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The Cellular Function of MASH1 in Autonomic Neurogenesis

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Summary

Using primary cultures and immortalized multipotential stem cell lines derived from wild-type and *Mash1* mutant neural crest cells, we have analyzed the cellular function of MASH1 in autonomic neurogenesis. We present evidence for the existence of a precursor expressing MASH1 and neuronal markers such as neurofilament, neuron-specific tubulin, and tetanus toxin receptor. This cell has a nonneuronal morphology. Differentiation of this precursor to neurons that express markers such as SCG10, peripherin, and neuron-specific enolase is dependent upon MASH1 function. These data imply that the differentiation of autonomic neurons from uncommitted neural crest cells occurs in several sequential steps. Moreover, they suggest that MASH1 does not commit multipotent cells to a neural fate, like its Drosophila *achaete-scute* counterparts, but rather promotes the differentiation of a committed neuronal precursor.

Introduction

Relatively little is known about the progression of cellular events that underlies the generation of neurons and glia from their progenitor cells in the developing nervous system. Lineage analysis has revealed that many neural progenitors are multipotent, able to give rise to both neurons and glia, in the CNS and PNS of both vertebrates and invertebrates (Anderson, 1989; McConnell, 1991; Udolph et al., 1993; Condron and Zinn, 1994; Jan and Jan, 1994). However, there is also evidence for the existence of progenitors with more restricted developmental capacities (Duff et al., 1991; Luskin et al., 1993; Lo and Anderson, 1995). These observations have suggested that the generation of cellular diversity during neural development involves a progressive or stepwise restriction in the developmental capacities of progenitor cells, analagous to what is thought to occur during the segregation of hematopoietic lineages (Anderson, 1989; McKay, 1989; Sieber-Blum, 1990; Le Douarin et al., 1991). However, the control of this restriction process remains poorly understood.

In invertebrate systems such as Drosophila melanogaster or Caenorhabditis elegans, it has been possible to identify genes that act at different stages in a neurogenic developmental pathway (Ghysen and Dambly-Chaudiere, 1989; Sternberg et al., 1992). Thus, for example, in Drosophila the initial segregation of a neural precursor from uncommitted neuroectodermal cells is controlled by proneural genes, such as *achaete–scute* (*ac–sc*), while other genes such as *asense* may act at later stages to control the further division and differentiation of the neural precursor (Jan and Jan, 1994). However, the analysis of mutant phenotypes needs to be complemented with mechanistic studies to understand fully the developmental function of a given gene. In Drosophila and C. elegans, such studies have been limited by the lack of cell isolation techniques and in vitro culture systems.

The development of the PNS provides an experimentally accessible model system to dissect the genetics and cell biology of vertebrate neurogenesis. The PNS develops from the neural crest, a transient population of migratory precursor cells that derives from the dorsal margins of the neural tube (Le Douarin, 1982). A number of mutations, both naturally occurring and engineered, affect the development of neural crest derivatives in the PNS (Marusich and Weston, 1991). Moreover, many molecular markers are available to identify neural crest cells and their derivatives at different developmental stages. In addition, culture systems have been established for mammalian (Boisseau and Simonneau, 1989; Smith-Thomas and Fawcett, 1989; Morrison-Graham et al., 1990; Stemple and Anderson, 1992; Ito et al., 1993) as well as avian (Cohen and Konigsberg, 1975) neural crest cells. Together, these advances present the opportunity to investigate the cellular function of genes required for neural crest development.

One gene that is essential for the development of a subset of neural crest derivatives is *Mash1*, a basic–helix-loop-helix (bHLH) transcription factor (Johnson et al., 1992) that is a mammalian homolog of the *ac–sc* genes (Johnson et al., 1990). MASH1 is specifically and transiently expressed in subsets of neuronal precursors, in both the CNS and PNS (Lo et al., 1991; Guillemot and Joyner, 1993). Analysis of homozygous embryos containing a targeted mutation in *Mash1* has revealed that this gene is required for the generation of both peripheral autonomic (sympathetic, parasympathetic, and some enteric) and olfactory sensory neurons (Guillemot et al., 1993). However, this analysis did not define a cellular function for MASH1; thus, MASH1 could function in the commitment of multipotent neural crest cells to a neuronal fate, or it could be required at some stage in the differentiation, proliferation, or survival of developmentally restricted precursors.

Using primary cultures and immortalized cell lines from both wild-type and *Mash1* mutant neural crest, we present evidence for an autonomic neuronal precursor that expresses a number of neuron-specific genes, such as neurofilament and neuron-specific β-tubulin, and that is MASH1 independent. The subsequent differentiation of this precursor to neurons expressing other markers, such as peripherin and SCG10, requires MASH1. Isolation and reculture of these precursors suggest that they are most

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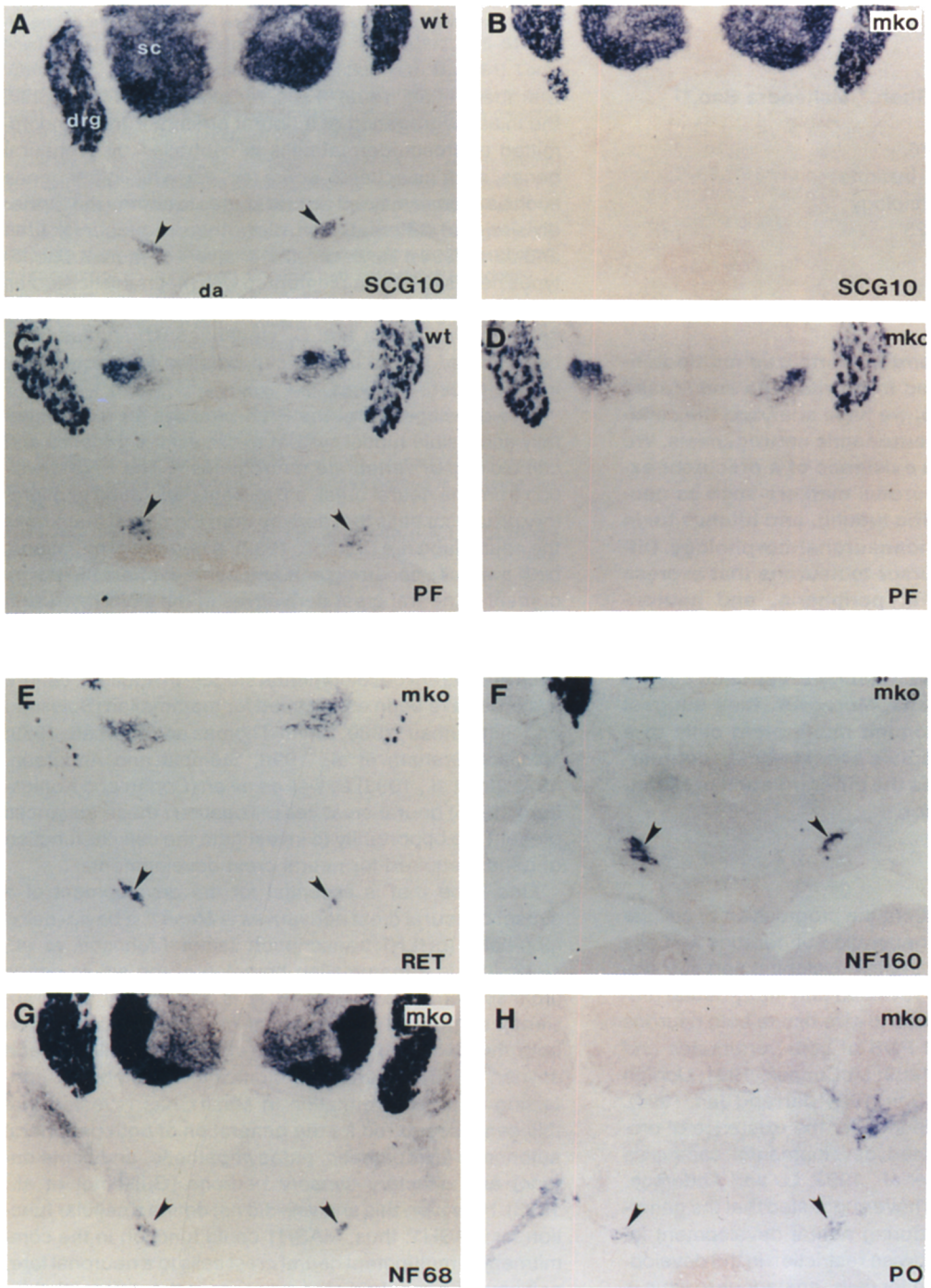

Figure 1. Cells Expressing Some Neuronal Markers and Glia Are Found in the Sympathetic Anlagen of *Mash1*$^{-/-}$ Embryos In Vivo

Near-adjacent serial transverse sections through an anterior region of a wild-type E12 embryo (A and C) and two E12 *Mash1*$^{-/-}$ (mko) embryos (B, D, and E–H) were processed for in situ hybridization, revealing the expression of mRNAs encoding SCG10 (A and B), peripherin (PF; C and D), c-RET (E), NF160 (F), NF68 (G), and P_0 (H). The sympathetic anlagen are marked by arrowheads. sc, spinal cord; drg, dorsal root ganglia; da, dorsal aorta.

likely committed to a neuronal fate. The phenotype of *Mash1* mutant embryos can therefore be explained by the arrest of neuronal development at this precursor stage, in various autonomic ganglia. These results identify both a novel intermediate in the autonomic neuronal differentiation pathway and the developmental step at which MASH1

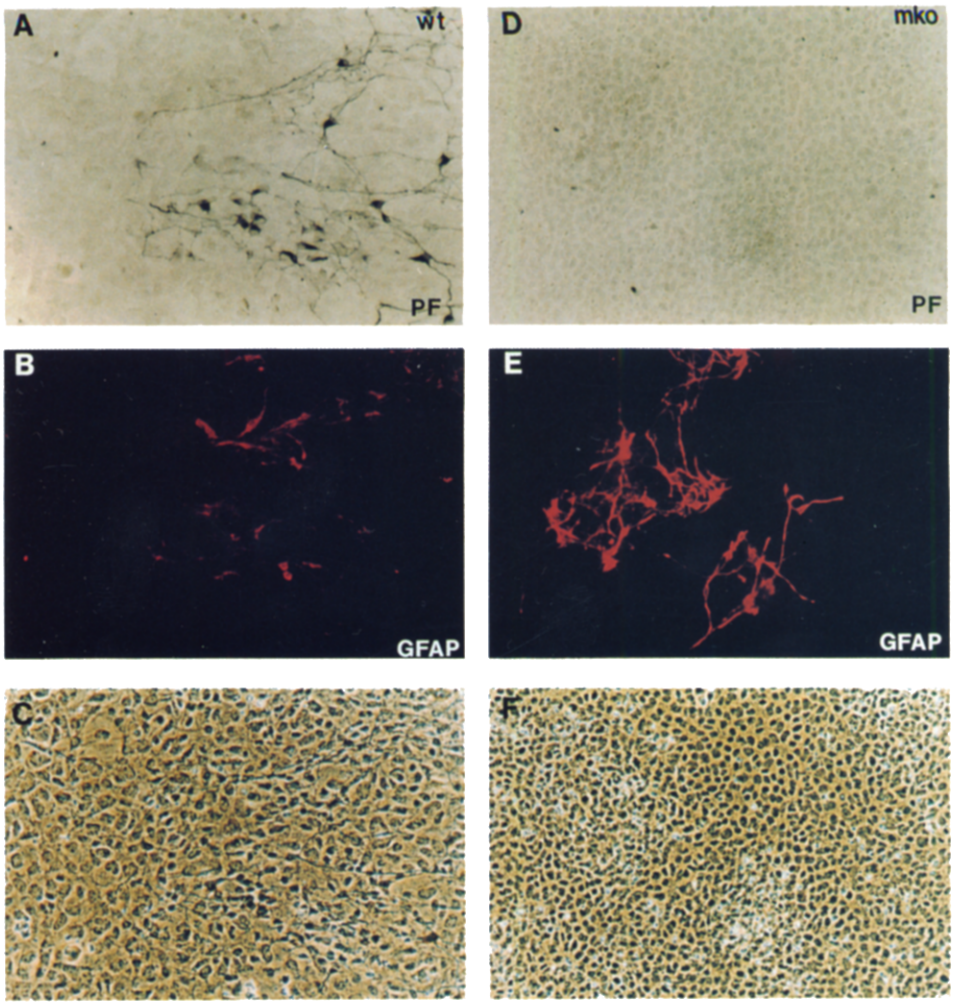

Figure 2. *Mash1*$^{-/-}$ Neural Crest Explants Do Not Produce Neurons but Give Rise to Glial Cells

Neural crest explants grown for 7 days were fixed and labeled with antibodies to peripherin (PF), and staining was visualized using a horseradish peroxidase–conjugated secondary antibody and bright-field optics (A and D). The same explants were then stained with anti-GFAP and visualized using a fluorescent phycoerythrin-conjugated secondary antibody (B and E). (A)–(C) show identical fields of neural crest explants derived from wild-type (wt) mice. (D)–(F) represent identical fields of *Mash1*$^{-/-}$ neural crest explants (mko). (C) and (F) are phase-contrast views. Note the absence of peripherin^{+} neurons in mko explants (D).

function is required. Furthermore, they indicate that the cell biological function of MASH1 is distinct from that of its Drosophila *ac–sc* homologs.

Results

Sympathetic Ganglia of *Mash1* Mutant Mice Express a Subset of Neuronal Markers

Previously, it was shown that neurons fail to develop in the sympathetic ganglia of *Mash1* mutant mice, as demonstrated by markers such as tyrosine hydroxylase, SCG10 (Guillemot et al., 1993; also see Figures 1A and 1B), and the PNS-specific intermediate filament protein peripherin (Figures 1C and 1D). Surprisingly, however, we found that two other neuronal markers, the 160 and 68 kDa subunits of neurofilament (NF160 and NF68, respectively), were expressed in the mutant ganglia (Figures 1F and 1G) at levels similar to those detected in wild-type embryos (data not shown). As shown previously, the mutant ganglia also expressed c-RET, an orphan receptor tyrosine kinase that is expressed by both autonomic neurons and their precursors (Pachnis et al., 1993; Lo and Anderson, 1995). The expression of P_0, a marker of peripheral glial cells, confirmed that glia develop in the mutant sympathetic ganglion anlagen (Figure 1H), as suggested earlier using less specific markers (Guillemot et al., 1993).

The finding that a subset of neuronal markers is expressed in the sympathetic ganglia of *Mash1* mutant mice raised the question of the nature of the cells expressing these markers. The presence of multiple markers could imply the existence of a single cell population coexpressing these markers, or a mixed population of cells each expressing a subset of the markers. The cells could represent neurons of a different lineage; they could represent arrested neuronal precursors; or they could represent an aberrant cell type that forms as a consequence of the loss

of MASH1 function. These possibilities could be distinguished by examining the phenotype of *Mash1* mutant neural crest cells in dissociated cultures.

Mash1 Mutant Neural Crest Cells in Primary Culture Express NF160 but Not Peripherin and Have a Nonneuronal Morphology

We first examined the phenotype of *Mash1* mutant neural crest cells in primary explants that had been allowed to differentiate for several days. Explants from wild-type embryos developed both peripherin$^+$ neurons (Figure 2A) and glia (detected by expression of glial fibrillary acidic protein [GFAP]; Figure 2B). (In addition, the explants contained other nonneuronal cells, including undifferentiated neural crest stem cells [Figures 2C and 2F].) In contrast, explants from *Mash1* mutant embryos failed to develop neurons (Figure 2D) but did develop glia (Figure 2E). This result suggested that the phenotype of *Mash1* null mutants observed in vivo could be reconstituted in vitro.

The absence of any neurons at all in mutant explants was unexpected, because sensory neurons, which also derive from the neural crest, develop in vivo independently of MASH1 function (Guillemot et al., 1993). This observation suggests that the neurons which develop in our cultures under these conditions are exclusively autonomic. The differentiation of sensory neurons in vitro may require the neural tube, which is routinely removed from these explant cultures and has been shown to be a necessary source of differentiation or survival factors for sensory neurons in avian embryos (Kalcheim and Le Douarin, 1986).

Although *Mash1* mutant explants did not contain peripherin$^+$ neurons, they did contain numerous NF160$^+$ cells (data not shown), like autonomic ganglia in mutant embryos. To visualize the morphology of these NF160$^+$ cells more clearly, explants were dissociated and replated at a lower density, then fixed and stained for NF160 together with GFAP. This revealed that the NF160$^+$ cells in mutant cultures did not have a neuronal morphology (Figure 3B, NF160), but rather had a flattened, fibroblast-like morphology and contained bundles of elongated NF160$^+$ filaments (Figure 3B, arrows). Moreover, these NF160$^+$ cells were clearly distinct from the GFAP$^+$ cells observed in the same cultures (Figure 3B, GFAP), indicating that they were not merely glial cells that had begun to express some neuronal properties. Furthermore, closer inspection of wild-type cultures revealed that they, too, contained some NF160$^+$ cells with a nonneuronal morphology (Figure 3A, arrows; and data not shown). This suggested that the NF160$^+$ cells seen in *Mash1* mutant cultures were not simply an aberrant cell type caused by the mutation but rather a normal cell type that forms in wild-type cultures as well.

Together, these data indicated that the explant cultures reconstituted the *Mash1* mutant phenotype of autonomic ganglia in vivo: they contained NF160$^+$ cells and glia but not peripherin$^+$ or SCG10$^+$ (data not shown) neurons. Furthermore, the ability to visualize the morphology of the NF160$^+$ cells clearly in mutant cultures indicates that they are not neurons of another lineage. Such nonneuronal NF160$^+$ cells can be identified in wild-type cultures as well,

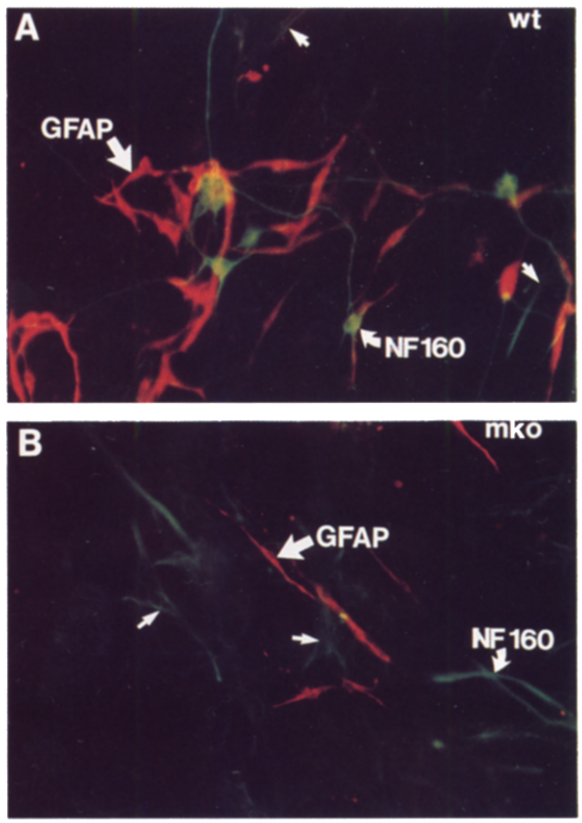

Figure 3. NF160$^+$ Nonneuronal Cells Are Present in *Mash1*$^{-/-}$ Neural Crest Cultures

Neural crest cells from wild-type (A) and *Mash1*$^{-/-}$ (mko; B) explants were dissociated, replated, and allowed to differentiate for another 6 days on poly-D-lysine/fibronectin in differentiation medium. Anti-NF160 and anti-GFAP antibodies followed by secondary antibodies conjugated to fluorescein (for NF160) or phycoerythrin (for GFAP) were used to label neuronal cells and glia, respectively. Note that the NF160$^+$ cells in mko (B) have a flat, nonneuronal morphology. Note also that the NF160$^+$ cells in the mko culture are distinct from the GFAP$^+$ cells. Small arrows in (B) indicate additional NF160$^+$ nonneuronal cells with elongated neurofilament bundles; note that similar cells are present in wild-type cultures (A, small arrows). These cells have a flattened, fibroblast-like morphology in phase-contrast views (not shown).

suggesting that they may be neuronal precursors whose further differentiation is arrested in the absence of MASH1. To test this hypothesis, it was necessary to characterize these cells further and to demonstrate directly their conversion into neurons.

Immortalized Cell Lines from Wild-Type and *Mash1* Mutant Neural Crest Cells Reproduce the Phenotype Seen in Primary Cultures

To manipulate the NF160$^+$ nonneuronal cells, we required greater numbers of cells than are available from primary explants. We therefore took advantage of recently developed methods for immortalizing mouse neural crest stem cells (M. R. et al., unpublished data) and applied them to explants from *Mash1*$^{-/-}$ embryos as well. To identify these cells, we relied on their expression of two antigens, the low affinity nerve growth factor receptor (p75^{LNGFR}) and nestin, previously shown to be expressed by neural crest

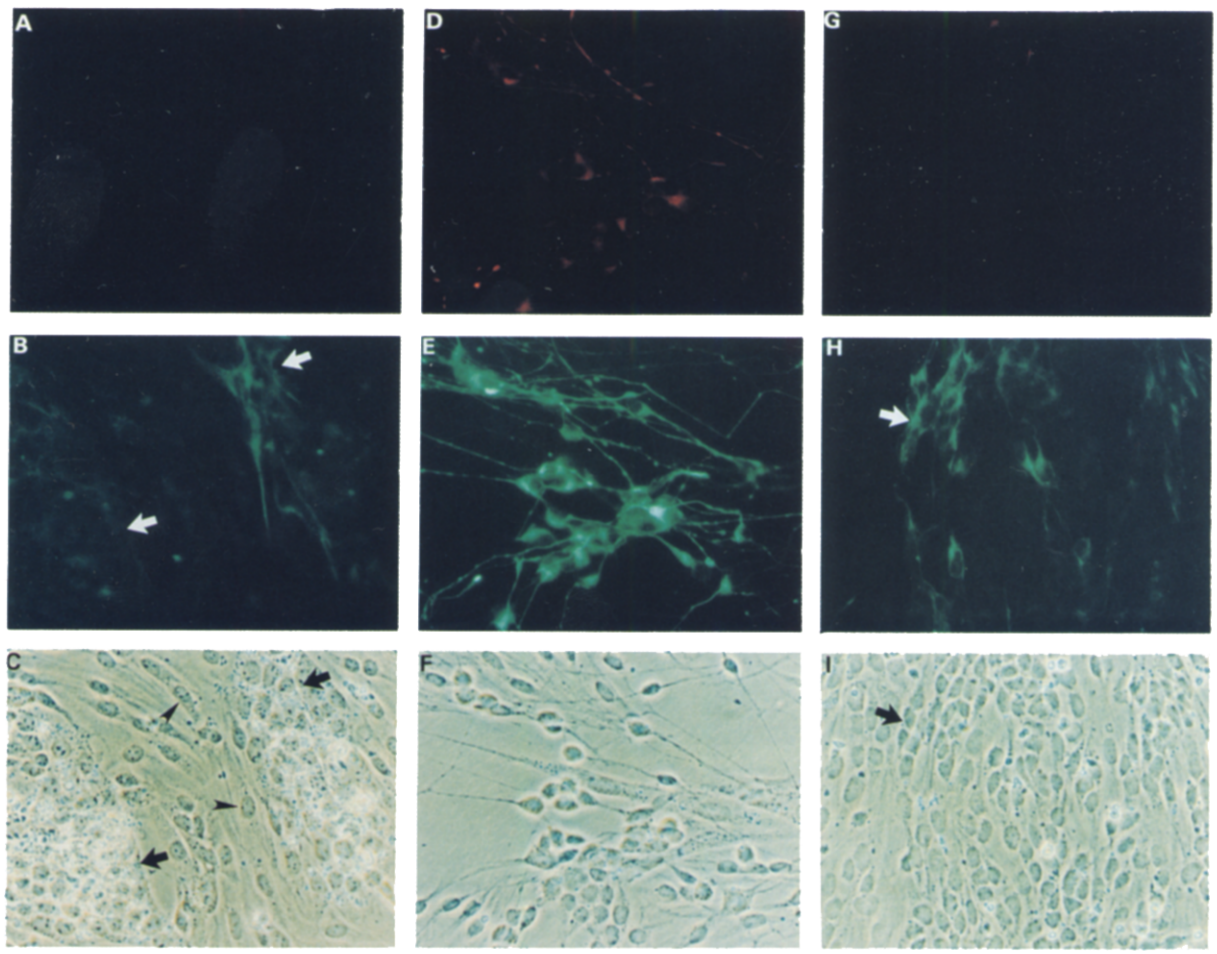

Figure 4. NF160$^+$ Cells in Immortalized Cell Lines from Wild-Type and *Mash1* Mutant Neural Crest Are Similar to Those Observed in Primary Cultures

(A–C) Mko-4 (*Mash1* mutant) cultures differentiated for 5 days were fixed and double labeled for peripherin (A) and NF160 (B). Note that the NF160$^+$ cell clusters (B and C, arrows) are peripherin$^-$ and are interspersed with NF160$^-$ nonneuronal cells (C, arrowheads), many of which are GFAP$^+$ glia (data not shown).

(D–F) Monc-1 (wild-type) cultures differentiated for 5 days. Note the presence of NF160$^+$ cells (E) with a neuronal morphology (F) that coexpress peripherin (D).

(G–I) After 2 days of differentiation, NF160$^+$ (H), peripherin$^+$ (G) cells with a nonneuronal morphology (I) can also be seen in wild-type (Monc-1) cultures.

stem cells in the rat (Stemple and Anderson, 1992), and on their fibroblast-like morphology and lack of expression of lineage markers such as neurofilament and GFAP (M. R., unpublished data). Cells of a similar morphology and antigenic phenotype were identified in *Mash1* knockout neural crest cultures as well (data not shown).

Clonal lines were established from both wild-type and *Mash1* mutant immortalized neural crest cells by infection with a retrovirus harboring v-*myc* (see Experimental Procedures) and are called Monc-1 (Mouse neural crest-1) and Mko-4 (*Mash1* knockout-4), respectively. Monc-1 cells can be propagated under conditions where they remain undifferentiated, or they can be replated under different conditions (see Experimental Procedures) where they differentiate after 5 days to neurons expressing both peripherin (Figure 4D) and NF160 (Figure 4E) as well as glia (data not shown). At 2 days, however, NF160$^+$, peripherin$^-$ nonneuronal cells can be seen in these wild-type cultures (Figures 4G–4I). The fact that these NF160$^+$ nonneuronal cells appear earlier than the neurons is consistent with the idea that they are neuronal precursors.

In contrast to Monc-1 cells, Mko-4 cells plated under differentiation-promoting conditions for 5 days did not generate peripherin$^+$ neurons (Figure 4A) but did produce NF160$^+$ cells (Figure 4B, arrows) that exhibited a nonneuronal morphology (Figure 4C). These cells appeared similar to those that developed after 2 days in wild-type Monc-1 cultures (Figures 4G–4I), providing further evidence that they are not an aberrant phenotype produced by the mutation. The NF160$^+$ cells typically formed dense clusters that were interspersed among nonneuronal cells (Figure 4C, arrowheads). When such cultures were double labeled for NF160 and GFAP, the two cell populations appeared mutually exclusive (data not shown), again indicating that the

Table 1. Expression of Markers in Wild-Type and *Mash1*$^{-/-}$ Immortalized Neural Crest Stem Cell Lines

	Monc-1		Mko-4	
Marker	U	D	U	D
Panneuronal markers				
MASH1-dependent				
SCG10	−	+	−	−
Peripherin	−	+	−	−
NSE	−	+	−	−
MASH1-independent				
NF160	−	+	−	+
NF68	−	+	−	+
N-CAM (5A5)	−	+	−	+
Ttx receptor	−	+	−	+
c-RET	−	+	−	+
Cl. III β-tubulin	−	+	−	+
Stem cell markers				
LNGFR	+	−(−/+)	+	−
Nestin	+	−	+	−

Comparison of marker expression in Monc-1 and Mko-4 as assayed by immunocytochemistry. Positive expression (+) in differentiated Monc-1 indicates marker expression either in neurons (MASH1-dependent markers) or in neurons and neuronal precursors (MASH1-independent markers); positive expression in differentiated Mko-4 refers to expression in precursors only. U, undifferentiated; D, differentiated.

NF160$^+$ cells are not glia that express some neuronal markers. Together, these data indicate that the Monc-1 and Mko-4 cell lines reproduce the phenotypes seen in primary explants of wild-type and *Mash1* mutant neural crest cells, respectively.

We next examined the expression of a panel of additional neuron-specific markers in differentiated Monc-1 and Mko-4 cultures. These markers fell into two classes: one class was expressed in both mutant and wild-type cells; the other was expressed in wild-type cells but not in the mutant. The first class included NF68, neuron-specific β-tubulin, neural cell adhesion molecule (N-CAM), and tetanus toxin receptor (Table 1). In all cases, these markers were expressed in the same morphologically identifiable cell clusters as expressed NF160, as illustrated by tetanus toxin receptor (Figure 5). The second class of markers included peripherin, SCG10, and neuron-specific enolase (Table 1); these markers were not expressed in the clusters in Mko-4 cultures but were expressed by neurons in Monc-1 cultures (data not shown). These data indicate that neuron-specific genes can be divided into two categories according to their dependence on MASH1 function. Moreover, expression of the MASH1-independent subset of genes is apparently insufficient to confer a neuronal morphology.

Coexpression of c-RET and MASH1 in NF160$^+$ Clusters

The foregoing data were consistent with the idea that the NF160$^+$ cells represent precursors that express some panneuronal markers, but whose progression to a fully differentiated neuronal phenotype is dependent upon MASH1. This implies that in wild-type cultures these putative precursors should express MASH1, or at least derive from a MASH1-expressing lineage. To address this issue, double labeling was performed with antibodies to NF160 and MASH1. We found that 100% of NF160$^+$ cell clusters contained MASH1$^+$ cells; conversely, 85% ± 5.7% of MASH1$^+$ cells were associated with NF160$^+$ clusters (n = 2 independent experiments). Moreover, many individual cells coexpressing both MASH1 and NF160 could easily be detected (Figures 6A and 6B), although the high cell density within clusters precluded precise quantitation.

The coexpression of MASH1 and NF160 suggested that the NF160$^+$ cells in mutant cultures should represent precursors that would normally express MASH1. However, it was not possible to demonstrate this directly since the targeted mutation in *Mash1* completely eliminates the coding sequence. We therefore needed an independent marker to link the NF160$^+$ cells in mutant cultures to the MASH1$^+$, NF160$^+$ cells in wild-type cultures. This marker was provided by c-RET. Unlike NF160, N-CAM, and tetanus toxin receptor, which are panneuronal markers, c-RET expression is tightly associated with that of MASH1 (Lo et al., 1994). For example, in a population of c-RET$^+$ autonomic precursors isolated by fluorescence-activated cell sorting (FACS) from embryonic gut, 87% of the cells expressed MASH1 (Lo and Anderson, 1995). The only other cells in the PNS that express c-RET are a small subset of postmitotic sensory neurons (Pachnis et al., 1993); however, since sensory neurons are not generated under the culture conditions used here (as mentioned earlier), c-RET provides a marker of MASH1-expressing autonomic precursors. Importantly, in *Mash1*$^{-/-}$ mutant embryos, c-RET$^+$ cells are still found in the sympathetic ganglia (see Figure 1E), indicating that c-RET expression is not dependent upon MASH1.

In wild-type cultures, c-RET-immunoreactive cells coexpressed both MASH1 (Figure 6D) and NF160 (Figure 6F). Of the c-RET$^+$ clusters, 99.2% ± 0.8% were associated with MASH1$^+$ cells (n = 2 independent experiments). All NF160$^+$ clusters (100%) contained c-RET$^+$ cells, while 74% of c-RET$^+$ cells were associated with NF160$^+$ clusters. (That some c-RET$^+$ cells do not express NF160 most likely reflects the fact that the former marker is first expressed before the latter [Lo et al., 1994].) Similarly, in mutant Mko-4 cultures, 98% of NF160$^+$ clusters contained c-RET$^+$ cells, while 76% of c-RET$^+$ cells were associated with NF160$^+$ clusters (Figures 6G and 6H). Individual cells coexpressing c-RET and NF160 could be seen in both wild-type and mutant clusters (Figures 6F and 6H, arrowheads), although the fact that both antigens are cytoplasmic obscures this coexpression in many cases. The expression of c-RET by NF160$^+$ cells in mutant cultures suggests that these cells correspond to wild-type precursors that would normally express MASH1.

Neurotag$^+$ Cell Clusters Contain Neuronal Precursors

If, as suggested by the foregoing data, the NF160$^+$ nonneuronal cells in mutant cultures represented arrested neuronal precursors, their counterparts in wild-type cultures should be capable of neuronal differentiation. To test this idea, we used the tetanus toxin receptor as a surface

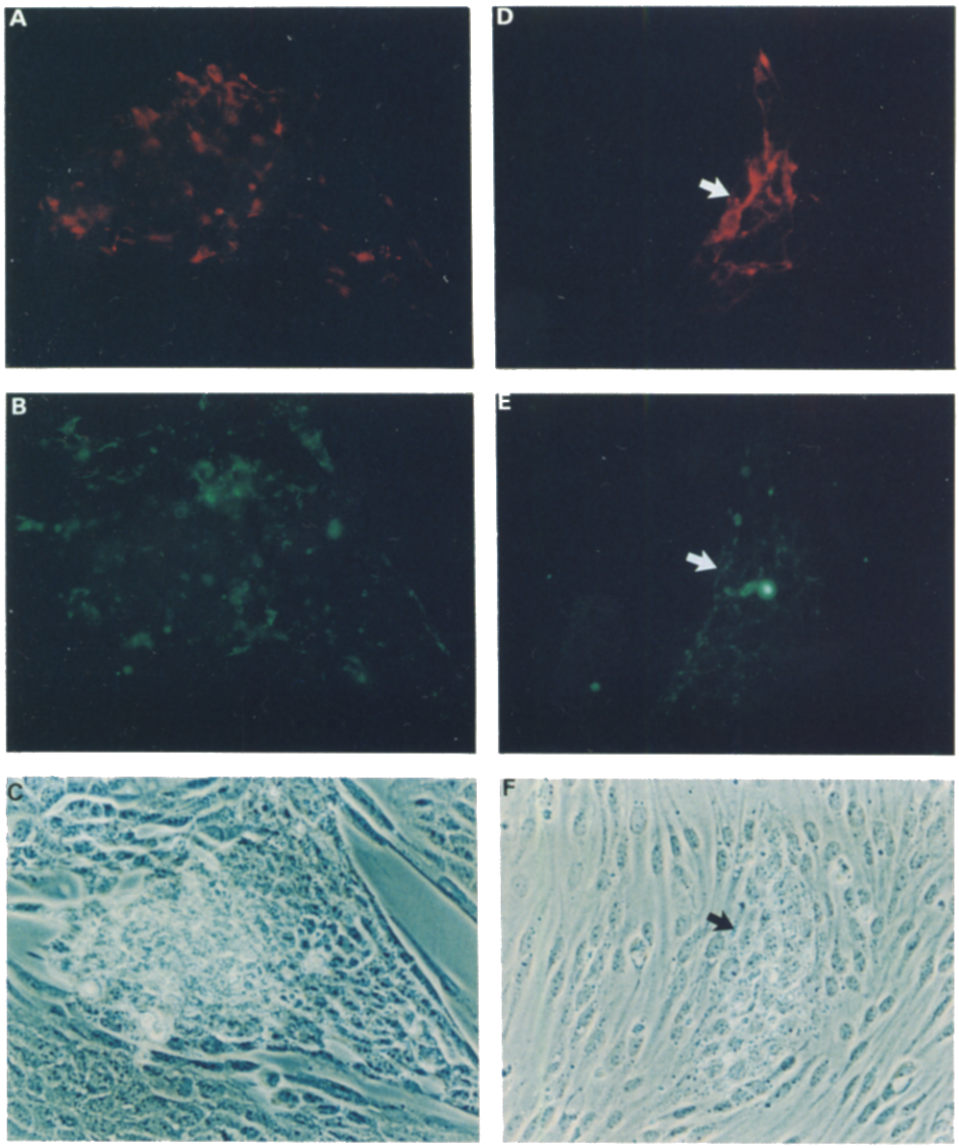

Figure 5. NF160$^+$ Clusters in Differentiated Monc-1 and Mko-4 Cultures Express Tetanus Toxin Receptor

Monc-1 (A–C) and Mko-4 (D–F) cells were differentiated for 2 days, and living cells were labeled with Neurotag and FITC-conjugated tetanus toxoid derivative (B and E). Cells were then fixed and double labeled for NF160 (A and D). Note that most of the Neurotag$^+$ cells in clusters of Monc-1 as well as Mko-4 (arrow) coexpress NF160.

marker to live-label these cells and follow their development into neurons. Receptor-bearing cells can be labeled using a fluorescein isothiocyanate (FITC)–conjugated derivative of tetanus toxoid (Raju and Dahl, 1982) called Neurotag. As mentioned above, Neurotag$^+$ cells colocalized with NF160$^+$ cells in both mutant and wild-type cultures (see Figures 5A–5D). When Neurotag$^+$ clusters in wild-type (Monc-1) cultures were followed, they generated neurons as well as dead cells (detected by propidium iodide staining; data not shown). In contrast, in mutant (Mko-4) cultures, Neurotag$^+$ clusters never generated neurons; rather, many of the cells died. These observations indicated that Neurotag$^+$ cells are unable to generate neurons in mutant cultures. However, owing to the cell death observed, it was not possible to conclude unequivocally by this in situ analysis that Neurotag$^+$ cells are neuronal precursors in wild-type cultures. To do this, it was necessary to isolate the Neurotag$^+$ population from wild-type cultures and follow the fate of individual cells.

Isolated Neurotag$^+$ Cells Are NF160$^+$ Neuronal Precursors

When Monc-1 cultures were dissociated to single cells 2 days after being placed under differentiation-promoting conditions (see Figures 4G and 4H), 95% (n = 302 cells counted) of the NF160$^+$ cells were Neurotag$^+$; furthermore, 78% of the NF160$^+$ cells coexpressed MASH1. This indicated that Neurotag labels virtually all NF160$^+$ cells, including the MASH1$^+$ cells. When Neurotag$^+$ cells were isolated by FACS, 73.2% of the cells were NF160$^+$ immedi-

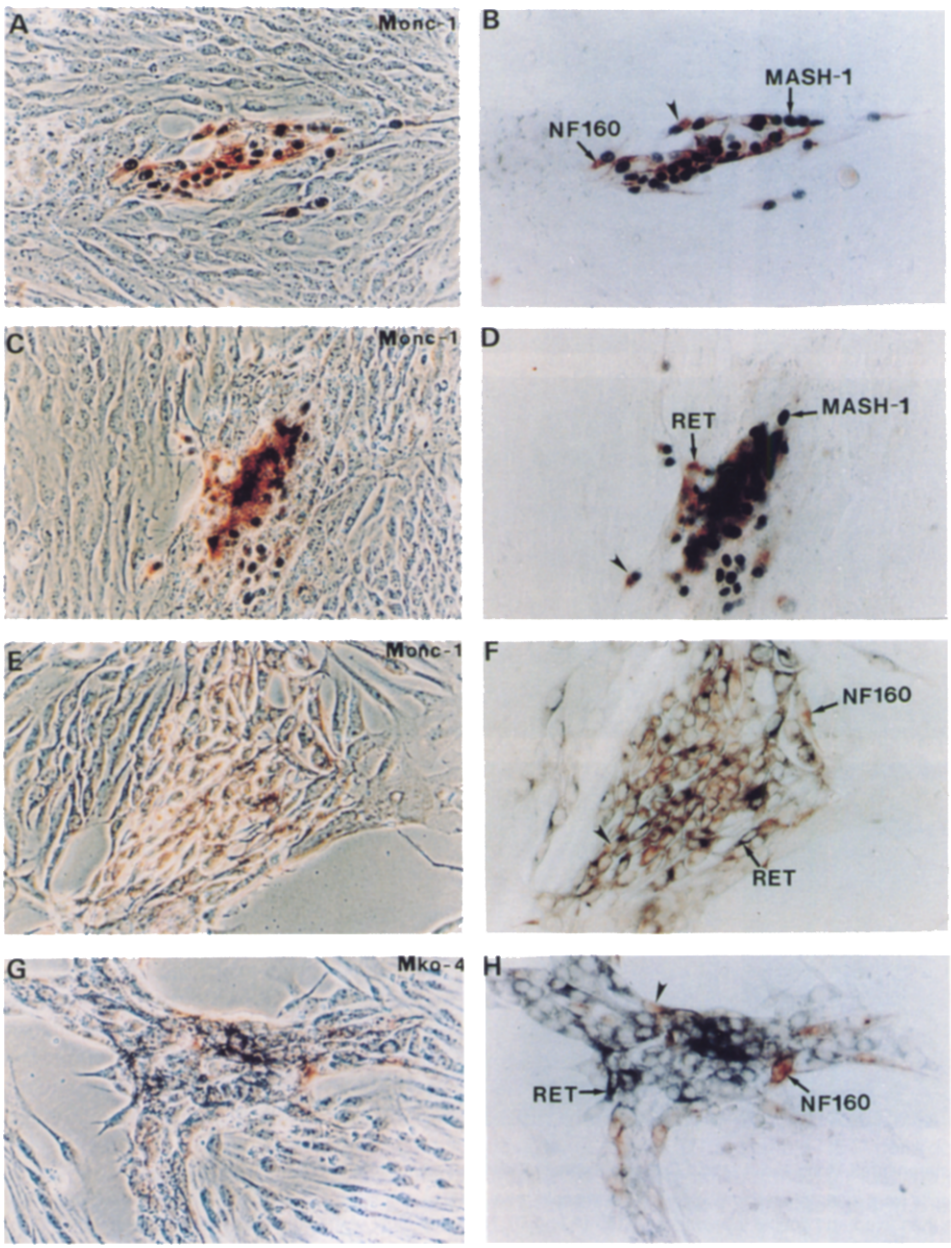

Figure 6. Colocalization of MASH1 and c-RET in NF160$^+$ Cell Clusters

Monc-1 (A–F) and Mko-4 (G and H) cells were differentiated for 4 days and then double labeled for NF160 and MASH1 (A and B), c-RET and MASH1 (C and D), or c-RET and NF160 (E–H). In (A), (B), and (E)–(H), the NF160 staining is visualized by a red precipitate, while the counterstain (c-RET or MASH1) is purple. In (C) and (D), the c-RET staining is red, and MASH1 is purple. Arrowheads in (B), (D), (F), and (H) indicate examples of double-labeled cells. (A), (C), (E), and (G) represent phase-contrast micrographs of the bright-field views shown in (B), (D), (F), and (H), respectively. Note that NF160$^+$ cell clusters coexpress c-RET in both Monc-1 (F) and Mko-4 (H) cells. Double labeling of individual cells is obscured by the fact that both markers are cytoplasmic, although in some cases a perinuclear accumulation of c-RET staining can be seen on a more diffuse background of NF160 staining (F and H, arrows).

ately after sorting, while only 25.5% of these cells were peripherin$^+$ (average of 3 independent experiments). This indicates that the Neurotag-isolated cells are enriched for the NF160$^+$, peripherin$^-$ cells seen in clusters in the mass cultures. The small percentage of the cells that are NF160$^-$ may represent the occasional Neurotag$^+$ cells that lie outside of the NF160$^+$ clusters (see Figures 5A and 5B; and data not shown).

To maintain the viability of FACS-purified Neurotag$^+$ cells, it was necessary to reculture them on a monolayer of unlabeled, differentiated Monc-1 cells. To distinguish the isolated Neurotag$^+$ cells from the bulk Monc-1 population, the cells were labeled with the lipid-soluble dye PKH26 prior to Neurotag labeling and cell sorting. When isolated double-labeled cells (Figure 7A) were cultured on Monc-1 feeder layers and then fixed and stained for periph-

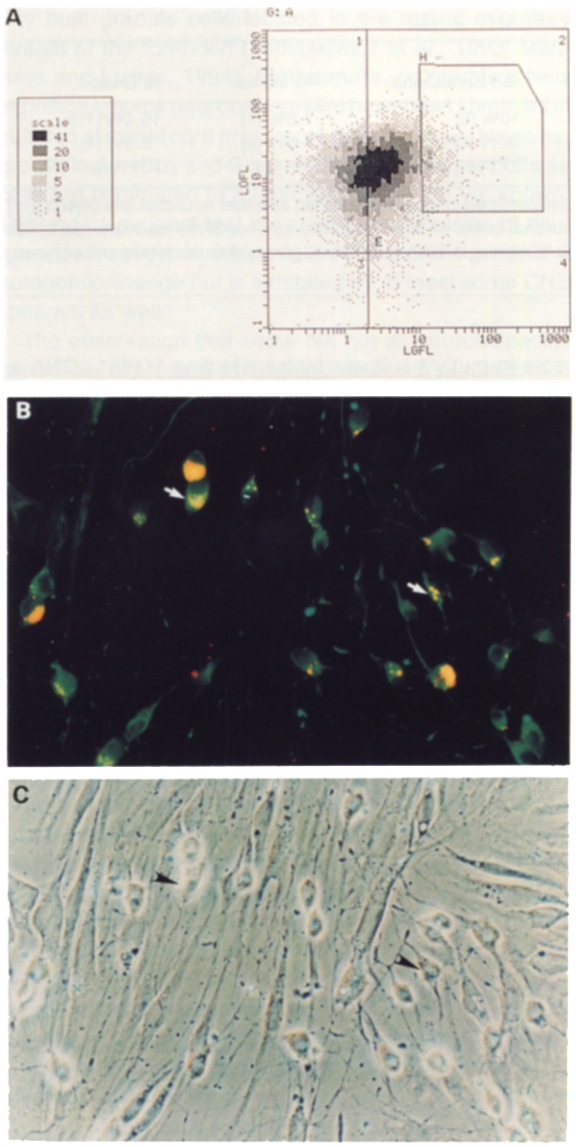

Figure 7. The NF160$^+$, Peripherin$^-$ Cell Type in Monc-1 Is a Neuronal Progenitor

Monc-1 cells were differentiated for 2 days as described before and labeled with Neurotag and the fluorescent dye PKH26 (see Experimental Procedures). Neurotag$^+$, PKH26$^+$ cells were isolated by FACS and plated onto unlabeled Monc-1 cells in differentiation-promoting conditions. The cultures were then allowed to differentiate for 5 days and labeled with anti-peripherin antibody. A typical FACS profile is shown in (A). Neurotag$^+$, PKH26$^+$ cells (about 5% of input cells) were collected from gate H. (B) shows a double exposure of peripherin$^+$ (green), PKH26$^+$ (orange) neurons (arrows) that differentiated from the sorted cells. (C) represents the phase-contrast view of the micrograph shown in (B).

erin after several days, many of the PKH26$^+$ cells had differentiated into peripherin$^+$ neurons (Figures 7B and 7C, arrows). These data suggested that the isolated Neurotag$^+$ cell population indeed contains neuronal precursors.

To assess quantitatively the differentiation capacity of the isolated Neurotag$^+$ population, a clonal analysis was performed (see Experimental Procedures). This analysis indicated that many of the Neurotag$^+$ cells divided to generate clones of peripherin$^+$ neurons similar to those found in unsorted Monc-1 cultures (36.8% ± 3%; Table 2). Since 73% of the cells were NF160$^-$ and only 25% were peripherin$^+$ immediately after sorting (see above), at least some of the Neurotag$^+$ cells that generated peripherin$^+$ neurons must have been initially NF160$^+$ and peripherin$^-$. Moreover, the value of 36.8% is likely to be an underestimate, because a portion of the Neurotag$^+$ cells generated clusters that contained peripherin$^+$ cells, but in which individual PKH26$^+$, peripherin$^+$ cells could not be distinguished due to high cell density. These cluster-forming cells were therefore assigned to a different category (27.3% ± 10%; Table 2, clusters). However, if these two categories are combined, 64% of the isolated cells adopted a neuronal fate.

Some of the Neurotag$^+$ cells (21.8% ± 13.6%; n = 3 experiments; Table 2) gave rise to cells with a nonneuronal morphology; these may have been glia or glial precursors. Such nonneuronal cells may derive from the subset of Neurotag$^+$ cells that were NF160$^-$ at the time of plating (see above). More importantly, however, such nonneuronal cells were rarely if ever found in clones that also contained neurons (Table 2, mixed). Since the culture conditions are permissive for both neuronal and glial differentiation, and yet most Neurotag$^+$ cells appear to give rise exclusively to neuronal progeny, the data suggest that these NF160$^+$ cells are likely committed to a neuronal fate.

Discussion

The process whereby multipotent neural stem cells generate their various differentiated derivatives remains poorly understood. We have taken a combined genetic and cell biological approach to analyze the cellular function of MASH1, a gene essential for the differentiation of autonomic neurons from uncommitted neural crest cells. We have shown that autonomic neurogenesis proceeds via a precursor that expresses a subset of neuronal genes and whose further differentiation requires MASH1 (Figure 8). This finding supports the idea that the genesis of autonomic neurons from undifferentiated neural crest cells occurs in several sequential steps. The behavior of isolated neuronal precursors indicates that they are most likely committed to a neuronal fate. This in turn suggests that MASH1 does not restrict multipotent cells to a neuronal fate but rather promotes the differentiation of committed neuronal precursors, perhaps analagous to the role of the myogenic bHLH protein myogenin in muscle development (Weintraub et al., 1991).

In Vitro Reconstitution of Wild-Type and Mutant Neural Crest Development

The in vitro reconstitution of neural crest cell differentiation from wild-type and *Mash1* mutant embryos has allowed us to infer a cellular function for MASH1 in a way that would not have been possible simply from an analysis of the *Mash1* mutant phenotype in vivo. Specifically, the identification of NF160$^+$ and c-RET$^+$ cells in sections of mutant embryos could not determine whether these markers were expressed in the same cells or in different cells

Table 2. Clonal Analysis of Neurotag$^+$ Cells

	# Clones Examined	% PF$^+$ Neurons	% in PF$^+$ Clusters	% Nonneuronal	% Mixed	% Dead
Experiment 1	61	36% (22)	36% (22)	10% (6)	1.6% (1)	16.4% (10)
Experiment 2	43	39.5% (17)	18.6% (8)	28% (12)	4.6% (2)	9.3% (4)
Experiment 3	40	35% (14)	27.5% (11)	27.5% (11)	0% (0)	10% (4)

Clonal analysis of FACS-isolated PHK26$^+$, Neurotag$^+$ cells. Clones derived from identified cells were classified as either neuronal (peripherin [PF]$^+$, associated with neuronal clusters, nonneuronal, mixed (neuronal plus nonneuronal cells), or dead. The total number of clones examined in each experiment is given in the first column. In the other columns, the percentage of clones in each category is given, with the total number listed in parentheses.

or whether they represented neurons of a different lineage, an arrested neuronal precursor, or an aberrant cell type that forms as a consequence of the mutation. The in vitro analysis has allowed us to determine that NF160 and c-RET define a single cell population rather than two distinct cell types; that these NF160$^+$, c-RET$^+$ cells are not neurons of a different lineage, but rather cells that have an undifferentiated, nonneuronal morphology; that cells with this morphology and antigenic phenotype are also found in wild-type cultures, indicating that the cells in mutant cultures are not an aberrant phenotype caused by the loss of MASH1 function; and that the NF160$^+$ nonneuronal cells in wild-type cultures are in fact proliferating, autonomic neuronal precursors.

We attempted to rescue the arrested neuronal precursors in mutant cultures by transfecting *Mash1* cDNA expression constructs into Mko-4 cells. Although transfected cells expressing exogenous MASH1 in their nuclei could clearly be identified by antibody staining, none of these cells differentiated to neurons (unpublished data). There are a number of possible explanations for this failure to rescue the *Mash1* mutant phenotype in vitro, including the level, timing, and duration of MASH1 expression, the presence of inhibitors, and the need for cofactors; these are currently being investigated.

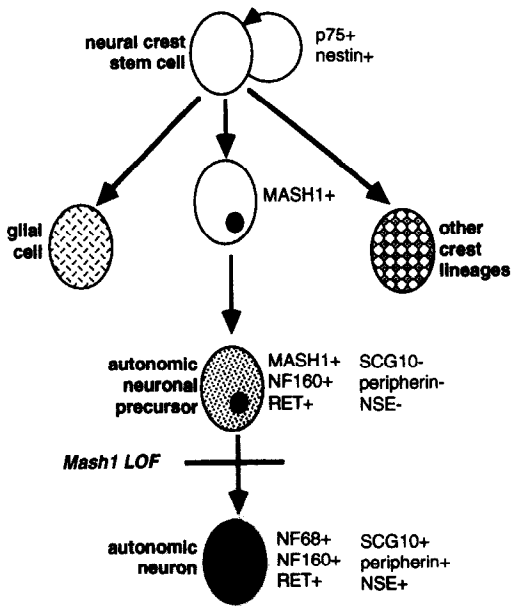

Figure 8. Model Representing the Function of MASH1 in the Context of Trunk Neural Crest Differentiation

Neural crest stem cells give rise to glial, sensory, and autonomic neuronal sublineages. Initially, these cells give rise to cells that express MASH1, but appear otherwise undifferentiated (N. S., L. S., and D. J. A., unpublished data). Such cells may retain some multipotency (Lo and Anderson, 1995). Subsequently, MASH1$^+$ cells express a subset of neuronal markers, including NF160, in a MASH1-independent manner. Cells expressing these markers appear committed to a neuronal fate. The progression of this precursor to a fully differentiated neuron expressing additional neuronal markers (e.g., SCG10, peripherin, and neuron-specific enolase) requires MASH1.

Our data indicate that the NF160$^+$ cells seen in the mutant are arrested neuronal precursors that eventually die. This conclusion is based on a combination of in vivo and in vitro data using both normal and immortalized neural crest cells. The ability to immortalize neural crest cells permitted us to demonstrate directly a conversion of wild-type NF160$^+$ precursors to neurons, something that would not have been possible with primary cultures alone, owing to the limited number of cells available. Furthermore, the identification of NF160$^+$, peripherin$^-$ cells in nonimmortalized primary crest cultures indicates that these precursors are not an artifact of immortalization. Finally, the detection of cells expressing NF160 but not peripherin in sections of *Mash1*$^{-/-}$ embryos indicates that the NF160$^+$, peripherin$^-$ cells seen in vitro are not a culture artifact. Thus, the in vivo and in vitro approaches we have employed complement each other and are internally consistent.

Two Classes of Neuron-Specific Genes Can Be Distinguished by Their Dependence on MASH1 Function

A surprising result from this study was that the products of neuron-specific genes could be divided into two categories according to their dependence on MASH1 function. Those in the first category, including NF68, NF160, N-CAM, neuron-specific β-tubulin, c-RET, and tetanus toxin receptor, were expressed in *Mash1* mutant cells and in wild-type neuronal precursors; those in the second category, including peripherin, SCG10, and neuron-specific enolase, were expressed only in differentiated neurons. This result is unexpected because NF68, NF160, and neuron-specific β-tubulin have generally been considered to be markers of terminally differentiated neurons. The results presented here indicate that, at least in the autonomic lineage, these proteins are already expressed in proliferating neuronal precursors. Recent studies have demonstrated β-tubulin and N-CAM expression in proliferating precursors of olfac-

tory bulb granule cells located in the rostral migratory stream of the forebrain (Tomasiewicz et al., 1993; Menezes and Luskin, 1994). Furthermore, proliferative cells expressing some neuronal markers have been identified in cultures of spinal cord precursors grown in basic fibroblast growth factor (Ray and Gage, 1994). Whether or not these cells are committed to a neuronal fate is not yet known. These data suggest that the stepwise expression of neuron-specific genes is not a unique feature of the peripheral autonomic lineage but is exhibited by at least some CNS lineages as well.

The observation that some but not all neuron-specific genes are expressed in arrested neuronal precursors in *Mash1* mutants implies that the expression of the genes that define a neuronal phenotype is not controlled by a single genetic program, but rather by a series of "subprograms." These subprograms could run either in series or in parallel. We favor the former simply because of the fact that expression of the MASH1-independent genes precedes that of the MASH1-dependent genes in wild-type cultures. However, this does not imply that the two subprograms are obligatorily coupled. In other experiments, we have provided evidence that subprograms controlling the expression of neurotransmitter-synthesizing enzymes and of some neuronal genes (such as SCG10) can be experimentally uncoupled by manipulation of the neural crest cells' environment (Groves et al., 1995). If the expression of the final neuronal phenotype in a given lineage indeed reflects the operation of different subprograms that are controlled by different environmental signals, it could provide a way to generate cellular diversity in both development and evolution, by using different combinations of subprograms to generate related but distinct cellular phenotypes, in different lineages or in different organisms.

MASH1 and Neural Crest Cell Lineage Segregation

In the present study, we have shown that MASH1 function is required in a committed NF160$^+$ neuronal precursor derived from migratory trunk neural crest cells in vitro. Previously, we have identified committed neuronal or neuroendocrine progenitor cells in two different populations of postmigratory neural crest–derived cells in vivo. One progenitor, called NP, gives rise only to neurons and has been identified in a population of c-RET$^+$ cells isolated from the E14.5 gut (Lo and Anderson, 1995). Another progenitor, called the SA progenitor, gives rise to both sympathetic neurons and chromaffin cells and has been isolated using several different antibodies from E14.5 adrenal glands (Michelsohn and Anderson, 1992) or sympathetic ganglia (Carnahan and Patterson, 1991). The relationship between these various progenitor cell types is not yet clear because they have been isolated from different tissues with different antibodies and, in some cases, cultured under different conditions. Moreover, progenitors in the gut derive from the vagal neural crest, whereas the SA lineage derives from the trunk neural crest. Nevertheless, all three progenitors represent lineages that require MASH1 function in vivo. A simple interpretation is that MASH1 function is required at a similar stage of neurogenesis in several distinct autonomic sublineages. Further studies will be required to determine whether the progenitors representing these sublineages are interconvertible or committed to producing different types of neurons.

In addition to its expression in committed neuronal precursors, MASH1 appears to be expressed in more primitive progenitors as well. In trunk neural crest primary cultures, for example, expression of MASH1 is first detected prior to the onset of NF160 expression, in morphologically undifferentiated cells expressing nestin and p75 (N. S., L. S., and D. J. A., unpublished data). Similarly, MASH1 is expressed in most of the the c-RET$^+$ cells isolated from fetal gut, and this population contains some multipotent cells (proNPs) as well as the committed NP cells mentioned above (Lo and Anderson, 1995). Together, these data indicate that MASH1 can be expressed by cells at several different stages of lineage commitment. However, our data demonstrate an essential function for this gene only in committed neuronal precursors. The apparent lack of a requirement for MASH1 in more primitive cells may reflect the presence of other, functionally redundant bHLH genes or simply the fact that the protein begins to accumulate to detectable levels before it actually carries out its requisite function.

Evolution of *ac-sc* Gene Function

The sequence of the MASH1 bHLH domain is highly related to those of the *ac–sc* complex genes in Drosophila (Johnson et al., 1990). Moreover, the expression of MASH1, like that of AC–SC, is restricted to the developing nervous system, where it appears transiently in subsets of precursor cells (Lo et al., 1991; Guillemot and Joyner, 1993). This parallel evolutionary conservation of amino acid sequence and cell type specificity of expression suggested an evolutionary conservation of function as well, a conclusion supported by the fact that the *Mash1* knockout prevents the development of specific subsets of neurons (Guillemot et al., 1993). However, the cellular analysis of the *Mash1* mutant phenotype presented here suggests that this apparent conservation obscures a difference in the cell biological functions controlled by *Mash1* and the *ac–sc* genes.

Mutations in *ac* and *sc* prevent the initial generation of the sensory mother cell, a multipotent progenitor of neurons and several different nonneuronal cell types (Ghysen and O'Kane, 1989). By contrast, the present studies indicate that MASH1 acts after the segregation of neuronal and glial lineages, to control the differentiation of a precursor that is likely committed to a neuronal fate. The *ac–sc* complex contains another bHLH gene related to *ac* and *sc*, called *asense*, that appears to be expressed immediately after the sensory mother cell has formed and therefore may act downstream of *ac–sc* (Brand et al., 1993; Jarman et al., 1993). However, the cells in which *asense* is expressed are still multipotent, so this Drosophila gene may still act at a comparatively earlier stage in its lineage than does MASH1. These data suggest that MASH1 exerts a different cell biological function than do its Drosophila *as–sc* homologs (Ghysen and Dambly-Chaudiere, 1989), de-

spite its similarities in sequence and expression. This difference is not due to intrinsic structural features of *Mash1*, however, as this mammalian gene efficiently complements the *ac–sc* mutations in Drosophila (A. Singson, J. Posakony, and D. J. A., unpublished data).

Together, these results indicate that, although *Mash1* and *ac-sc* perform different cellular functions, these genes are functionally interchangeable. This implies that cellular context may determine the biological roles played by different bHLH proteins, as much as primary structure. This conclusion is underscored by the fact that MASH2, which is 95% identical to MASH1 (Johnson et al., 1990), controls the development of extraembryonic membranes (Guillemot et al., 1994). Moreover, MASH2 complements *ac–sc* mutations in Drosophila as efficiently as MASH1 (A. Singson, unpublished data). The evolutionary conservation of AC–SC and MASH1 amino acid sequence must bear some relationship to the fact that these genes both function during neurogenesis. However, the cellular functions of these bHLH genes may have diverged as a consequence of evolutionary changes in the regulatory sequences controlling the time and place of their expression.

Upstream and Downstream of MASH1

In both Drosophila neurogenesis and mammalian myogenesis, bHLH genes act in cascades (Jan and Jan, 1993). It is therefore likely that there are additional bHLH genes acting in neural crest development, both earlier and later than MASH1. A novel bHLH gene, *eHAND/Th1* (Cserjesi et al., 1995; Hollenberg et al., 1995), is expressed in the same autonomic lineage as *Mash1*. Preliminary data indicate that *eHAND/Th1* mRNA is not expressed in *Mash1*$^{-/-}$ embryos, suggesting that it functions downstream of MASH1 (L. S., P. Cserjesi, E. N. Olson, and D. J. A., unpublished data). It is also likely that there are other bHLH genes that act earlier than MASH1 and function more analagously to *ac–sc* in Drosophila. However, such genes have yet to be identified in vertebrates. Extensive searches in mammals have failed to identify additional AC–SC homologs besides MASH1 and MASH2 (K. Zimmerman, J. E. Johnson, and D. J. A., unpublished data). XASH-3, another AC–SC homolog identified in Xenopus (Zimmerman et al., 1993; Turner and Weintraub, 1994), is expressed earlier than XASH-1, the Xenopus MASH1 homolog (Ferreiro et al., 1992), but searches for mammalian XASH-3 homologs have thus far been unsuccessful (K. Zimmerman and D. J. A., unpublished data). The availability of clonal cell lines blocked at the step before MASH1 function is required may facilitate the identification of novel bHLH genes that act at earlier stages in neural crest development.

Experimental Procedures

Culture of Primary Mouse Neural Crest Cells

Timed pregnant mice with a mixed C57Bl/6J × 129/Sv//Ev background carrying a null allele in the *Mash1* locus were obtained from the breeding facility at the California Institute of Technology. Mouse neural crest cells were isolated from gestational day 9 (E9) embryos and cultured essentially as previously described for rat neural crest cell cultures (Stemple and Anderson, 1992); details of minor modifications are available upon request. Secondary cultures were replated at a density of about 4000 cells per 35 mm dish (Stemple and Anderson, 1992) and differentiated in standard medium supplemented with 10% fetal bovine serum (FBS) and 5 μM forskolin (differentiation medium).

Genotyping Mice with a Targeted Null Mutation in the *Mash1* Locus

To distinguish *Mash1*$^{-/-}$ embryos from *Mash1*$^{+/-}$ and *Mash1*$^{+/+}$ embryos, the neural tube (from neural crest cultures; see above) or a forelimb (in the in situ hybridization experiments described below) was genotyped by polymerase chain reaction as described (Blaugrund et al., 1996), with slight modifications (details available upon request).

Generation of Immortalized Neural Crest Stem Cell Lines

A detailed characterization of immortalized neural crest stem cells will be published elsewhere (M. R. and D. J. A., unpublished data). In brief, neural crest cells were infected with a retroviral vector harboring the avian v-*myc* and neomycin resistance genes (Birren and Anderson, 1990). At 24 hr after infection, cells were placed under neomycin selection (500 μg/ml) for a period of 4 days. Neomycin-resistant clones expressing LNGFR immunoreactivity were isolated and expanded. Clonal cell lines Monc-1 (wild-type neural crest stem cell line) and Mko-4 (*Mash1*$^{-/-}$ neural crest stem cell line) were used to perform the experiments described in this study. To differentiate Monc-1 and Mko-4, cells were trypsinized and replated onto dishes sequentially coated with poly-D-Lysine (0.5 mg/ml) and fibronectin (0.25 mg/ml) in standard medium (Stemple and Anderson, 1992) containing 10% FBS and 5 μM forskolin. Neuronal and glial differentiation occurred within 2–5 days.

Immunocytochemistry

Labeling of cell surface antigens on living cells was performed as described in Stemple and Anderson (1992), using a monoclonal rat anti-mouse LNGFR antibody (IgG) (M. R. and D. J. A., unpublished data); a monoclonal anti-N-CAM antibody 5A5 (IgM) (from hybridoma cells obtained from Developmental Studies Hybridoma Bank; Dodd et al., 1988); and Neurotag, a FITC-labeled tetanus toxoid derivative (Boehringer Mannheim; Raju and Dahl, 1982). Phycoerythrin-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) were used.

Labeling of intracellular antigens was performed as described (Stemple and Anderson, 1992; Shah et al., 1994) using the following reagents: rabbit polyclonal anti-SCG10 antibody (used at a 1:800 dilution; Stein et al., 1988); mouse monoclonal anti-peripherin antibody (IgG) (1:200 dilution; Chemicon; Parysek et al., 1988); monoclonal anti-neuron-specific enolase antibody (IgG) (1:10 dilution; Chemicon; Marangos and Schmechel, 1987); monoclonal anti-NF160 antibody NN18 (IgG) (1:40 dilution; Sigma Immuno Chemicals); monoclonal anti-NF68 antibody NR4 (IgG) (1:100 dilution; Sigma Immuno Chemicals; Cochard and Paulin, 1984); monoclonal anti-Class III β-tubulin antibody TuJ1 (IgG) (1:500 dilution; Geisert and Frankfurter, 1989); monoclonal anti-nestin antibody Rat 401 (IgG) (1:2 dilution; Developmental Studies Hybridoma Bank; Friedman et al., 1990); and rabbit polyclonal anti-GFAP antibody (1:500 dilution; Accurate Chemical and Scientific Corporation; Jessen et al., 1990). Detection of c-RET and MASH1 in fixed cells was performed as described (Shah et al., 1994; Lo and Anderson, 1995). Staining was developed with either fluorescent secondary antibodies or horseradish peroxidase–conjugated goat anti-mouse IgG (Chemicon) and goat anti-rabbit IgG (Vector Laboratories) using nickelous sulfate and diaminobenzidine (NiDAB) or 3-amino-9-ethyl carbazole (AEC) as substrates.

Isolation and Culture of Neuronal Precursors

Monc-1 cells were allowed to differentiate for 2 days and then labeled with FITC-conjugated Neurotag (see above). Subsequently, cells were removed from the culture dishes by trypsinization, washed once in medium containing 10% FBS, and labeled with the fluorescent dye PKH26 using the PKH26 Red Fluorescent General Cell Linker Kit (Sigma Immuno Chemicals; Horan and Slezak, 1989), according to the manufacturer's instructions. Neurotag$^+$, PKH26$^+$ cells were isolated on an Epic Elite Fluorescent Activated-Cell Sorter (Coulter) using a multiparametric gate based on fluorescent intensity, size, density, and granularity. To increase their survival, Neurotag$^+$, PKH26$^+$ cells were plated onto a monolayer of unlabeled Monc-1 cells that had previously

been cultured under differentiating conditions for 6–12 hr. Cultures were fixed 5 days after plating of the sorted cells and permeabilized by a freeze–thawing method (Temple and Davis, 1994) prior to immunocytochemical analysis of intracellular markers.

In Situ Hybridization

Nonradioactive in situ hybridization with digoxigenin-labeled cRNA probes was carried out on frozen sections of paraformaldehyde-fixed mouse embryos according to Birren et al. (1993). Detailed protocols are available upon request. Antisense cRNA probes used in this study were the following: SCG10 (Stein et al., 1988), peripherin (Parysek et al., 1988), NF160, NF68 (Julien et al., 1986), c-RET (Pachnis et al., 1993), and P_0 (Lemke et al., 1988).

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