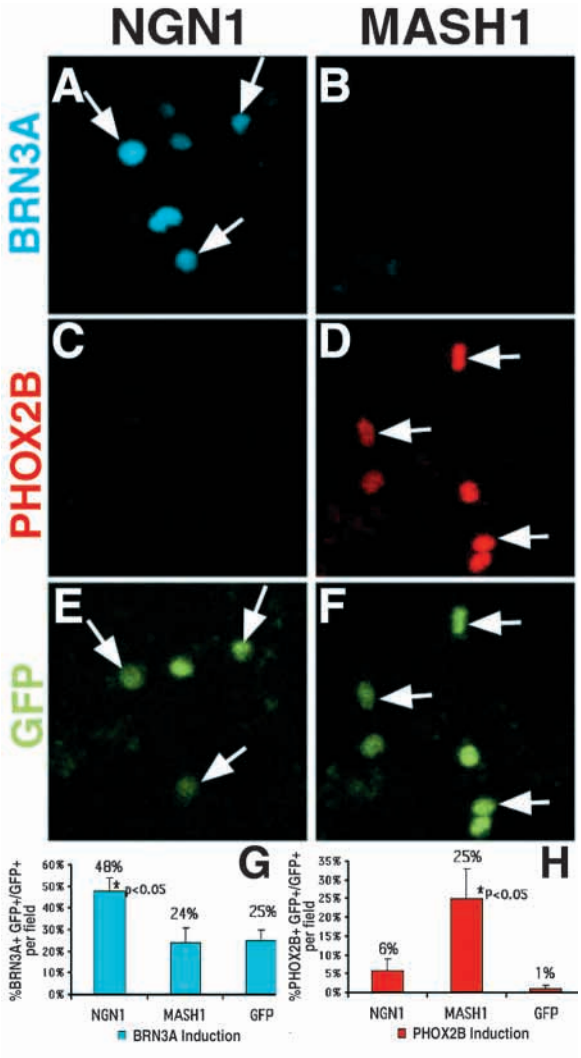


Fig. 4. Exogenous NGN1 and MASH1 promote sensory and autonomic neurogenesis, respectively, at 10 ng/ml BMP2. Cultures were infected with retroviruses encoding NGN1-IRES-GFP (A,C,E) or MASH1-IRES-GFP (B,D,F), grown for 3 days in 10 ng/ml BMP2 and triple-labeled with antibodies to BRN3A (A,B), PHOX2B (C,D) and GFP to detect infected cells (E,F; arrows). NGN1-infected cells that express BRN3A (A,E, arrows) do not co-express PHOX2B, while MASH1-infected cells that express PHOX2B (D,F, arrows) do not co-express BRN3A. (G,H) The percentage of cells infected with the indicated retroviruses that express BRN3A (G) or PHOX2B (H) is indicated. *P<0.05 indicates significantly different from control GFP-infected cells. Note that the percentage of NGN1-infected cells expressing PHOX2B in H (6%) is not significantly different from the GFP control (1%; one-way ANOVA followed by post-hoc analysis, HSD=1.33<4.34). The results were pooled from eight experiments. For additional data, see Fig. 6, Table 1 and Table 2.

(or PE) and Cy5. Data were collected by directly counting the positive cells from the pictures. In some cases, Topro-3 (nucleic acid dye, Molecular Probes) were used to reveal the total cells in a given field.

RESULTS

Neural crest cells develop in dissociated neural tube cultures grown in low concentrations of BMP2

To compare the PNS neuronal subtypes generated by constitutive expression of NGNs and *Mash1* in vitro, we first sought to identify and characterize a cultured neural progenitor population that is competent to generate both sensory and autonomic neurons de novo. NCSCs isolated from E10.5 rat neural tube explants can generate autonomic neurons (Shah et al., 1996), but these stem cells have never been observed to generate sensory neurons, either in vitro or in vivo (White et al., 2001). Neural tube explant cultures also contain a

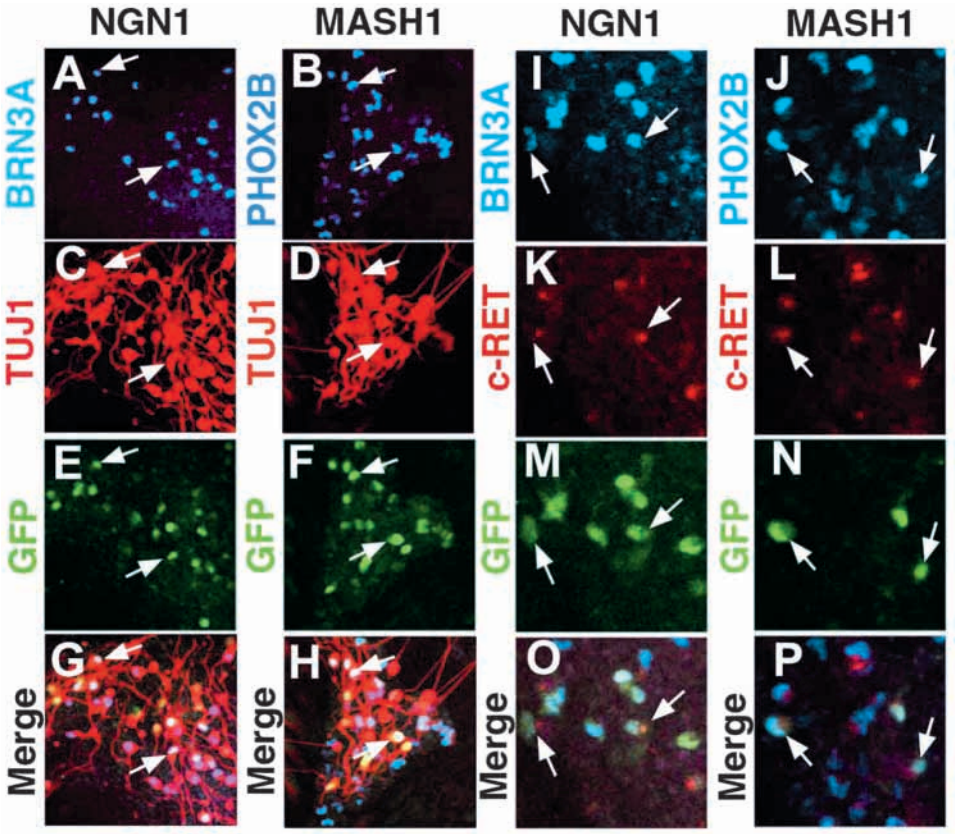


Fig. 5. NGN1- and MASH1-infected cells that express BRN3A or PHOX2B co-express pan-neuronal and peripheral neuron-specific markers. Cultures infected with the retroviruses indicated above the columns were grown for 3 days in 10 ng/ml BMP2, fixed and triple-labeled with antibodies to BRN3A (A,I) or PHOX2B (B,J); TuJ1 (C,D) or RET (K,L); and GFP (E,F,M,N). Arrows indicate infected cells labeled by all three antibodies. 'Merge' indicates superposition of the three overlying panels in the same column. I-P are shown at twice the magnification of A-H.

subpopulation of sensory neuron precursors; however, such precursors appear determined for a sensory fate and lineally distinct from NCSCs (Greenwood et al., 1999). We therefore reasoned that dissociated E10.5 neural tube cultures might contain an earlier population of premigratory neural crest cells, which are unspecified with respect to sensory and autonomic neuronal subtypes (Bronner-Fraser and Fraser, 1988; Bronner-Fraser and Fraser, 1989).

The formation of the neural crest from the neural tube is known to require a dorsalization function that is provided by BMP family members expressed in the dorsal neural tube and/or dorsal epidermis, such as BMP2, BMP4, BMP7 and GDF7 (Liem et al., 1995; Liem et al., 1997; Lee and Jessell, 1999). Consistent with this idea, BMP2 (and BMP4) has been shown to promote the appearance of cells expressing the neural crest marker $p75^{LNTR}$ in dissociated cultures of E10.5 neural tubes (Mujtaba et al., 1998). These cells can differentiate to autonomic-like neurons. However those studies did not establish whether these $p75^{+}$ crest-like cells also generate sensory neurons. To address this question, we examined the expression of markers of sensory precursors and sensory neurons in such dissociated neural tube (dNT) cultures.

We first characterized the formation and differentiation of neural crest cells in such cultures using a new monoclonal antibody (mAb) to the HMG-box protein SOX10 (Kuhlbrodt et al., 1998; Southard-Smith et al., 1998). Unlike p75, which is expressed not only by neural crest cells, but also eventually by neural tube-derived cells (Kalyani et al., 1997), SOX10 is neural crest-specific (Britsch et al., 2001), as confirmed by staining sections of E10.5 rat embryos with the anti-SOX10 mAb (Fig. 1F, arrowheads).

We next examined the development of $SOX10^{+}$ cells in dNT cultures grown in the absence or presence of different concentrations of BMP2. In the absence of exogenously added BMP2, growth of dissociated neural tube cells for 24 hours resulted in the formation of dense cellular reaggregates that were predominantly $SOX10^{-}$ (Fig. 1A, arrows). At the margins of these reaggregates, however, more dispersed cells were visible that were often $SOX10^{+}$ (Fig. 1A,B; arrowheads). As the concentration of BMP2 was increased, the proportion of $SOX10^{+}$ cells increased (Fig. 1A,C,E). The location of the relatively more dispersed $SOX10^{+}$ cells at the margins of the dense $SOX10^{-}$ clusters suggested that these two cell populations probably represent neural crest and re-aggregated neural tube cells, respectively.

Identification of sensory neuron precursors in dNT cultures

The presence of neural crest cells in dNT cultures raised the question of whether these cells might include sensory neuron precursors. To investigate this, we first examined the expression of NGN2, which marks a subset of such precursors in vivo (Ma et al., 1999). In sections of E10.5 rat embryos, NGN2 is expressed by a small subset of cells at the dorsal margin of the neural tube (Fig. 1G, arrows), as well as in more numerous $SOX10^{+}$ cells in the neural crest migration pathway (Fig. 1G,H; arrowheads). However the majority of migrating $SOX10^{+}$ crest cells are $Ngn2^{-}$. At later stages, very few $Ngn2^{+}$ neural crest cells co-express *SOX10* (not shown), suggesting that the HMG-box factor is rapidly downregulated in sensory precursors. In addition to neural crest-derived cells, the anti-NGN2 antibody stained many cells in the ventral neural tube,

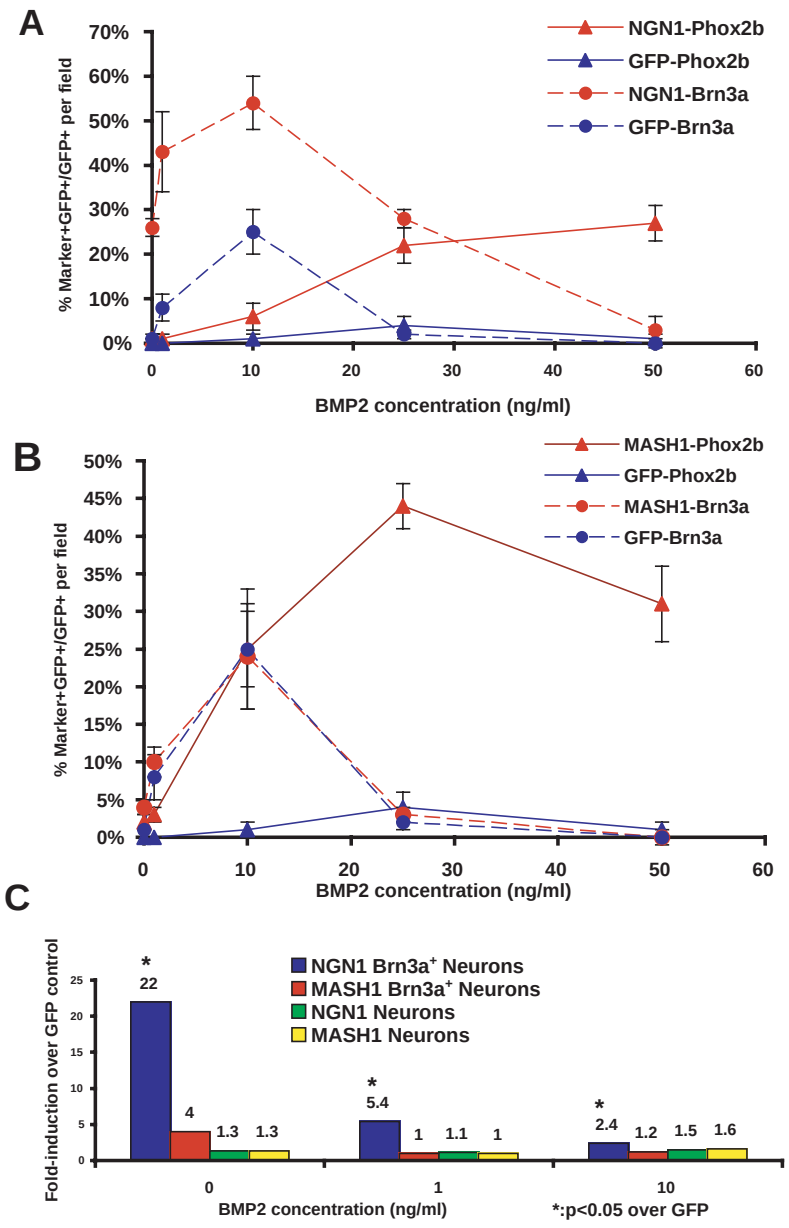
Table 1. Specific enhancement of BRN3A expression and neurogenesis by NGN1

	NGN1 infected cells (%)		MASH1 infected cells (%)		GFP infected cells (%)	
BMP2 concentration (ng/ml)	BRN3A$^+$ neurons	Neurons	BRN3A$^+$ neurons	Neurons	BRN3A$^+$ neurons	Neurons
0	22±7	89±3	4±2	85±4	1±1	68±5
1	43±2	94±2	8±1	88±2	8±1	84±2
10	59±6	84±8	29±9	86±10	25±3	55±6

Dissociated neural tube cultures were fixed 3 days after infection with the indicated viruses, and triple-labeled with antibodies to the Myc epitope tag or GFP (to identify infected cells), TuJ1 (to identify neurons) and BRN3A. 'Neurons' indicates the percentage of infected cells expressing TuJ1. 'BRN3A$^+$ neurons' indicates the percentage of infected cells expressing TuJ1 that also expressed BRN3A. The data are pooled from four experiments. Note that at all concentrations of BMP2, the increase in BRN3A expression produced by NGN1 is greater than the increase in neuronal differentiation produced by NGN1. Note also that the enhancement of neurogenesis produced by MASH1 is similar to that produced by NGN1, but MASH1 does not increase BRN3A expression over its level in GFP-infected controls.

which include motoneuron precursors (Scardigli et al., 2001) (Fig. 1G).

In dNT cultures grown for 48 hours in 10 ng/ml BMP2, and then double-labeled with anti-NGN2 and anti-SOX10, small clusters of SOX10$^+$ cells containing a few NGN2$^+$ cells could be observed (Fig. 1I-K, arrows). In addition, there were NGN2$^+$ cells that did not co-express SOX10 in dense aggregates, as well as more dispersed, SOX10$^+$, NGN2$^-$ cells (Fig. 1K). Approximately 8% of NGN2$^+$ cells co-expressed SOX10. These data paralleled the distribution of SOX10-expressing and NGN2-expressing cells in the neural crest in vivo, and suggest that the NGN2$^+$, SOX10$^+$ cells in dNT cultures grown in 10 ng/ml BMP2 may be sensory neuron precursors. Similar results were obtained in cultures grown in 1 ng/ml BMP2 (not shown). However, the frequency of SOX10$^+$, NGN2$^+$ cells increased fourfold between 1 and 10 ng/ml BMP2 (from an average of 11 cells per 20× field to 47 cells per 20× field; n=8 and 10 fields counted, respectively).



Sensory neurons differentiate in dNT cultures grown in low BMP2

We next determined whether overt sensory neurogenesis occurred in dNT cultures grown in 10 ng/ml BMP2. After 3 days in vitro, such cultures contained numerous neurons, as revealed by staining with antibodies to pan-neuronal

Fig. 6. Neuronal subtype-inducing properties of NGN1 and MASH1 at different concentrations of BMP2. At each concentration of BMP2, cultures infected with NGN1 (A), MASH1 (B) or control GFP (A,B) retroviruses were fixed and double-labeled with antibodies to BRN3A or PHOX2B, and GFP. The percentage of infected (GFP$^+$) cells co-expressing BRN3A or PHOX2B is plotted. The data are pooled from 11 experiments, although not all BMP2 concentrations were tested in every experiment. For additional quantification see Table 2. Note that in B, although the percentage of MASH1-infected cells expressing PHOX2B in 10 ng/ml BMP2 (~25%; red triangles) is similar to the percentage of MASH1- or GFP-infected cells expressing BRN3A (red and blue dots), this similarity is coincidental; PHOX2B and BRN3A are never co-expressed (not shown). (C) The percentage of BRN3A$^+$ and neuronal (TuJ1$^+$) cells among NGN1-infected cells is directly compared. Note that the enhancement of the sensory marker is always greater than the enhancement of neuronal differentiation. The fold-induction of BRN3A expression declines with increasing BMP concentration because the level of baseline BRN3A expression increases (see A); see also Table 2. *P<.05; other values are not significantly different from each other.

Table 2. Relative enhancement of BRN3A and PHOX2B expression by NGN1 and MASH1 at different concentrations of BMP2

BMP2 concentration (ng/ml)	NGN1: BRN3A*	NGN1: PHOX2B*	NGN1: BRN3A/PHOX2B†	MASH1: BRN3A*	MASH1: PHOX2B*	MASH1: PHOX2B/BRN3A‡
0	26	1 (NS)	26	1 (NS)	1 (NS)	1 (NS)
1	5.4	1 (NS)	5.4	1 (NS)	3	3
10	2.15	1 (NS)	2.15	1 (NS)	25	25
25	14	5.5	2.8	1 (NS)	11	11
50	1 (NS§)	27	0.04	1 (NS)	31	31

*The percentage of NGN1- (or MASH1-) infected cells expressing BRN3A (or PHOX2B) was divided by the percentage of control GFP-transduced cells expressing the same marker (see Fig. 6 for percentages).

†The ratio of the increase in BRN3A by NGN1 to its increase in PHOX2B (column 1/column 2).

‡The ratio of the increase in PHOX2B by MASH1 relative to its increase in BRN3A (column 5/column 4).

§NS, no significant enhancement of marker expression by NGN1 or MASH1 relative to GFP was observed.

The data were pooled from 11 experiments. Note that at 25 ng/ml BMP2, both MASH1 and NGN1 enhance PHOX2B expression, but NGN1 is not as effective as MASH1. However, NGN1 still enhances BRN3A, while MASH1 does not. At 50 ng/ml BMP2, NGN1 and MASH1 enhance PHOX2B equally well.

markers such as NeuN (Mullen et al., 1992) and neuron-specific β-III tubulin (Geisert and Frankfurter, 1989) (Fig. 2B and data not shown). To determine whether any of these neurons might be sensory, we stained them with antibodies to the POU homeodomain transcription factor BRN3A, which, in the PNS, marks sensory neurons and their immediate precursors in vivo (Gerrero et al., 1993; Fedtsova and Turner, 1995) and in vitro (Greenwood et al., 1999). Such staining revealed that many BRN3A$^+$ cells co-expressed NeuN or β-III tubulin (Fig. 2A-C, arrows and data not shown). Many of these neurons were located in cell clusters or aggregates resembling peripheral ganglia, and were closely associated with SOX10$^+$ cells (Fig. 2G-I).

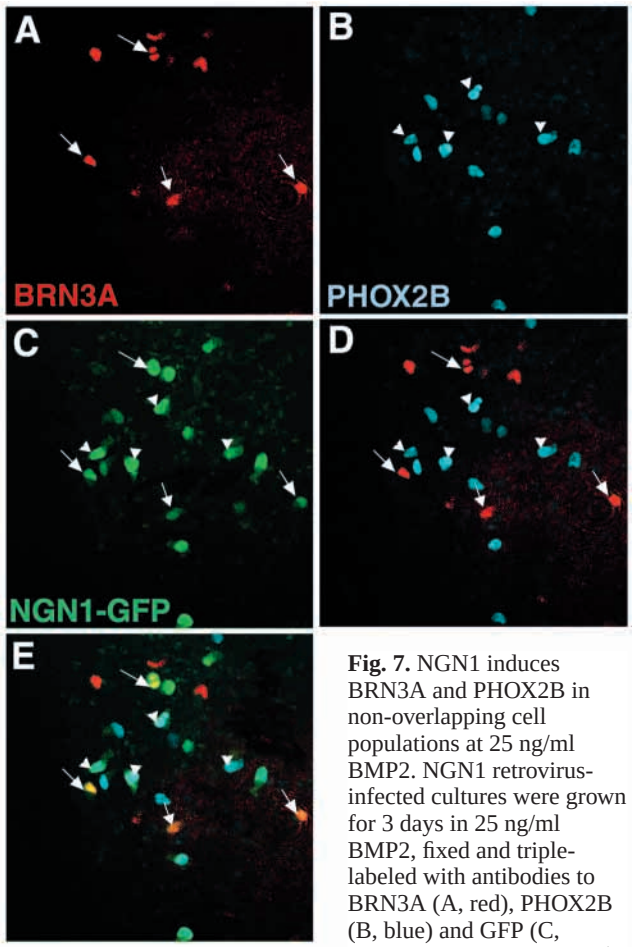


Fig. 7. NGN1 induces BRN3A and PHOX2B in non-overlapping cell populations at 25 ng/ml BMP2. NGN1 retrovirus-infected cultures were grown for 3 days in 25 ng/ml BMP2, fixed and triple-labeled with antibodies to BRN3A (A, red), PHOX2B (B, blue) and GFP (C, green). (D,E) The BRN3A$^+$, GFP$^+$ (arrows) and PHOX2B$^+$, GFP$^+$ cells (arrowheads) are non-overlapping.

Since BRN3A is also expressed by some dorsal interneurons in the spinal cord (White and Anderson, 1999), we also counterstained for peripherin, which is expressed at hight levels in peripheral neurons compared with central neurons (Parysek et al., 1988; Parysek and Goldman, 1988), and for RET, which is expressed by motoneurons (which are BRN3A$^-$) and not by dorsal interneurons (Pachnis et al., 1993). Many of the BRN3A$^+$ neurons co-expressed peripherin and/or RET (Fig. 2D-F, arrows and data not shown), suggesting that they are indeed peripheral sensory neurons. Taken together, these data suggest that dNT cultures grown in 1 or 10 ng/ml BMP2 contain progenitor cells that can differentiate into sensory neurons.

Reciprocal differentiation of sensory and autonomic neurons in different concentrations of BMP2

In low concentrations of BMP2 (1 or 10 ng/ml), very few autonomic neurons, identified by expression of the paired homeodomain protein PHOX2B (Pattyn et al., 1997; Pattyn et al., 1999), were observed to develop (Fig. 3B,G). However, as the concentration of BMP2 was raised above 10 ng/ml, increasing numbers of PHOX2B$^+$ cells were observed, while the number of BRN3A$^+$ cells concomitantly decreased (Fig. 3G). At 50 ng/ml BMP2, very few BRN3A$^+$ cells were observed in comparison with PHOX2B$^+$ cells (Fig. 3D-G). Co-expression of BRN3A and PHOX2B in the same cells was never observed (Fig. 3F). Like the BRN3A$^+$ cells, these PHOX2B$^+$ cells co-expressed pan-neuronal markers such as NeuN, and peripheral neuron markers such as RET or peripherin, indicating that they are likely to be autonomic neurons (not shown). Consistent with this identification, cultures in which such PHOX2B$^+$ neurons developed also contained cells expressing MASH1 at earlier times (data not shown).

Taken together, the foregoing data suggest that dNT cultures contain progenitors to sensory and autonomic neurons. The fact that such PNS neurons appear to differentiate in a reciprocal manner at different concentrations of BMP2 is, furthermore, consistent with the idea that they may develop from a common progenitor. Attempts to test this hypothesis directly by clonal analysis or retroviral lineage tracing were unsuccessful, however, for technical reasons. Nevertheless, at

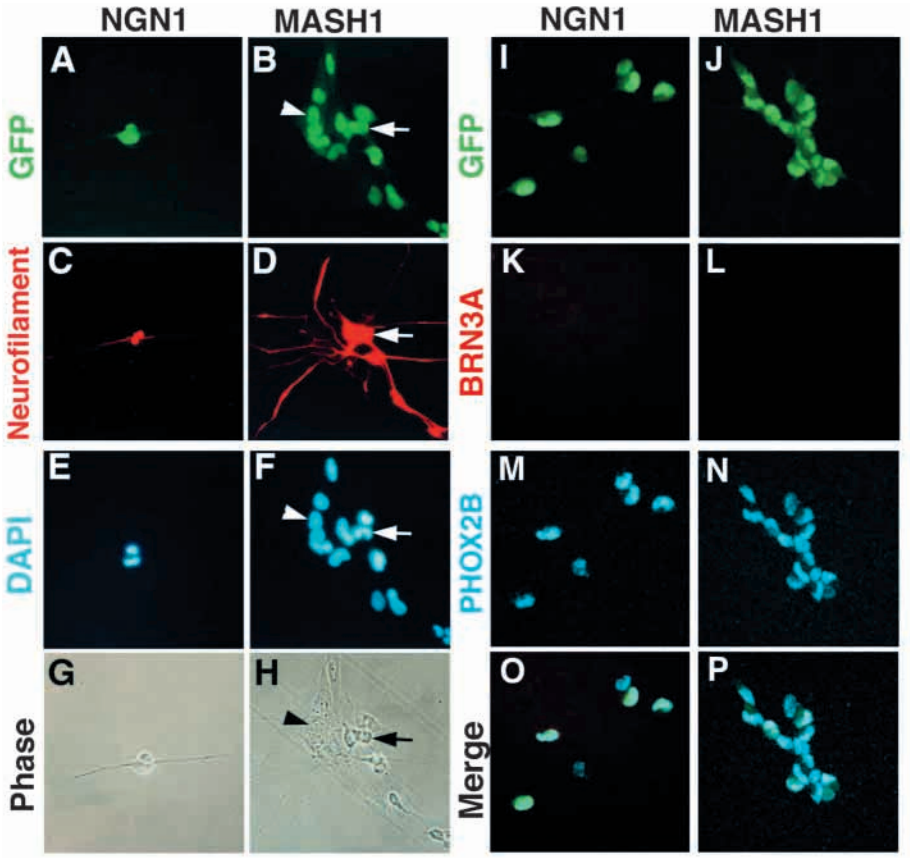


Fig. 8. NGN1 and MASH1 both induce autonomic neurogenesis in NCSC clonal cultures. Colonies were grown for 4 days after infection with the retroviruses indicated above the columns, before fixation and labeling. (A-H) Representative colonies from NGN1- and MASH1-infected cultures labeled with antibodies to NF160 (C,D), GFP (A,B) and with DAPI to visualize cell nuclei (E,F); (G,H) Phase-contrast views of the fields shown in A,C,E and B,D,F, respectively. Arrows in B,D,F,H indicate a neuron; arrowheads in B,F,H indicate a non-neuronal cell. All cells in the NGN1 colony illustrated are neurons. No neurons have differentiated at this time point in control GFP-infected cultures (not shown). (I-P) Colonies from a similar experiment triple-labeled with antibodies to GFP (I,J), BRN3A (K,L) and PHOX2B (M,N); 'Merge' indicates superposition of the three labels.

the population level, the dNT cultures were competent for both sensory and autonomic neurogenesis, and thus provided a system in which to test the neuronal subtype(s) promoted by constitutive expression of NGNs and *Mash1*.

NGNs and MASH1 promotes sensory and autonomic neurogenesis, respectively, at low concentrations of BMP2

To mis-express NGNs and *Mash1* in dNT cultures, we cloned their coding sequences into pseudotyped, replication-incompetent retroviruses, where their expression is under the control of the viral LTRs. These constructs also contained an IRES-GFP cassette to mark infected cells (see Materials and Methods). We first compared the neuronal subtypes promoted by these proneural genes at 10 ng/ml BMP2 (Fig. 4). Under these conditions, dNT cells infected with *Ngn1*- or *Ngn2*-expressing retroviruses predominantly expressed the sensory marker BRN3A (Fig. 4A,C,E), while those infected with the *Mash1*-expressing virus primarily expressed the autonomic marker PHOX2B (Fig. 4B,D,F). These BRN3A$^+$ or PHOX2B$^+$ cells co-expressed pan-neuronal markers such as βIII-tubulin (Fig. 5A-D,G-H, arrows), as well as RET (Fig. 5I-L; O-P, arrows). The latter marker confirms their identity as peripheral sensory and autonomic neurons.

Importantly, the proportion of neurons expressing BRN3A was more than twice as high among NGN1-infected cells as among control GFP-infected cells (Table 1, 10 ng/ml BMP2). By contrast, the overall extent of neuronal differentiation was only 1.5-fold higher in NGN1-infected cultures (Table 1). At 0 and 1 ng/ml BMP2, this difference was even more pronounced (Table 1 and Fig. 6C). These data suggest that the enhancement of BRN3A expression by NGN1 cannot be explained simply by its enhancement of generic neuronal differentiation. Consistent with this, MASH1 enhanced neuronal differentiation to a similar extent as NGN1, yet the percentage of BRN3A$^+$ neurons among MASH1-infected cells was never significantly higher than among control GFP-infected cells (Table 1). Thus, in low concentrations of BMP2, NGNs enhanced sensory but not autonomic differentiation, while MASH1 induced only autonomic neurogenesis (but did not suppress sensory differentiation).

The absolute fraction of NGN-infected cells expressing BRN3A increased as a function of BMP2 concentration between 0 and 10 ng/ml (Fig. 6A, red dots). However the enhancement of BRN3A expression by NGNs over control decreased in parallel (Fig. 6C and Table 2). This decrease reflects an increasing frequency of BRN3A expression among control GFP-infected cells (Fig. 6A, blue dots). These data suggest that there is a limit to the ability of exogenous NGN1 to enhance BRN3A expression above control levels. This could reflect a limit in the number of precursors that can differentiate to BRN3A$^+$ neurons, and/or in the amount of exogenous NGN expression needed to activate BRN3A expression. Consistent with the latter explanation, at 10 ng/ml BMP2 there was a fourfold increase in the number of cells expressing endogenous NGN2 (data not shown).

NGNs promote autonomic neurogenesis at high concentrations of BMP2

Above 10 ng/ml BMP2, the percentage of BRN3A$^+$ cells

Table 3. MASH1 and NGN1 differentially affect cell cycle withdrawal in NCSCs

Virus	Number of cells/clone	% Neurons	% BrdU-labeled
NGN1	4.7±1.0	66±4	26±2
MASH1	14.8±3.9	35±4	54±4*
GFP	15.3±1.1	0±0	66±3*
R(MASH1/NGN1)	3.0	0.5	2.1

NCSCs were infected with the indicated viruses and plated at clonal density. After 3 days, 1.75 μM BrdU was added. The cells were fixed 24 hours later and triple-labeled for GFP, BrdU and NF-M. The average percentage of neurons and of BrdU-labeled cells was determined in 70-72 randomly selected 20× or 10× fields from two independent experiment. The average number of cells/clone was determined at the same time point, and compiled from three independent experiments. Numbers represent the mean±s.e.m. The difference in the percentage of BrdU-labeled cells in MASH1- and NGN1-infected colonies is significant (*t*-test; $P<0.01$).

*The difference in BrdU-labeling index between MASH1- and GFP-infected clones is not significant ($P>0.1$).

†R(MASH1/NGN1) indicates the ratio of the values obtained in MASH1-infected versus NGN1-infected colonies.

among NGN1-infected cells decreased, while that of PHOX2B$^+$ cells increased (Fig. 6A, compare red dots with red triangles), paralleling the trend in uninfected cultures (Fig. 4). Nevertheless, the proportion of NGN-infected cells expressing PHOX2B was always higher than among control GFP-infected cells at 25 and 50 ng/ml BMP2 (Fig. 6A, red versus blue triangles; Table 2). At 25 ng/ml BMP2, a similar percentage of NGN1-infected cells expressed either BRN3A or PHOX2B (Fig. 6A, red symbols). However, triple-labeling of NGN1-infected cultures with anti-BRN3A, anti-PHOX2B and anti-GFP antibodies indicated that the sensory and autonomic markers were never co-expressed by the same infected cells (Fig. 7, arrows versus arrowheads). At 50 ng/ml BMP2, NGN1 only promoted autonomic neurogenesis, and did not enhance BRN3A expression significantly above control (Fig. 6A, red symbols; Table 2).

In contrast to NGN1, MASH1 always enhanced autonomic and not sensory neurogenesis, irrespective of BMP2 concentration (Fig. 6B, red symbols). For example, at 10 ng/ml BMP2, MASH1 stimulated PHOX2B expression about 25-fold relative to control (Figs 4H, 6B, red and blue triangles; Table 2). As the concentration of BMP2 was raised to 25 ng/ml, the enhancement of PHOX2B expression by MASH1 was slightly diminished (Table 2), reflecting the increased frequency of PHOX2B expression among control GFP-expressing cells (Fig. 6B, blue triangles). Nevertheless, at 50 ng/ml BMP2, MASH1 still enhanced expression of the autonomic marker substantially above control levels (Fig. 6B, triangles and Table 2).

At 25 ng/ml BMP2, NGN1 enhanced autonomic differentiation to a lesser extent than MASH1, whereas at 50 ng/ml BMP2 it did so to about the same extent as did MASH1 (~30-fold; Table 2). The enhancement of PHOX2B expression by NGN1 and MASH1 does not simply reflect the promotion of generic neuronal differentiation, as both bHLH factors enhanced neurogenesis only approx. twofold under these conditions. Furthermore, the promotion of autonomic neurogenesis by NGN1 does not appear to be mediated by induction of MASH1: for example, out of 336 NGN1-infected cells examined at 25 ng/ml BMP2, none co-expressed

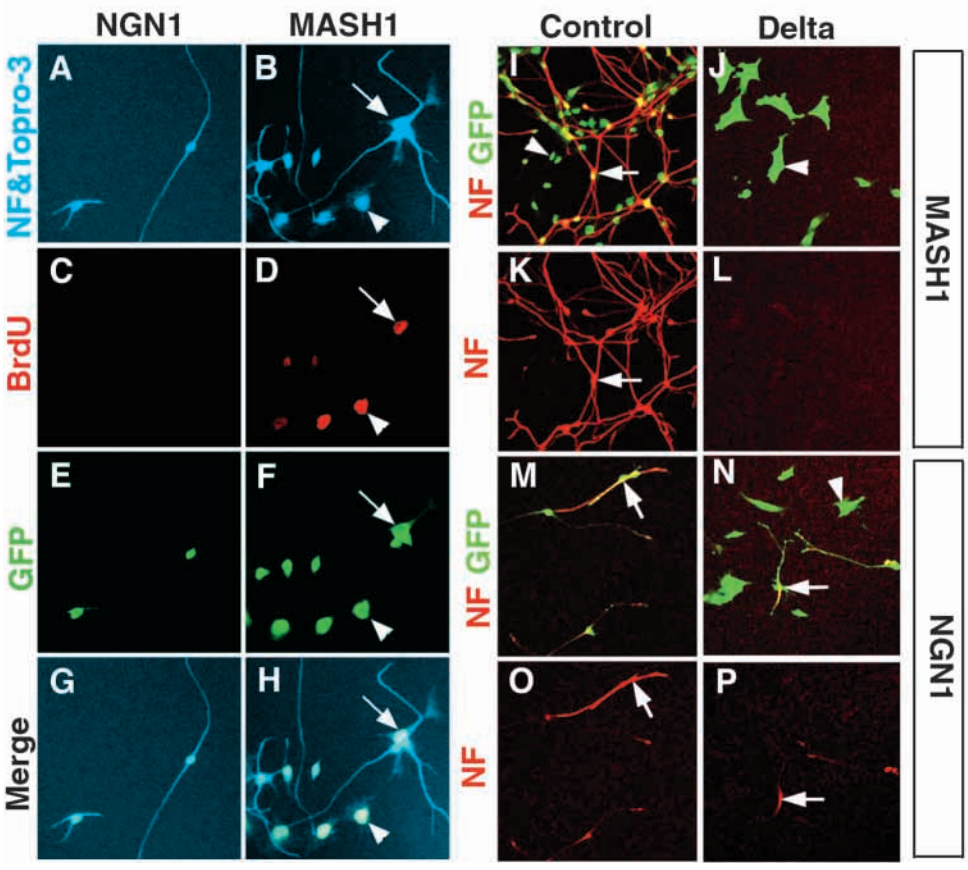


Fig. 9. Differences in cell cycle withdrawal-promoting activity and Notch sensitivity between NGN1 and MASH1 in NCSCs. (A-H) BrdU labeling in NGN1- or MASH1-infected colonies pulsed for 24 hours 3 days after infection. Colonies were labeled antibody to NF-M and the nuclear dye Topro-3 (A,B), anti-BrdU (C,D) and anti-GFP (E,F). Arrows in B,D,F,H indicate a neuronal cell, arrowheads indicate a non-neuronal cell (see Table 4 for quantification). (I-P) MASH1 is more sensitive than NGN1 to inhibition by Delta-Fc. Colonies were double-labeled with anti-GFP (green) and anti-NF-M (red). In K,L,O,P only the NF staining is shown for clarity. Arrows indicate process-bearing, NF-M$^+$ neurons, arrowheads indicate NF-M$^-$ non-neuronal cells. Note that there are still some neurons in NGN1-infected colonies treated with Delta-Fc (P) (see Table 4 for quantification).

Table 4. MASH1 is more sensitive than NGN1 to post-transcriptional inhibition by Notch signaling in NCSCs

	% Neuron-only clones			% Mixed clones			% Neurons/clone		
	Control	Delta	Fold-R	Control	Delta	Fold-R	Control	Delta	Fold-R
MASH1	19±5	1±0	19	32±4	9±5	3.5	29±6	3±1	9.7
NGN1	49±6	7±7	7	13±4	11±3	NS	54±5	13±3	4.1

NCSCs were infected with MASH1 or NGN1 retroviruses, plated at clonal density and exposed 3-4 hours after plating to 10 nM Delta-Fc in the absence (Control) or presence (Delta) of anti-Fc cross-linking antibody. (Delta-Fc activity is dependent on crosslinking and therefore omission of anti-Fc serves as a control (Morrison et al., 2000). Clones were scored after 4 days as Neuron-only or Neuron+Non-Neuronal (Mixed). Non-neuronal only clones are not listed but represent the difference between the total percentage of the other two clone types and 100%. '% Neurons/clone' indicates the average percentage of neurons per infected (GFP^+) clone. No neurons were observed in uninfected or control GFP-infected clones at this time point (not shown). 'Fold-R' is fold-reduction, calculated as Control/Delta. The data are presented as the mean±s.e.m. and are derived from two independent experiments; at least 200 clones were counted for each condition. The differences between Control- and Delta-treated samples is significant (P<0.02) for all cases except Mixed clones in NGN1-infected cultures (NS, not significant). The difference between MASH1- and NGN1-infected samples is also significant in all categories (P<0.02).

endogenous MASH1 2 days post-infection. Thus, at high concentrations of BMP2, NGNs are converted to inducers of autonomic neurogenesis in dNT cells.

NGNs and MASH1 each promote autonomic neurogenesis in neural crest stem cells

The foregoing data indicated that NGNs can specifically promote sensory neurogenesis in dNT cells at 0-10 ng/ml BMP2, while under these same conditions MASH1 promotes only autonomic neurogenesis. We were therefore interested to know whether NGNs could similarly promote sensory neurogenesis in NCSCs. NCSCs are multipotent self-renewing stem cells isolated from E10.5 neural tube explants that can generate multiple classes of autonomic neurons, glia and smooth muscle cells (Stemple and Anderson, 1992; Shah et al., 1996). MASH1 promotes autonomic neurogenesis in these cells (Lo et al., 1998). However, conditions that promote sensory neurogenesis in dNT cultures (e.g. 10 ng/ml BMP2) do not promote sensory neurogenesis in NCSC cultures (not shown).

When NCSCs were infected with either the NGN1 or MASH1 retroviruses, both proneural genes strongly enhanced generic neuronal differentiation after 4 days, a time at which virtually no neuronal differentiation was observed in control GFP-infected cultures (Fig. 8A-D, arrow and data not shown). When such infected colonies were triple-labeled for GFP, PHOX2B and BRN3A expression, however, in both NGN1- and MASH1-infected colonies, the infected cells only expressed PHOX2B and never BRN3A (Fig. 8K-N). As was the case in dNT cultures, the extent of PHOX2B induction by both bHLH factors was greater than the extent of induction of neurogenesis (49% $PHOX2B^+$ versus 37% $TuJ1^+$ cells for MASH1-infected cultures (n=162 cells in 10 colonies); 70% $PHOX2B^+$ versus 50% $TuJ1^+$ cells for NGN1-infected cultures (n=76 cells in 12 colonies), consistent with previous studies of MASH1 in these cells (Lo et al., 1998). Similar results were obtained in the absence or presence of 10 ng/ml BMP2, or with an NGN2 virus (not shown). Thus, NGNs exclusively promote autonomic neurogenesis in NCSCs, even under culture conditions that favor sensory neurogenesis in dNT cultures.

NGNs and MASH1 exhibit different cell cycle arrest-promoting activities and sensitivities to lateral inhibition in NCSCs

The observation that MASH1 and the NGNs each promote autonomic neurogenesis in NCSCs raised the question of whether these neural bHLH factors exhibit any detectable differences in activity in this stem cell context. We noted a difference in the size and composition of colonies infected with the two proneural genes. NGN-infected colonies tended to be smaller than MASH1-infected colonies by about a factor of three, and contained a higher percentage of neurons, on average (Fig. 8C-F and Table 3). In addition, a higher proportion of NGN-infected colonies consisted only of neurons [49% versus 19% for MASH1; Fig. 8C,E,G; Table 4 (% Neuron-only clones – Control)]. Conversely, the proportion of mixed colonies was much higher in MASH1-infected cultures [Figs 8D,F,H, 9A,B; Table 4 (% Mixed clones, Control)], and the size of such colonies tended to be larger (by ~1.5- to 2-fold) as well. These data suggested that NGNs might promote cell cycle exit and neuronal differentiation more effectively or more rapidly than MASH1, in NCSCs.

To determine whether the smaller size of NGN-infected colonies indeed reflected a difference in proliferation, we performed BrdU-labeling experiments. MASH1-infected colonies pulsed for 24 hours with BrdU 3 days after infection had more than twice as many labeled cells as did NGN1-infected colonies (Table 3; Fig. 9A-D). Interestingly, this difference in BrdU incorporation was smaller when labeling was performed 24 hrs after infection (74±4% versus 64±2% $BrdU^+$ cells in MASH1 versus NGN1-infected colonies, respectively). These data suggest that NGN1-infected NCSCs may differentiate and drop out of division sooner than MASH1-infected NCSCs. Consistent with this, when BrdU labeling was performed 3 days after infection, in both NGN1- and MASH1-infected cultures, most of the $BrdU^+$ cells were undifferentiated non-neuronal cells (Fig. 9B,D,H; arrowhead and data not shown), although there were many more such cells in MASH1-infected cultures. In addition, there was a ninefold higher frequency of $BrdU^+$ neurons in MASH1- than in NGN1-infected colonies (26±6% versus 3±1%, respectively), suggesting that forced expression of MASH1 is more compatible with continued proliferation in differentiating neuroblasts, than is that of NGN1. Little or no apoptotic cell death was detected by DAPI staining, suggesting that differential apoptosis does not contribute to the observed difference in colony size (not shown).

While the average number of neurons per clone was similar in MASH1- and NGN1-infected colonies (2.2±0.5 versus 1.8±0.3, respectively), MASH1-infected colonies contained many more non-neuronal cells (Fig. 9A-D; Table 3). One explanation for this is that MASH1 function might be more susceptible to lateral inhibition mediated by local Notch signaling within such colonies (Kubu et al., 2002). To test this,

we exposed MASH1- and NGN1-infected colonies to an Fc-fusion form of the Notch ligand Delta (Delta-Fc), which has previously been shown to inhibit neurogenesis in NCSCs (Morrison et al., 2000). The ligand was added ~2 hours post-infection to ensure that Notch signaling would be activated before the cells had a chance to differentiate into neurons in response to the bHLH factors.

Strikingly, Delta-Fc inhibited MASH1-induced neurogenesis more strongly than NGN1-induced neurogenesis (Fig. 9I-P; Table 4). The Notch ligand virtually abolished neuron-only colonies in MASH1-infected cultures, whereas it reduced them in NGN1-infected cultures by sevenfold (Table 4). The differential effect of Delta-Fc was also evident in mixed colonies: in MASH1-infected cultures the percentage of such colonies was reduced by 3.5-fold, whereas there was no significant reduction in NGN1-infected cultures (Table 4). Together, these data suggest that MASH1 is more sensitive than NGN1 to Notch-mediated inhibition of neurogenesis in NCSCs.

DISCUSSION

It has been suggested that the NGNs, unlike their *Drosophila* homolog *ato*, do not play a role in specifying neuronal identity in the vertebrate PNS (Hassan and Bellen, 2000). We have directly compared the subtype(s) of peripheral neurons promoted by constitutive expression of NGNs and *Mash1* in vertebrate PNS progenitor cells. In dNT cultures dorsalized by low concentrations of BMP2, NGNs promoted sensory neurogenesis while MASH1 promoted autonomic neurogenesis. Thus, in the appropriate progenitor cell context, the NGNs and *Mash1* each promote distinct neuronal subtype identities. However, in other contexts, both proneural genes promote autonomic differentiation, analagous to the behavior of their *Drosophila* homologs *ac-sc* and *ato* in the fly PNS (Jarman et al., 1993; Jarman and Ahmed, 1998). Our data also reveal differences in Notch sensitivity and cell cycle arrest-promoting activity between these vertebrate proneural genes, which may explain why some neural lineages employ MASH1 and others the NGNs.

The role of NGNs in the specification of sensory neuron identity

The observation that NGNs can promote either sensory or autonomic neurogenesis in dNT cells, depending on the ambient level of BMP2, suggests that these bHLH factors collaborate with other dominant determinants of neuronal identity whose expression is promoted by different BMP2 concentrations (Fig. 10B – ID_{Sn}, ID_{An}, etc.). Thus, BMP2 appears to be the primary determinant of a sensory versus autonomic fate in dNT cells. However, the fact that NGNs can quantitatively enhance the expression of sensory-specific markers over and above their effect to promote neurogenesis, suggests that levels of *Ngn1/2* expression may limit the proportion of dNT cells that can acquire a sensory fate (Fig. 10B). That this effect of NGNs is specific is supported by the fact that MASH1 does not promote a sensory identity under the same conditions. Consistent with this, substitution of the *Mash1*-coding sequence for that of the endogenous *Ngn2* gene fails to rescue sensory neurogenesis in vivo (Parras et al., 2002). Thus, NGNs have a sensory-promoting function that MASH1 lacks.

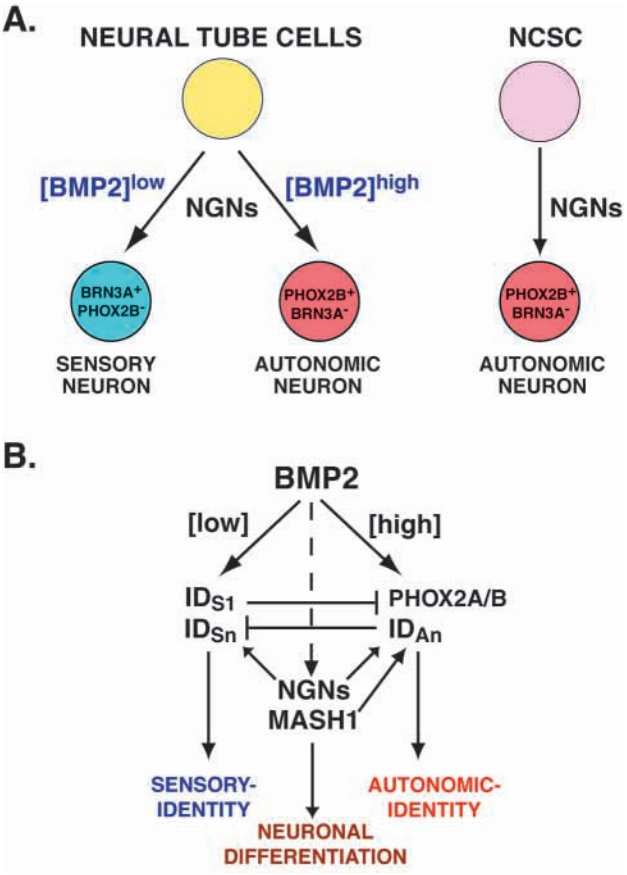


Fig. 10. Role of NGNs in the specification of neuronal identity in the PNS. (A) Summary of the results obtained with NGN1 retrovirus in dNT cells (left) and NCSCs (right). (B) Model to explain results obtained in dNT cultures. $ID_{S1, n}$ indicates postulated sensory neuron identity co-determinants; ID_{An} indicates postulated autonomic identity co-determinants; the former are thought to be induced by low, and the latter by high concentrations of BMP2 (Fig. 3). The existence of such identity co-determinants is inferred from the fact that BMP2 dominantly controls the neuronal subtype promoted by NGNs, depending on its concentration (Fig. 6 and Table 2). The fact that NGNs can significantly enhance either sensory or autonomic differentiation above that in control GFP-infected cells (Fig. 6) implies that NGNs promote either the expression and/or function of the collaborating identity co-determinants. Blunt symmetrical arrows: the reciprocal inhibition between sensory and autonomic identity co-determinants is inferred from the fact that NGN1 promotes sensory and autonomic fates in a mutually exclusive manner at 25 ng/ml (Fig. 7). The induction of NGNs by BMP2 (broken arrow) is inferred from the observation that increasing BMP2 concentrations enhance the expression of endogenous NGN2. MASH1 promotes autonomic but not sensory neurogenesis at all concentrations of BMP2, because it cannot upregulate, or collaborate with, sensory identity co-determinants.

At high concentrations of BMP2, NGNs promoted autonomic and not sensory neurogenesis, while at intermediate BMP2 concentrations *Ngn*-infected cells expressed either sensory or autonomic markers, but never both. The induction of autonomic neurogenesis by NGNs is unlikely to be mediated by endogenous MASH1, as an approximately equal proportion

of NGN-infected cells expressed PHOX2B or BRN3A at 25 ng/ml BMP2 (Table 2) and yet endogenous MASH1 was not expressed in any NGN virus-infected cells at 2 days post-infection. The mutual exclusivity of sensory and autonomic marker expression suggests that there may be a reciprocal inhibition between the sensory and autonomic identity co-determinants with which NGNs interact (Fig. 10B, blunt arrows). Consistent with this notion, in the spinal cord, different homeodomain determinants of neuronal identity expressed in adjacent progenitor domains reciprocally inhibit each other's expression (Muhr et al., 2001).

Specification of autonomic identity

Unlike the NGNs, MASH1 promoted exclusively autonomic neurogenesis at virtually all concentrations of BMP2 tested. Such an observation might imply that MASH1 is sufficient to instruct an autonomic identity. However, MASH1 was unable to promote autonomic neurogenesis in the absence of BMP2, suggesting that it interacts with additional co-determinants of autonomic identity that are induced by BMP2 (Fig. 10B, ID_{An}). The existence of autonomic identity determinants independent of MASH1 is further suggested by the observation that *Ngn2* can promote autonomic neurogenesis in the absence of endogenous *Mash1* function in vivo (Parras et al., 2002).

Strong candidates for such autonomic identity co-determinants are PHOX2A (ARIX – Mouse Genome Informatics) and PHOX2B, paired homeodomain proteins that share identical DNA-binding domains (Pattyn et al., 1997) and that activate autonomic-specific genes in the PNS (Swanson et al., 1997; Lo et al., 1998; Yang et al., 1998; Lo et al., 1999; Stanke et al., 1999). Like *Mash1* (Guillemot et al., 1993) *Phox2b* is essential for autonomic neurogenesis in vivo (Pattyn et al., 1999) but its expression is independent of *Mash1* (Hirsch et al., 1998), and vice-versa (Pattyn et al., 1999). Another candidate for such a co-determinant is the bHLH factor eHAND, which also promotes autonomic neurogenesis when mis-expressed in chick embryos (Howard et al., 2000).

In complementary experiments, Guillemot and colleagues have shown that when *Ngn2* is substituted for the *Mash1*-coding sequence by homologous recombination, it is able to rescue the sympathetic autonomic phenotype of *Mash1*$^{-/-}$ mice, and does not induce sensory-specific markers in sympathetic ganglia (Parras et al., 2002). These results are consistent with our observation that NGNs promote autonomic and not sensory neurogenesis in dNT cultures at high concentrations of BMP2, or in NCSCs, and provide further evidence that this effect is unlikely to be mediated via induction of endogenous MASH1. *Mash1* is first expressed when neural crest cells migrate to the dorsal aorta, which expresses BMPs (Reissman et al., 1996; Shah et al., 1996; Schneider et al., 1999). Our results suggest that *Ngn2* does not induce sensory markers in autonomic ganglia when substituted for *Mash1* (Parras et al., 2002), because the *Mash1* transcriptional control elements constrain its expression to progenitors already restricted to an autonomic fate by autonomic identity co-determinants. However, our results also show that the inability of NGNs to override such autonomic determinants in vivo does not imply that these proneural genes play no role in promoting a sensory identity in the context where they normally function.

Specification of neuronal identities by proneural genes in *Drosophila* and in vertebrates

Comparative GOF studies in the *Drosophila* PNS first established that the proneural genes *achaete-scute* and *atonal* coupled neural precursor selection (analagous to generic neuronal differentiation) to neuronal subtype specification (Jarman et al., 1993; Chien et al., 1996; Jarman and Ahmed, 1998). It has recently been argued that in the vertebrate PNS, these two functions have become uncoupled (Hassan and Bellen, 2000; Brunet and Ghysen, 1999). According to this 'uncoupling' hypothesis, NGNs promote only precursor selection/generic neuronal differentiation and do not determine subtype identity; other, downstream-acting *ato* homologs are postulated to control identity specification (Bermingham et al., 2001; Gowan et al., 2001). Conversely, MASH1 is argued to specify subtype identity but not precursor selection in the PNS. The latter process is postulated to be controlled by other undiscovered proneural genes (Hassan and Bellen, 2000).

The observations presented here do not support this 'uncoupling' hypothesis. In dNT cells dorsalized by low concentrations of BMP2, NGNs and MASH1 each promote both generic neuronal differentiation, and the expression of distinct neuronal subtype-specific markers. These data argue that MASH1 and the NGNs each play a role in both precursor selection and neuronal identity. However, NGNs can promote either sensory or autonomic neurogenesis, depending on cellular context, while MASH1 exclusively promotes autonomic neurogenesis. Similarly, in *Drosophila*, forced expression of *ac-sc* promotes exclusively ES organ formation in the PNS, while forced expression of *ato* can promote either ES organ or CD organ formation, depending on context (Brand et al., 1993; Jarman et al., 1995; Jarman and Ahmed, 1998) (A. Jarman, personal communication). Thus, in the fly and vertebrate PNS, the function of proneural genes in promoting both generic neuronal differentiation and neuronal subtype specification appears to be conserved.

Different features of vertebrate proneural gene function may underlie cellular differences in the neural lineages in which they function

Our data unexpectedly revealed functional differences between MASH1 and NGNs that are unrelated to identity specification. Specifically, we observed a greater sensitivity of MASH1 to the inhibitory effects of NOTCH signaling on neurogenesis, and less rapid promotion of cell cycle withdrawal. Interestingly, as *Mash1* and the NGNs are driven by the same retroviral transcriptional control elements, these differences must reflect post-transcriptional rather than transcriptional mechanisms (Kageyama and Nakanishi, 1997; Artavanis-Tsakonas et al., 1999). Consistent with this notion, in *Xenopus* XASH3, when expressed from micro-injected mRNA, is more sensitive to inhibition by activated Notch than is NeuroD, another *ato*-related gene (Chitnis and Kintner, 1996).

Neural bHLH factors, like their myogenic counterparts (Crescenzi et al., 1990), are known to promote cell cycle-withdrawal in conjunction with differentiation (Farah et al., 2000; Novitch et al., 2001). The difference in proliferation between MASH1- and NGN-infected NCSCs may therefore reflect intrinsic differences in the cell cycle withdrawal-promoting activities of the two bHLH factors. Alternatively, it may be secondary to their different sensitivities to Notch-

mediated inhibition of neurogenesis. As lateral inhibition is known to occur within differentiating NCSC colonies (Kubu et al., 2002), MASH1-infected colonies may contain more proliferating cells than do NGN-infected colonies simply because they are more easily inhibited from undergoing neurogenesis by endogenous Notch signaling. Consistent with this, exogenous activation of Notch signaling by treatment with Delta-Fc more than doubled the average size of NGN1-infected colonies (Fig. 9M,N). However, we also observed that the proportion of $BrdU^+$ neurons was ninefold higher in MASH1-infected than in NGN-infected cultures. These latter data suggest that NGNs may have an intrinsically stronger ability than MASH1 to promote cell cycle withdrawal in differentiating peripheral neuroblasts. Consistent with this idea, there was reduced proliferation in the sympathetic ganglia of mouse embryos in which the *Ngn2*-coding sequence was substituted for that of *Mash1* by homologous recombination (Parras et al., 200).

These observations may explain the paradox of why some neural lineages have evolved to use *Mash1*, when *Ngn2* can functionally substitute for it in vivo (Parras et al., 2002). During normal development, sensory neurons withdraw from the cell cycle and differentiate more rapidly than do sympathetic neurons (Rohrer and Thoenen, 1987). Sympathetic neuroblasts also continue to proliferate for several days after extending processes and expressing neuronal genes (Anderson and Axel, 1986; Rohrer and Thoenen, 1987; DiCicco-Bloom et al., 1990). Similarly, in the forebrain NGNs are used by cortical progenitors (Fode et al., 2000) that exit the cell cycle and differentiate shortly after leaving the ventricular zone (Chen and McConnell, 1995; Noctor et al., 2001), while MASH1 is used by striatal interneuron progenitors (Fode et al., 2000) that migrate to the cortex and olfactory bulb before differentiating (reviewed by Wilson and Rubenstein, 2000). These in vivo observations, and the functional differences we observe between *Mash1* and the NGNs in vitro, suggest that MASH1 is employed in lineages where an extended period of proliferative amplification of undifferentiated neuronal precursors is required, while NGNs are used where more rapid differentiation without intervening proliferation is needed. The mechanistic basis of these functional differences will be interesting to determine.

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