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Early specification of sensory neuron fate revealed by expression and function of neurogenins in the chick embryo

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SUMMARY

The generation of sensory and autonomic neurons from the neural crest requires the functions of two classes of basic helix-loop-helix (bHLH) transcription factors, the Neurogenins (NGNs) and MASH-1, respectively (Fode, C., Gradwohl, G., Morin, X., Dierich, A., LeMeur, M., Goridis, C. and Guillemot, F. (1998) *Neuron* 20, 483-494; Guillemot, F., Lo, L.-C., Johnson, J. E., Auerbach, A., Anderson, D. J. and Joyner, A. L. (1993) *Cell* 75, 463-476; Ma, Q., Chen, Z. F., Barrantes, I. B., de la Pompa, J. L. and Anderson, D. J. (1998 *Neuron* 20, 469-482). We have cloned two chick NGNs and found that they are expressed in a subset of neural crest cells early in their migration. Ectopic expression of the NGNs in vivo biases migrating neural crest cells to localize in the sensory ganglia, and induces the expression of sensory neuron-appropriate markers in non-sensory crest derivatives. Surprisingly, the NGNs can also induce the expression of multiple pan-neuronal and sensory-specific markers in the dermomyotome, a mesodermal derivative. Taken together, these data suggest that a subset of neural crest cells may already be specified for a sensory neuron fate early in migration, as a consequence of NGN expression.

Key words: Chick embryo, Neurogenin, Neural crest, Sensory lineage, Expression

INTRODUCTION

The neural crest gives rise to the neurons and glia of the peripheral nervous system (PNS), which is subdivided into two major branches: sensory and autonomic. A major question in crest development is to understand how and when these two neurogenic lineages become specified. Transplantation experiments in avian embryos have suggested that some neural crest cells become restricted to an autonomic fate shortly after migration (Le Douarin, 1986; Le Lievre et al., 1980). Experiments in cell culture have provided indirect evidence for early commitment of some crest cells to a sensory fate (Sieber-Blum, 1989a; Ziller et al., 1983). However the mechanisms underlying segregation of the sensory and autonomic lineages have remained ill-defined, largely due to the lack of early markers for sensory neuron precursors.

We have approached this problem by identifying transcription factors in the basic helix-loop-helix (bHLH) family (Guillemot, 1995; Kageyama and Nakanishi, 1997; Lee, 1997), which are differentially expressed by autonomic and sensory progenitors (Anderson, 1997). For example, *Mash-1*, a mammalian homolog of the *Drosophila* proneural genes *achaete-scute (ac-sc)* (Johnson et al., 1990), is expressed by precursors of autonomic but not of sensory neurons (Guillemot and Joyner, 1993; Lo et al., 1991). Targeted mutagenesis in mice has shown that MASH-1 is essential for the development of sympathetic, parasympathetic and a subset of enteric neurons (Blaugrund et al., 1996; Guillemot et al., 1993; Hirsch et al., 1998; Sommer et al., 1995). Forced expression of MASH-1 in cultured neural crest stem cells (NCSCs) induces the differentiation of neurons that express autonomic markers, such as the paired homeodomain transcription factor Phox2a (Valarché et al., 1993) and the GDNF receptor c-RET (Lo et al., 1998; Pachnis et al., 1993). Whether MASH-1 imparts an autonomic identity on such cells, or simply activates a program of neurogenesis in precursors that are already specified for an autonomic fate, is not yet clear.

Recently, a novel subfamily of bHLH factors has been identified, called the Neurogenins (NGNs), which are related to another *Drosophila* proneural bHLH factor called ATONAL (Gradwohl et al., 1996; Ma et al., 1996; McCormick et al., 1996; Sommer et al., 1996). *ngn-1* and *-2* are expressed in precursors of sensory neurons in both the cranial and trunk regions of the murine neural crest, as well as in placodal precursors of cranial sensory neurons. Targeted inactivation of these genes has indicated that they are required early in the generation of different subsets of cranial and trunk sensory neurons (Fode et al., 1998; Ma et al., 1998; Q. Ma et al., unpublished). In the absence of *ngn* function, precursors of cranial sensory neurons fail to delaminate from the placodal epithelium (Fode et al., 1998). This cellular defect is correlated with a failure to express downstream bHLH genes such as *neuroD* (Lee et al., 1995), *MATH-3* (Takebayashi et al., 1997) and *NSCL-1* and *-2* (Begley et al., 1992; Göbel et al., 1992;

Fode et al., 1998; Ma et al., 1998). Thus, the NGNs are necessary for peripheral sensory neurogenesis.

Misexpression studies have been previously carried out with the NGNs in lower vertebrate embryos (Blader et al., 1997; Ma et al., 1996; Olson et al., 1998). Although these studies demonstrated a proneural function for the NGNs within the ectoderm, relatively little was revealed about the extent to which the NGNs specify neuronal subtype. Misexpression of two other *atonal*-related genes, *Xath-5/Math-5* and *NeuroD*, in the retina has shown that they can promote differentiation of certain retinal neuron subtypes (Brown et al., 1998; Kanekar et al., 1997; Yan and Wang, 1998), although *NeuroD* likely functions downstream of proneural genes (Fode et al., 1998; Ma et al., 1998, 1996). Similar experiments in *Drosophila* have shown that proneural genes such as *ac-sc* and *atonal* contribute to the specification of neuronal identity in both the embryonic PNS and CNS (Jarman and Ahmed, 1998; Jarman et al., 1993; Skeath and Doe, 1996).

We wished to determine whether expression of the NGNs is sufficient to instruct a sensory neuron fate in uncommitted neural crest cells. To test this hypothesis, we have isolated two avian NGNs, and shown that they are expressed in a subset of migrating neural crest cells. Misexpression of the NGNs in premigratory crest cells biases their final location to the dorsal root sensory ganglia (DRG). The NGNs can also induce the ectopic expression of sensory neuron markers in non-sensory crest derivatives, including sympathetic ganglia and presumptive glial cells along the peripheral nerve. Surprisingly, at least five pan-neuronal and five sensory neuron-specific markers could be induced by NGNs in the dermamyotome, a mesodermal derivative. Taken together, these results suggest that expression of endogenous *ngns* in a subset of neural crest cells may specify them for a sensory fate early in migration. They also indicate that the ability of NGNs to activate a program of sensory neuron-specific gene expression in heterologous cells is not restricted to ectodermal derivatives.

MATERIALS AND METHODS

Animals

Chick eggs were obtained from local suppliers and from SPAFAS (Norwich, CT). Animals were incubated in a forced draft incubator at 38°C. All staging was done as previously described (Hamburger and Hamilton, 1951).

Isolation of chick neurogenin cDNAs

The bHLH regions of the chick neurogenins were amplified using degenerate RT-PCR from E3.5 cDNA. cDNA was prepared using RNAzol (Teltest, Inc.)-extracted total RNA and Gibco's Superscript kit. Primer sequences corresponded to the following conserved residues within the neurogenin bHLH regions: RNRNRMH (5′ GN-AAC/T-A/CGN-AAC/T-C/AGN-ATG-CA 3′) and NYIWAL (antisense 5′ AG/A-NGC-CCA-A/G/T/AT-G/ATA-A/GTT 3′). PCR products were gel purified, and cloned into pBluescript. Two clones with homology to the mouse neurogenin bHLH regions were used as probes to screen a mixed stage chick spinal cord cDNA library (gift of T. Jessell). From the 2×10^6 clones screened, 45 chick neurogenin-2 clones and one chick neurogenin-1 clone were obtained. The longest clones were chosen for further analysis. Additionally, one chick NeuroDL clone (corresponding to 370-790 bp in the published sequence; Roztocil et al., 1997) and one chick NSCL-2 clone (1.3 kb, this study) were also isolated. All sequencing was done on an ABI sequencer, and GCG software was used to generate alignments. Accession numbers are as follows: AF123883 (c-ngn-1), AF123884 (c-ngn-2) and AF123885 (cNSCL-2).

In situ hybridization

Hybridization of whole-mount specimens and frozen sections was carried out as previously described (Birren et al., 1993; Nieto et al., 1996). Detailed protocols are available upon request. Most probes were hybridized at 65°C (except trkC and NeuroD-L, which require a 70°C hybridization temperature). Slides were incubated overnight at 4°C in either pre-absorbed anti-digoxigenin (1/2000) or anti-FITC (1/4000) antibodies (Boehringer Mannheim). Riboprobes were synthesized using labeled nucleotides from Boehringer Mannheim and RNA polymerases from Promega. Probes used in this study include cSCG10 (Groves et al., 1995); chick neurogenins 1 and 2 and chick NSCL-2: digest with *Sac*I and transcribe with T7 (this study); chick NeuroDL: digest with *Eco*RI and transcribe with T3 (Roztocil et al., 1997; this study); mash-1, m-ngn-1 and n-Luc: digest with *Nco*I and transcribe with T7 (see section on viral constructs and Chen et al., 1998); c-trkA: cut with *Xho*I and transcribe with T7 (probe corresponds to amino acids 170-374; Backstrom et al., 1996); c-trkC: cut with *Eco*RI and transcribe withT7 (probe corresponds to amino acids 152-369; Garner and Large, 1995); phox2 and GATA-2 (Groves et al., 1995).

Immunohistochemistry

For most epitopes, embryos were fixed by immersion in 4% paraformaldehyde overnight at 4°C. In order to detect either the HB9 or ISL2 epitopes, animals were fixed for 2 hours on ice. Immunohistochemistry on whole-mount specimens and frozen sections were carried out using standard protocols. Primary antibodies were used at the following dilutions: anti-HNK-1 (undiluted, ATCC); TuJ1 (1/500, Babco); 3A10 (1/5, Developmental Studies Hybridoma Bank); 270RM0 (1/100, gift of V. Lee); p27 (1/1000; Spafas); anti-myc (1/100, clone 9E10; Sigma); NeuN (1/500, Chemicon); ec8 (undiluted, Developmental Studies Hybridoma Bank); anti-mash-1 (undiluted, Lo et al., 1991); anti-axonin-1/TAG-1 (1/500, Developmental Studies Hybridoma Bank); anti-tyrosine hydroxylase (1/500, Boehringer Mannheim), HB9 and ISL2 (1/100, gift of T. Jessell). The following secondary antibodies were used at 1/200: goat-anti-mouse-IgG-HRP (Chemicon); goat-anti-mouse-IgM-HRP (Boehringer Mannheim); goat-anti-mouse-IgG-Cy5 (Jackson Immuno) and goat-anti-rabbit-Cy3 (Jackson Immuno).

Microscopy/photography

Cultured cells were viewed using an Olympus microscope. Sectioned material was viewed using either a Zeiss Axioskop or a BioRad MRC600 confocal microscope. Whole-mount specimens were viewed using a Leica Wild M8 microscope.

Viral construction and generation of high titer stocks

All constructs were generated by cloning the genes of interest into the shuttle vector, SLAX-MT (Chen et al., 1998), which provides a single myc epitope tag at the amino terminus of retrovirally expressed proteins, and subsequently into RCAS-B (Morgan and Fekete, 1996; Petropoulos and Hughes, 1991). Mouse neurogenin-1 (m-NGN-1), mouse neurogenin-2 (m-NGN-2) and rat MASH-1 were subcloned from previously described vectors (Lo et al., 1997; Ma et al., 1996). Chick neurogenin-1 (c-NGN-1) and a non-coding version of m-NGN-1 were PCR-amplified with Pfu polymerase (Stratagene), using primers that supplied appropriate restriction sites and the appropriate cDNAs as templates. To generate viral stocks, RCAS plasmids were transfected into primary chick embryonic fibroblasts via $CaPO_4$ transfection. Viral supernatant was collected from 5-7 days following transfection, and concentrated by ultracentrifugation (2-3 hours at 64,000 ***g*** in a fixed angle rotor). Viral titers in this study ranged from 10^8-10^9/ml.

Viral infection and analysis

Neural crest cell distribution assay

Premigratory neural crest were infected by injecting virus into the

lumen of the neural tube in animals at Hamburger-Hamilton (HH) stage 10-12 (Fekete and Cepko, 1993). Animals were returned to the incubator and harvested 40 hours after injection, at HH20-21. Representative cross sections through the forelimb region of each animal were examined via in situ hybridization for transgene expression; sections were taken through a minimum of 960 μm (roughly 3 body segments) of each animal. Crest distribution was quantitated by counting the total number of infected cells in the dorsal root (DRG) and sympathetic ganglia (SG). Alternatively, we examined the distribution of infected crest using whole-mount in situ hybridization, and quantitated crest distribution between the forelimb and hindlimb regions by scoring infected body segments for transgene expression in either the DRG, or in both the DRG and the SG. ANOVAs and pairwise student's *t*-tests were performed using the Microsoft Excel program.

Because the forelimb region of the experimental animals was at the neural plate stage at the time of infection, our analysis focused on crest cells that had been infected quite early relative to the onset of migration.

Infection of migrating neural crest cells and of mesodermal derivatives

Virus was injected unilaterally into somites at the forelimb level of animals at HH 13-15 (somites 15-19) (Christ and Ordahl, 1995). Animals were typically incubated for an additional 60-72 hours prior to harvesting. In experiments in which the expression patterns of ISL-2 and HB9 were scored, the animals were incubated for an additional 5-6 days. Serial sections were collected through the forelimb region of each infected animal. In order to determine the extent of infection, each animal was examined via in situ hybridization with probes to detect the relevant transgenes, or with antibodies to the viral protein, p27. Well-infected animals were scored for the ectopic expression of neuronal markers via immunohistochemistry (TuJ1, 3A10, 270RMO, TH, TAG-1/axonin-1, HB9, ISL2 or NeuN) or in situ hybridization (SCG10, NeuroD-L, NSCL-2, trkA, trkC or GATA-2).

Double labeling of mesodermal derivatives and either the neural crest or the ectoderm

Mesodermal derivatives were infected with either RCAS-m-ngn-1, RCAS-m-ngn-2 or RCAS-c-ngn-1 by unilateral injection into somites 15-19 in animals between HH13-15. Neural crest derivatives were labeled by injecting Cell Tracker DiI/CM (Molecular Probes) into the lumen of the neural tubes of these animals as previously described (Bronner-Fraser, 1996). Alternatively, the animals were returned to the incubator for an additional 1-2 hours, at which time DiI/CM was used to label the overlying ectoderm. DiI/CM is a lipophilic, carbocyanine dye that is stable to paraformaldehyde fixation. Animals were incubated for an additional 72 hours prior to harvesting. Serial sections were collected through the forelimb region of each animal and examined for labeling with DiI/CM ectopic TuJ1 expression.

RESULTS

The chick neurogenins are similar to their murine counterparts

Two different classes of chick neurogenin cDNAs were isolated (see Materials and methods). A 1.3-kb

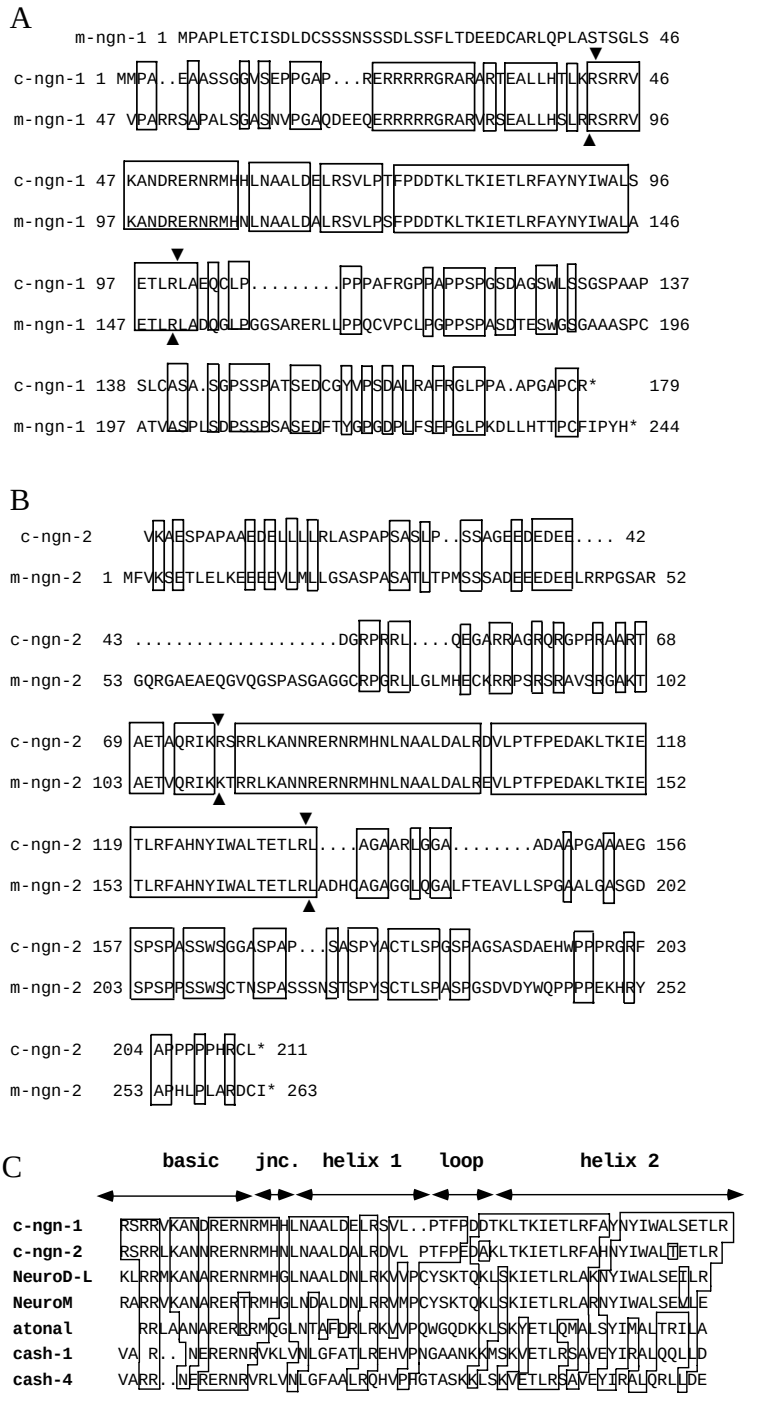

Fig. 1. Sequence analysis of chick neurogenin-1 and neurogenin-2. (A,B) The amino acid sequence of each chick gene is shown in alignment with its murine counterpart. Regions of identity are boxed, and the bHLH domains are indicated with arrowheads. (A) Chick neurogenin-1 (c-ngn-1) encodes a 241-amino-acid ORF that is 64% identical (75% similar) to mouse neurogenin-1. (B) Chick neurogenin-2 (c-ngn-2) encodes an ORF that lacks two N-terminal amino acids and that is 62% identical (77% similar) to mouse neurogenin-2 (m-ngn-2). (C) Multiple alignments of the bHLH domains from several chick neuronal bHLH proteins. Regions of identity are boxed. C-ngn-1 and c-ngn-2 are 50% identical (70% similar) to each other. Also shown are the bHLH regions of NeuroD-L and NeuroM (Roztocil et al., 1997), CASH-1 (Jasoni et al., 1994; Wang and Kirby, 1995), CASH-4 (Henrique et al., 1997) and chick atonal (Ben-Arie et al., 1996).

cDNA clone encodes a 179-residue open reading frame that is 93% identical to m-NGN-1 within the bHLH region, and 66% identical over the entire length of the protein (Fig. 1A). This sequence has been named chick neurogenin-1 (c-NGN-1). A second, 0.8-kb cDNA clone encodes an open reading frame of 211 residues that is is 95% identical to m-NGN-2 within the bHLH region, and 62% identical over the entire length of the protein (Fig. 1B), and has been named chick neurogenin-2 (c-NGN-2). The c-NGNs are more similar to each other (51% identity overall) than they are to other chick neural bHLH proteins (Fig. 1C).

Neither *c-ngn-1* nor *c-ngn-2* is detectably expressed in the earliest-migrating trunk neural crest cells at HH13 (Fig. 2A,B, arrowheads). Expression in the neural crest is first detected at HH14-15. At this stage, *c-ngn-2* is strongly expressed in a small number of cells, and *c-ngn-1* is expressed at a low level in very rare cells (not shown). By mid-migration at HH 16-17, both of the *c-ngns* are clearly expressed in migrating crest adjacent to the neural tube (Fig. 2D,E,G,H). The number of crest cells labeled by either of the *ngn* probes always appeared to be smaller than the number of crest cells labeled by HNK-1, an impression confirmed by analysis of the markers on adjacent serial sections (not shown). These observations indicate that the *ngns* are expressed by a subset of neural crest cells early in their migration.

At these stages, both of the *c-ngn* genes are also expressed strongly at the dorso-lateral margins of the neural tube, from which region neural crest cells delaminate (arrows; Fig. 2D,E,G,H). At HH 18 (31-35 somites), *c-ngn-2* is weakly expressed in the dorsal root sensory ganglia (DRG), and by HH 24 (E4) is undetectable (Fig. 2J and data not shown). In contrast, *c-ngn-1* is strongly expressed at HH 27 (Fig. 2K, arrowhead), and is still expressed through HH31 (E7) (data not shown). At these later stages, *c-ngn-1* is expressed in the DRG periphery, which consists of non-neuronal cells and/or neuronal precursors, while differentiating neurons are localized more towards the center of the DRG and appear negative for *c-ngn-1*.

Expression of both c-NGNs in the DRG is followed by that of chick NeuroD-like (c-NeuroD-L) (Roztocil et al., 1997) and a chick homolog of the mouse *NSCL* genes (Begley et al., 1992; Göbel et al., 1992) called c-NSCL-2, two additional bHLH proteins that are expressed subsequently to, and are dependent upon, NGNs in the mouse (Fode et al., 1998; Ma et al., 1996). c-NeuroD-L is first detected in sensory precursors at HH 16-17 (Fig. 2F), shortly after the cNGNs, while c-NSCL-

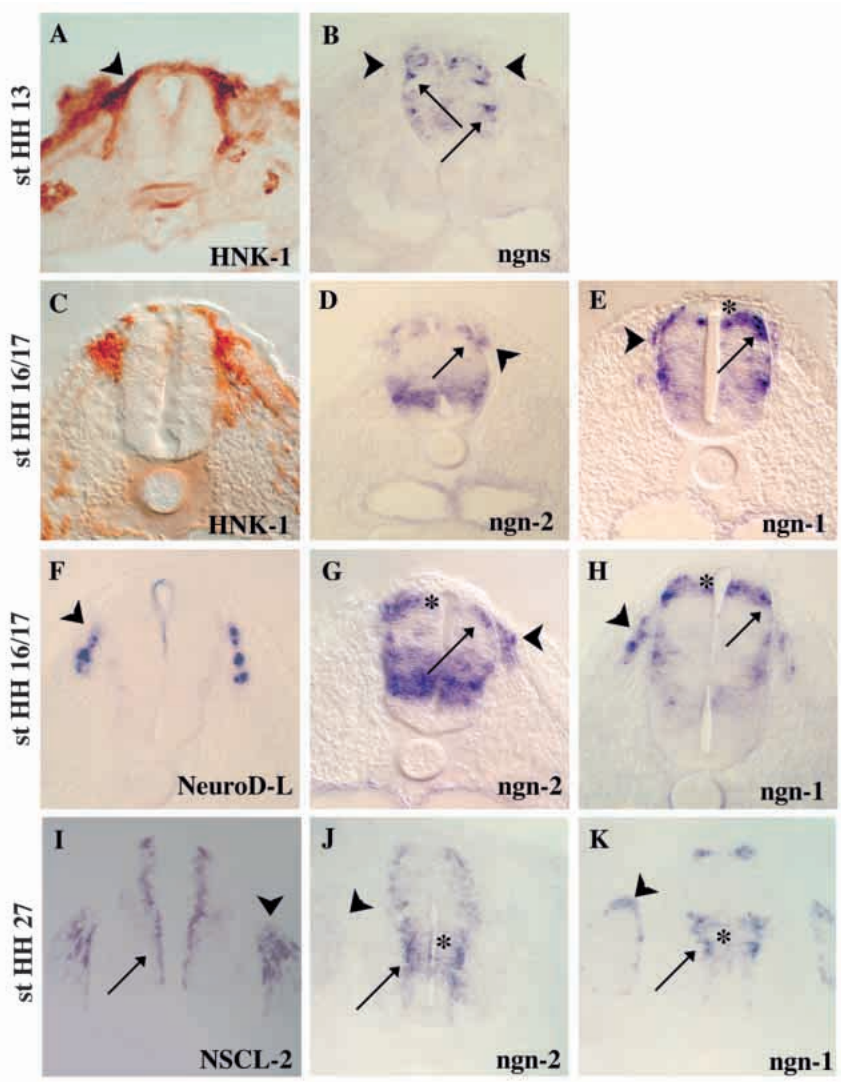

Fig. 2. Expression of the chick neurogenins in the trunk neural crest. All panels show cross sections through the forelimb region. (A,B) Neither of the neurogenins is expressed in the neural crest at the onset of migration, HH13. (A) Neural crest expressing the crest markers, HNK-1, are visible adjacent to the neural tube (arrowhead). (B) Expression of the *ngns* is seen at the lateral margins of the neural tube (arrow) but not in neural crest cells (arrowheads). (D,E) Both of the *ngns* are expressed in migrating neural crest cells (shown here at HH16/17, arrowheads). (C) HNK-1 expression is shown for comparison. (G,H) Expression of the *ngns* is seen in sensory precursors adjacent to the neural tube in slightly older animals (arrowheads). (F) Expression of NeuroD-L is also visible in sensory precursors at this stage (arrowhead). (D,E,G,H) At HH16/17, expression of the ngns within the neural tube is seen at the lateral margins (arrows) and in the ventricular zone (asterisks). (J) Expression of *ngn-2* in the DRG starts to fade at ganglion condensation, and is no longer visible by HH 27 (arrowhead). (K) Expression of ngn-1 persists in the DRG periphery, shown here at HH 27 (arrowhead). At this later stage, both genes are expressed within the neural tube in the ventricular (asterisk) and subventricular (arrow) zones. No expression is seen at the lateral margins of the neural tube. (I) At this stage, expression of NSCL-2 is seen in the DRG (arrowhead) and in the subventricular zone (arrow) of the neural tube.

2 is not detected until after HH 19 (shown at HH 27 in Fig. 2I, arrowhead). At no time are any of these bHLH factors detectably expressed in developing sympathetic ganglia (SG), in peripheral glial cells or in paraxial mesodermal derivatives surrounding the peripheral ganglia (data not shown).

Both *c-ngns* are also expressed in restricted regions of the neural tube, as are their rodent counterparts. While we did not examine this aspect of NGN expression in detail, at HH 16-17, *c-ngn-2* is expressed in the ventral neural tube (Fig. 2D,G) extending laterally from the ventricular zone to the pial surface, while *c-ngn-1* is more strongly expressed in the dorsal neural tube (Fig. 2E,H). By HH 27, both *c-ngns* are expressed in the ventral neural tube (Fig. 2J,K, asterisks), although *c-ngn-2* expression extends throughout the dorso-ventral axis. At these stages, when neural crest cell migration is complete, neither of the *c-ngns* is expressed at the lateral margins of the neural tube (Fig. 2J,K, arrows).

Forced expression of mouse *ngn-1* in ovo biases neural crest cell distribution towards the dorsal root sensory ganglia

In order to determine whether the expression of *ngns* in a subset of neural crest cells might cause their differentiation to sensory neurons, we infected premigratory neural crest cells in ovo with an RCAS(B) recombinant retrovirus encoding the open reading frame of mouse NGN-1 (see Materials and methods). Use of the rodent gene allowed us to clearly distinguish between expression of the transgene and expression of the endogenous chick *ngns*. As controls, we expressed either alkaline phosphatase (AP) (Fekete and Cepko, 1993), a nuclear-localized luciferase (nLuc) (Chen et al., 1998) or rat MASH-1.

Expression of both of the control transgenes, as well as of rodent *Mash-1*, can be readily detected in both infected dorsal root and sympathetic ganglia 40 hours after virus injection (Fig. 3B,G,H, arrows, and data not shown). By contrast, *m-ngn-1* expression is found predominantly in the dorsal root ganglia (DRG) and much less frequently in the sympathetic ganglia (SG), whether detected by in situ hybridization (Fig. 3A,C,E; cf. arrowheads versus arrows) or by immunohistochemistry with an antibody to the myc epitope tag included in the *ngn* transgene (Fig. 3F; cf. arrowheads versus arrows). Even in cases where *m-ngn-1* expression is detected in the SG, the proportion of infected cells in this location relative to the DRG is small (Fig. 3E,F; cf. arrows versus arrowheads; Table 1). By contrast, a much higher proportion of crest cells in the SG relative to the DRG is revealed by the pan-crest marker, HNK-1 (cf. Fig. 3C versus 3D, arrows).

Quantification of the number of infected cells in the DRG or SG in 16-20 representative sections from each of 19 infected embryos indicated that crest infected with m-NGN-1 show a 92%-8% DRG-SG distribution (Table 1). This difference is significantly different ($P<0.01$, ANOVA followed by pairwise Student's *t*-test) from that observed for crest infected with each

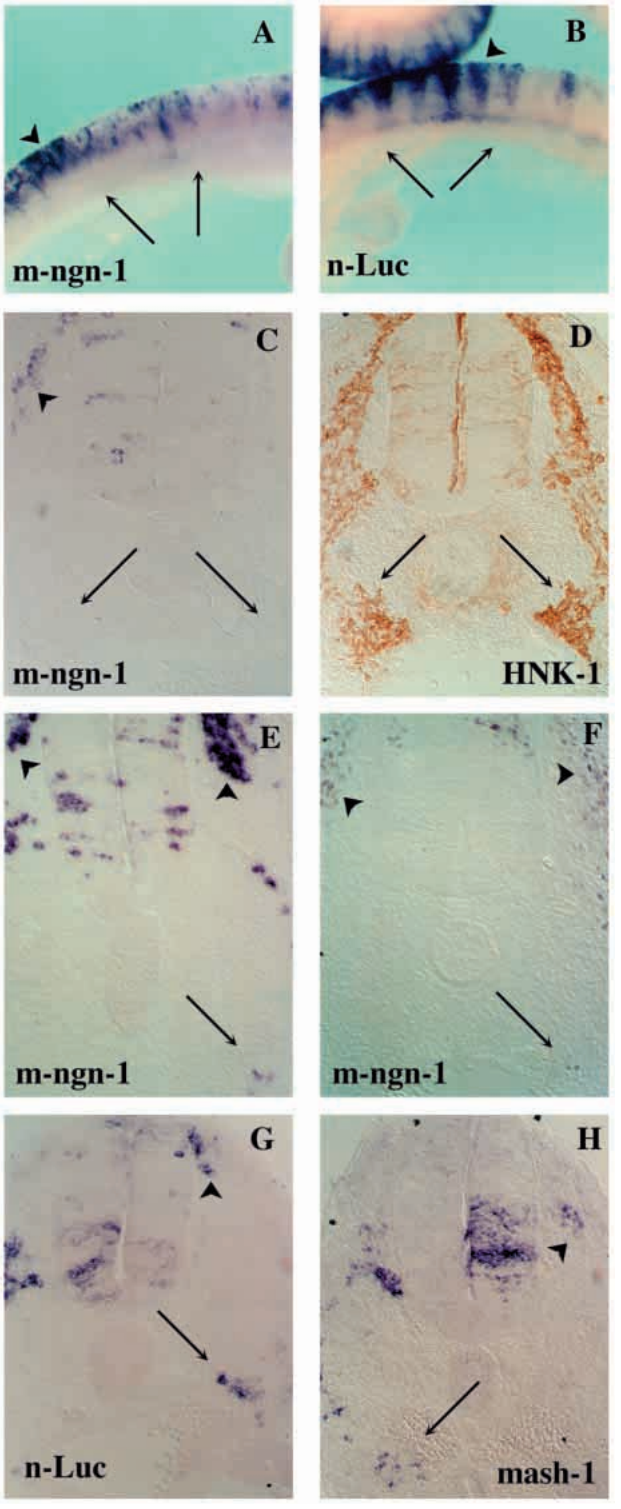

Fig. 3. Forced expression of m-NGN-1 skews crest distribution toward the dorsal root ganglia. Animals were infected via viral injection into the neural tube at HH10-12, and harvested 40 hours later at HH 21. Whole-mount preparations (A,B) and sections through the forelimb level (C-H) are shown. (A,C,E,F) Neural crest infected with RCAS-m-NGN-1 are preferentially seen in the dorsal root ganglia (DRG, arrowheads); few infected cells are seen in the sympathetic ganglia (SG, arrows). (D) A section adjacent to (C) is stained with anti-HNK-1 to detect neural crest cells. The SG are indicated with arrows. Crest infected with either the RCAS-n-Luc control virus (B,G) or RCAS-mash-1 (H) are seen in both the DRG and the SG. Transgene expression is detected via in situ hybridization in all panels except for (F), in which transgene expression is detected via immunohistochemistry to the myc epitope tag.

Table 1. Distribution of crest between the dorsal root and sympathetic ganglia

Virus	*n*	Average number of cells in 16-20 sections	%DRG	%SG
(A)				
m-NGN-1	19	110±77	92±13	8±13
AP	13	117±49	74±18 (*P=0.003)	26±18
nLuc	14	589±426	67±14 (*P<0.00001)	33±14
MASH-1	9	120±91	70±17 (*P=0.001)	30±17
(B)				
m-NGN-1	5	221±99	94±6	6±6
MASH-1	4	541±63	72±9	28±9

DRG, dorsal root ganglia; SG, sympathetic ganglia.
n, number of infected embryos.
*ANOVA, followed by pairwise Student's *t*-test.

Table 2. Distribution of neural crest in whole-mount preparations

Virus	*n*	%DRG	%DRG+SG
(A)			
m-NGN-1	16	81±13	19±13
MASH-1	8	48±15 (P<0.001)	52±15
nLuc	14	61±17 (P<0.01)	39±17
(B)			
m-NGN-1	16	88±12	12±12
stop-NGN-1	15	66±15 (P<0.01)	34±15

n, number of infected body segments.
See Materials and methods for details.

of the control viruses or with MASH-1, which show an approximately 70%-30% DRG-SG distribution (Table 1); this bias in control-infected embryos likely reflects the fact that the DRG are larger than the SG. The DRG bias of NGN-infected crest cells is observed for equivalent numbers of NGN- versus control-infected cells per embryo (Table 1A) and is maintained even when the average number of NGN-infected cells is doubled (Table 1B). This indicates that the biased distribution is unlikely to be an artifact of a smaller number of NGN-infected cells.

To extend these observations, we developed a simpler method of quantification using whole-mount preparations, in which we counted the percentage of body segments that contained m-NGN-1-expressing cells only in the DRG, versus in both the DRG and SG. This assay allowed us to examine crest distribution along the entire rostro-caudal axis of the trunk. There does not appear to be a significant DRG bias (relative to controls) in the distribution of NGN-infected crest cells at levels rostral to the forelimb (not shown). Since the first crest cells to emigrate contribute preferentially to SG, and since crest emigration proceeds from rostralmost to more caudal levels (Weston, 1963), it is likely that viral gene expression (which requires up to 18 hours post-infection; Homburger and Fekete, 1996) at these more rostral axial levels is not widespread until after many crest cells have already migrated ventral to the DRG. We therefore restricted our analysis to levels at and caudal to the forelimb region of the embryo. Using this simplified assay, the bias towards the DRG obtained upon infection with m-NGN-1 (81% DRG only versus 19% DRG+SG segments) is still significantly different from the control or MASH-1 viruses (61% and 48% DRG-only segments, respectively; Table 2A). We further demonstrated in a blind experiment that m-NGN-2 can also bias the distribution of crest cells towards the DRG (73% DRG only for m-NGN-2 (n=7) versus 55% DRG only for MASH-1 (n=5), P<0.01). Chick NGN-1 also appears to bias the distribution of infected cells (88% DRG only, n=10), although the scoring was ambiguous in some cases because it was difficult to distinguish transgene from endogenous *c-ngn-1* expression.

It was possible that the bias in distribution of infected neural crest cells simply reflected instability of the NGN mRNA in the SG. To address this issue, a mutant version of the m-NGN-1 mRNA was constructed, in which two stop codons were inserted downstream of the m-NGN-1 initiator methionine. Compared to wild-type m-NGN-1-infected embryos analyzed in parallel (88±12% DRG only segments, n=16, Table 2B), embryos infected with this mutant m-NGN-1 virus exhibited a significantly reduced percentage of segments with transgene expression in DRG only (66±19%; n=15; P<0.01; Table 2B). These data indicate that the DRG bias of *m-ngn-1*-infected crest cells requires the synthesis of m-NGN-1 protein.

Ectopic expression of m-NGN-1 induces the expression of sensory neuron markers within neural crest derivatives

The foregoing data indicated that expression of NGNs in neural crest cells biases their distribution towards the DRG, but did not address whether the infected cells had differentiated

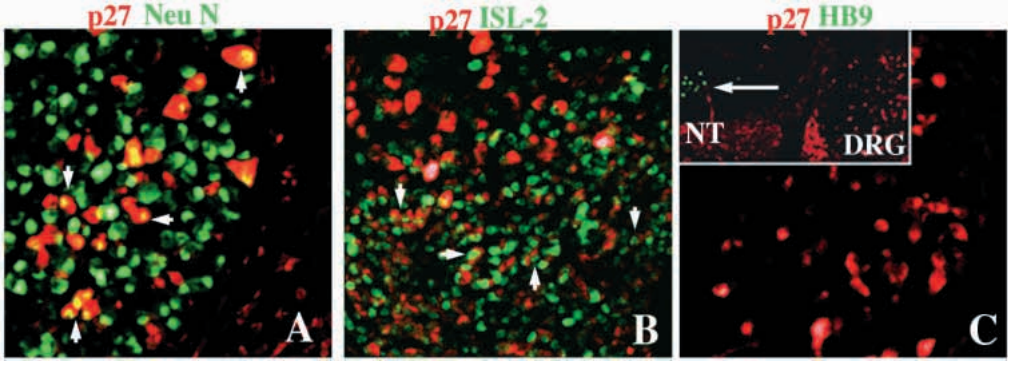

Fig. 4. Cells in the DRG infected with RCAS-m-NGN-1 express sensory neuron markers. Shown are adjacent sections through a DRG of an animal infected at HH13-15 and harvested at HH 34. In each panel, infected cells are detected via immunohistochemistry to a viral protein, p27 (red) and double stained for a neuronal marker (green). Cells expressing m-NGN-1 co-express (A) the pan-neuronal marker, Neu N (arrows) and (B) the sensory and motor neuron marker, ISL2 (arrows), but do not express (C) the motor neuron markers, HB9. The inset in C shows expression of HB9 in the adjacent neural tube (arrow).

appropriately for this environment. To determine whether these cells express a sensory neuron phenotype, they were double-labeled with antibodies to the viral envelope protein, p27, and one of several different neuronal markers. $p27^+$ cells in the DRG of embryos infected with the m-NGN-1 retrovirus co-express NeuN (Mullen et al., 1992), a pan-neuronal nuclear marker (Fig. 4A), indicating that they are neurons. Similar results were obtained with an antibody to neuron-specific tubulin, TuJ1 (not shown). Many $p27^+$ cells co-express ISL2 (Fig. 4B), a LIM-homeodomain protein expressed by trunk sensory, but not sympathetic neurons (Tsuchida et al., 1994). Because motor neurons also express ISL2, however, m-NGN-1 infected cells were also examined for expression of the motor neuron marker, HB9, which is normally not expressed by sensory neurons (Tanabe et al., 1998). No expression of HB9 is seen in $p27^+$ m-NGN-1-infected cells in the DRG (Fig. 4C). This combination of markers ($NeuN^+ISL2^+HB9^-$) therefore identifies m-NGN-1-infected DRG cells as sensory neurons.

The preceding data did not distinguish whether ectopic expression of NGN is simply permissive for sensory neuron differentiation in the DRG, or whether it can instruct such differentiation as well. To address this issue, we examined the consequences of *ngn* misexpression in other non-sensory crest-derived populations that normally do not express endogenous *c-ngns*, by unilaterally infecting crest cells as they were migrating through the somite. Expression of m-NGN-1, as well as of m-NGN-2 and c-NGN-1, induces the expression of the sensory-specific marker NSCL-2 in SG on the infected but not the uninfected side of the embryo (n=6) (Fig. 5B, cf. Fig. 5A; Table 3A). In contrast, neither n-Luc nor MASH-1 induces NSCL-2 expression in the SG (Table 3A). This indicates that misexpression of m-NGN-1 can induce the ectopic expression of at least one sensory marker in a location appropriate for autonomic neuronal precursors. Expression of NeuroD-L and ISL2, however, was not detected in m-NGN-1-infected SG, even though ectopic expression of these markers was detected in other locations in the same embryos (see below). We were unable to assay for expression of other sensory markers such as trkC or TAG-1/axonin-1 (see below) in this location, because they are expressed in early sympathetic neurons (Birren et al., 1993; DiCicco-Bloom et al., 1993) and on nerve fibers that normally pass through the region of the sympathetic ganglia (Furley et al., 1990; Wolfer et al., 1994), respectively.

To determine whether NGNs could induce expression of neuronal markers in a non-neuronal crest derivative, we examined cells along the peripheral nerve within the limbs of infected embryos. Surprisingly, cells associated with peripheral nerves (detected via TuJ1 staining; Fig. 5C) in m-NGN-1-infected embryos express the pan-neuronal markers SCG10 (Fig. 5D) and NeuN (data not shown). Moreover, in embryos infected with m-NGN-1 (n=5), m-NGN-2 (n=3) or c-NGN-1 (n=3) viruses these cells also express markers appropriate for sensory neurons: NeuroD-L, NSCL-2 and the neurotrophin-3 receptor trkC (Fig. 5E,F; Table 3B; expression of TAG-

Table 3. Ectopic induction of sensory neuron markers within neural crest derivatives

Virus	NSCL-2	NeuroD-L	trkC	ISL2
(A) Sympathetic ganglia				
m-NGN-1	3/5	0/4		0/3
m-NGN-2	2/3	0/3		nd
c-NGN-1	1/3	0/3		nd
MASH-1	0/3	0/2		0/2
nLuc	0/4	0/3		nd
wt or mock infected	0/5	0/2		0/2
	NSCL-2	NeuroD-L	trkC	ISL2
(B) Peripheral nerve				
m-NGN-1	4/5	6/6	3/4	0/3
m-NGN-2	3/3	3/3	2/3	nd
c-NGN-1	3/3	3/3	3/3	nd
MASH-1	0/2	2/3	0/1	0/2
nLuc	0/4	0/4	1/4	nd
wt or mock infected	0/4	0/1	0/3	0/3

wt, wild type; nd, not determined.
For details, see text.

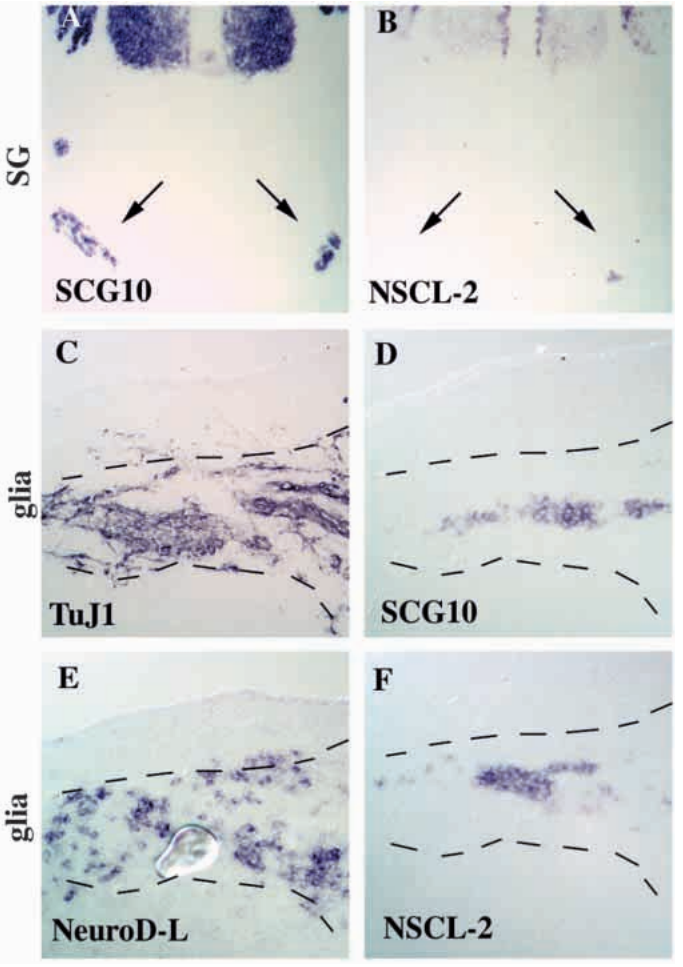

Fig. 5. Ectopic expression of sensory neuron markers in non-sensory crest derivatives. Animals were infected with RCAS-m-NGN-1 via viral injection into the crest migratory pathway at HH 13-15, and harvested at HH 27. (A,B) Adjacent sections through an animal with infection in the sympathetic ganglia (SG, arrows). (A) The SG are detected via the pan-neuronal marker, SCG10. (B) Ectopic expression of the sensory marker, NSCL-2, is seen in the SG on the infected side of the embryo. (C-F) Adjacent sections through an animal with infection in glial cells along the peripheral nerve. (C) The nerve is detected via TuJ1 staining and outlined. Cells associated with the nerve ectopically express the pan-neuronal markers, SCG10 (D) and the sensory neuron markers, NeuroD-L (E) and NSCL-2 (F).

1/axonin-1 was not assessed for the reasons mentioned above). No expression of any of these markers is seen in the peripheral nerve of wild-type or mock-infected embryos. Unlike m-NGN-1, ectopic expression of MASH-1 does not induce expression of NSCL-2 or trkC in peripheral nerve (not shown). These data suggest that ectopic expression of NGNs is not simply permissive for sensory neuron differentiation in the DRG, but that it can instruct at least some aspects of a sensory neuron fate in non-sensory neural crest derivatives.

Expression of m-NGN-1 induces expression of pan-neuronal and sensory-specific markers in the dermomyotome

The foregoing results prompted us to ask whether ectopic expression of the NGNs could elicit expression of neuronal markers in a non-neural crest-derived tissue in vivo. We therefore infected paraxial mesoderm with the various retroviral constructs. Expression of m-NGN-1 in the somite (Fig. 6B) is sufficient to induce the expression of five pan-neuronal markers in the dermomyotome: TuJ1; low molecular weight neurofilament, as detected by the 270RMO antibody (Lee et al., 1987), SCG10 (Anderson and Axel, 1985; Stein et al., 1988); NeuN and the neurofilament associated antigen, 3A10 (Storey et al., 1992) (Fig. 6C-E; Table 4A; data not shown). Moreover, TuJ1 antibody staining revealed apparent neuronal processes projecting from the infected somitic cells (Fig. 6D, arrows). Similar results were obtained with m-NGN-2 (n=7) and c-NGN-1 (n=6) (Table 4A). No ectopic marker expression is observed on the contralateral, uninfected side of NGN-infected embryos (n=10), and none of the markers is induced in embryos infected with the control n-Luc virus (n=3).

We also examined expression of sensory neuron markers in NGN-infected dermomyotome. Infection of the dermomyotome with m-NGN-1, m-NGN-2 or c-NGN-1 retroviruses induces the expression of NeuroD-L (n=11) and NSCL-2 (n=11) (Fig. 6F,G, arrowheads; Table 4B), as it did in the peripheral nerve. Although these markers are specific for sensory neurons in the PNS, since they are also expressed in the CNS (this study; Roztocil et al., 1997), we examined the expression of additional, more restricted sensory markers. m-NGN-1 (as well as m-NGN-2 and c-NGN-2) induces expression of trkC (n=8) (Fig. 6H, arrowhead; Table 4B) as well as of TAG-1/axonin-1 (n=10) (Fig. 6I, arrowhead), a cell-surface glycoprotein that is expressed by sensory and motor axons (Furley et al., 1990; Honig et al., 1998; Wolfer et al., 1994). M-NGN-1-infected dermomyotomal

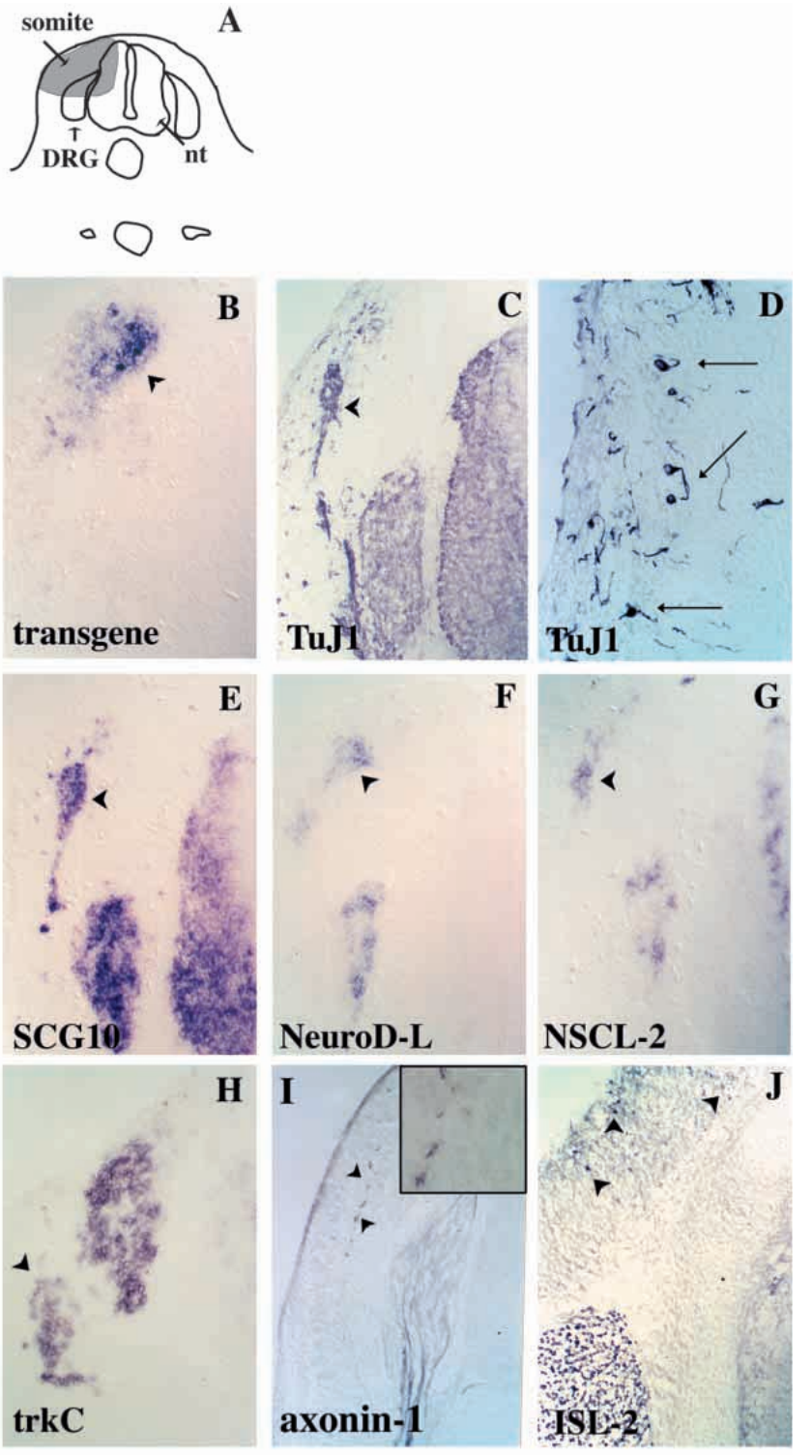

Fig. 6. Ectopic expression of pan-neuronal and sensory markers in the dermomyotome. (A) Schematic diagram. The shaded area represents the region of infected animals shown in (B-I). Animals were infected with RCAS-m-NGN-1 via unilateral viral injection into somites at forelimb level at HH 13-15, and harvested at HH 27 (B-H) or at HH 34 (I). Transgene expression in the dermamyotome is indicated with an arrowhead in one animal (B). Arrowheads indicate ectopic expression of (C) TuJ1, (E) SCG10, (F) NeuroD-L, (G) NSCL-2, (H) trkC, (I) axonin-1/TAG-1 (enlarged in inset) and (J) ISL-2. (D) Apparent neuronal processes projecting from the infected cells are revealed by TuJ1 antibody staining (arrows).

Table 4. Induction of neuronal markers in the dermomyotome

(A)	Pan-neuronal markers				
Virus	TuJ1	SCG10	270RMO	NeuN	3A10
m-NGN-1	9/10	6/7	5/6	5/6	2/2
m-NGN-2	7/7	3/3	2/3	3/3	nd
c-NGN-1	6/6	3/3	3/3	3/3	nd
MASH-1	4/8	0/6	4/6	1/6	nd
nLuc	0/3	0/3	0/3	0/3	0/2
(B)	Sensory markers				
Virus	NSCL-2	NeuroD-L	trkC	axonin-1	ISL2
m-NGN-1	5/6	5/5	3/3	3/5	2/3
m-NGN-2	3/3	3/3	3/3	5/7	nd
c-NGN-1	3/3	3/3	2/3	2/3	nd
MASH-1	0/5	0/4	0/2	0/2	0/2
nLuc	0/4	0/4	1/4	0/3	nd
wt or mock infected	0/4	0/1	0/3	0/2	0/3

wt, wild type; nd, not determined.
Values are number of embryos showing the marker out of the total number of embryos infected.
For details, see text.

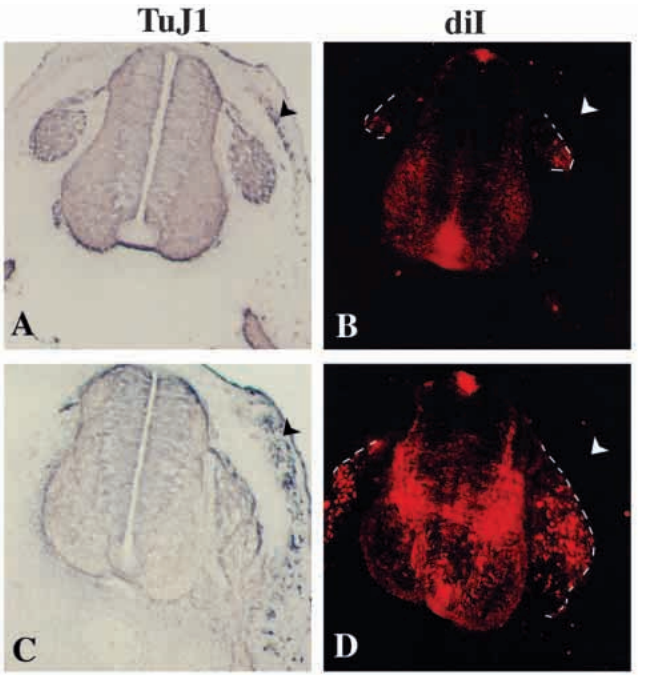

Fig. 7. Cells expressing neuronal markers in the dermomyotome are not of crest origin. At HH13-15, the dermamyotome was infected with RCAS-m-NGN-1 via unilateral viral injection into somites at forelimb level, and neural crest derivatives were labeled via DiI injection into the neural tube. Two animals are shown. Cells that ectopically express TuJ1 in the dermamyotome (A,C, arrowheads) are not labeled with DiI (B,D).

cells also express ISL2 (Fig. 6J, arrowheads), but not the motorneuron marker HB9 (data not shown); as mentioned earlier, this combination of transcription factor markers is sensory-specific. Thus, expression of the NGNs in the dermomyotome also induces the expression of five genes that are specific markers of sensory neurons in the PNS.

In contrast to the NGNs, ectopic expression of MASH-1 in the dermomytome induces the expression of neuron-specific tubulin (TuJ1) and NF-L (270RMO), but not of SCG10 and only weakly of NeuN (Table 4A). Furthermore, no ectopic expression of ISL2, trkC or TAG-1/axonin-1 is visible in MASH-1-infected embryos (Table 4B; data not shown). MASH-1 also fails to induce the expression of the sympathetic marker tyrosine hydroxylase (Cochard et al., 1979) in the dermomyotome, and only very weakly induces expression of GATA-2, another sympathetic specific marker (Groves et al., 1995; Yamamoto et al., 1990). Thus MASH-1 induces the expression of only two pan-neuronal markers, fails to induce sensory-specific markers and poorly induces one autonomic-specific marker in this mesoderm-derived tissue.

We considered the possibility that the sensory neuron-like cells induced by m-NGN-1 in the dermomyotome might derive from neural crest cells that were infected en route through the somite, and which then stopped migrating prematurely and differentiated ectopically. This scenario is highly unlikely since, given the 18 hour lag time between infection and widespread expression of virally encoded proteins (Homburger and Fekete, 1996; Martinsen and Bronner-Fraser, 1998), and the estimated 5 hours that it takes for a migrating crest cell to traverse the somite (Loring et al., 1982), even premigratory neural crest cells infected at the time of injection would not be expected to express the transgene until after they had migrated through the somite. Consistent with this, as shown above, crest cells deliberately infected in the somite successfully migrated to the SG and peripheral nerve.

We addressed this issue experimentally by labeling premigratory neural crest cells with Cell Tracker DiI/CM in the same embryos in which mesodermal derivatives were infected with m-NGN-1 retrovirus. If the ectopic neurons in the dermomyotome were crest-derived, they should also be DiI-labeled. Among four animals showing ectopic TuJ1 expression in the dermomyotome (Fig. 7A,C) as well as extensive DiI labeling of crest derivatives (Fig. 7B,D), none showed DiI^+ cells in the region of somitic TuJ1 expression. We also addressed the possibility that the neuronal cells in the dermomyotome were derived from overlying ectoderm which had been infected with NGN virus, by infecting the dermomyotome and then subsequently labeling the overlying ectoderm with DiI/CM. None of the animals with ectopic TuJ1 in the dermomyotome showed DiI^+ cells in the dermomyotome (n=7; not shown), indicating that the $TuJ1^+$ cells do not delaminate from the ectoderm. Thus, the neuronal like cells induced by ectopic expression of m-NGN-1 in the dermomyotome are unlikely to be of either neural crest or ectodermal origin, and are therefore most likely to be mesodermal cells.

DISCUSSION

The neural crest gives rise to sensory and autonomic neurons, but the time at which these two lineages first become specified is not clear. Although clonal analyses in vitro and in vivo have shown that premigratory and even some early migrating crest cells can generate both sensory and sympathetic autonomic neurons (Bronner-Fraser and Fraser, 1988, 1989; Duff et al.,

1991; Fraser and Bronner-Fraser, 1991), some crest cells in these experiments generated only sensory derivatives. Such observations do not, however, distinguish whether these cells were already restricted to a sensory fate, or rather were multipotent and had simply failed to express additional fates. Here we have identified the chick *neurogenins*, genes which in the mouse are essential for sensory neurogenesis (Fode et al., 1998; Ma et al., 1998; Q. Ma et al., unpublished), and have shown that they are expressed in a subset of migrating neural crest cells. Ectopic expression of the NGNs in premigratory crest cells biases their distribution to the DRG. Furthermore, ectopic expression of the NGNs in non-sensory crest derivatives and in paraxial mesoderm induces the expression of both pan-neuronal and sensory-specific markers. Taken together, these data suggest that expression of exogenous NGNs in neural crest cells is sufficient to determine some aspects of a sensory neuron phenotype. Therefore, the expression of endogenous *ngns* observed in a subset of migrating neural crest cells may indicate that some of these cells are already specified for a sensory fate.

Neurogenins are expressed by a subset of neural crest cells early in migration

In both the chick and the mouse (Q. Ma et al., unpublished), the *ngns* are expressed by migrating neural crest cells. However, in chick, the first crest cells to migrate in the trunk region at HH 13 do not express either *c-ngn-1* or *c-ngn-2*; nor are other known bHLH genes, such as *Cash1, Cash4* and *NeuroM* expressed in these cells (Jasoni et al., 1994; Wang and Kirby, 1994; Henrique et al., 1997; Roztocil et al., 1997). Rather, the *ngns* are expressed later, at HH 14/15. Interestingly, this timing roughly coincides with the onset of sensory neuron generation (Weston, 1963). At later stages, fewer crest cells express the NGNs than express the pan crest marker, HNK-1. These data suggest that NGN-expressing cells represent a subset of migrating neural crest cells. Consistent with this, in rat neural tube explant cultures *ngn*-expressing cells comprise only a small proportion of the crest cells that migrate onto the substrate (A. Greenwood and D. J. Anderson, unpublished).

Although an in vivo lineage tracing study demonstrated that some migrating neural crest cells are multipotent, greater than 25% of the clones in this study contributed only to the DRG (Fraser and Bronner-Fraser, 1991). Furthermore, studies of cultured avian crest cells have raised the possibility that there may exist some early-specified sensory neuron precursors (Sieber-Blum, 1989b; Ziller et al., 1983, 1987). In light of these data, the restricted expression pattern of the NGNs is interesting in that, at the very least, it provides a molecular correlate of what may be an early sensory lineage specification event. Moreover, as discussed below, our gain-of-function data suggest that there is a causal relationship between expression of the NGNs and specification of a sensory neuron fate. In the same way, the proneural gene *atonal*, a homolog of the *ngns*, specifies a particular sensory organ fate in *Drosophila* (Jarman et al., 1993).

Ectopic expression of neurogenins specifies neural crest cells for a sensory neuron fate

Ectopic expression of the NGNs in premigratory neural crest biases crest cell distribution toward the DRG. Furthermore, NGN-infected cells appear to differentiate appropriately for the DRG environment, based on their co-expression of pan-neuronal and sensory-specific markers. Because these data do not distinguish between permissive and instructive roles for the NGNs within the DRG, we examined the effects of misexpression of the NGNs on non-sensory crest derivatives. Upon infection with the NGN viruses, some cells in the sympathetic ganglia (SG) expressed the sensory neuron marker, NSCL-2, while non-neurogenic crest cells along the peripheral nerve ectopically expressed pan-neuronal and several sensory-specific markers (Fig. 8). These data suggest that NGNs can act instructively within such non-sensory crest derivatives (as well as in non-crest-derived mesoderm) to induce some aspects of a sensory phenotype. By extension, they are likely to act on crest cells within the DRG in an instructive manner as well.

An instructive influence of NGN could cause a positive bias of crest cell distribution towards the DRG, a negative bias away from the SG, or both. There are several possible cellular mechanisms that could underly such a biasing function. First, exogenous NGN could promote cell migration towards the DRG, or could inhibit ventral migration to the SG, for example by altering the expression of cell surface molecules that control homing or adhesion. Second, NGN could lead to the death of infected cells within the SG, for example by inducing dependence on a sensory-specific survival factor. Third, NGN could promote mitotic arrest that indirectly prevents crest cell migration beyond the DRG. Finally, NGN could bias a cell fate choice towards the sensory lineage, activating a differentiation program that consequently affects some or all of the foregoing cellular properties. It is currently difficult to experimentally distinguish between these various cellular mechanisms in this paradigm. Therefore we cannot say with certainty whether NGN promotes a sensory fate at the expense of an autonomic (sympathetic) fate. Nevertheless, if NGNs can induce ectopic expression of five different sensory neuron markers in mesodermal cells, then it seems highly likely that they can also induce sensory neuron differentiation in neural crest cells (where they normally function) as well. This interpretation is further strengthened by the ability of ATONAL, a *Drosophila* homolog of the NGNs, to promote determination of a particular sense organ identity in the fly (Jarman et al., 1993).

These gain-of-function and expression data, when taken together with loss-of-function data from the mouse, have implications for the process of lineage specification in the neural crest. In the mouse, all sensory neurons must derive from crest cells that eventually express one of the NGNs, since the DRG are completely absent in *ngn1/ngn2* double mutants (Q. Ma et al., unpublished). Although this does not necessarily mean that *all* NGN-expressing crest cells must differentiate to sensory neurons, it is likely that many of them do. Therefore, the expression of *ngns* identifies a subset of neural crest cells that are specified for a sensory neuron fate, in the sense that most if not all of them likely become sensory neurons (Davidson, 1990; Jan and Jan, 1994). This need not imply that these cells are fated *only* to generate sensory neurons, or that they are independent of environmental signals that promote sensory neuron differentiation. Whether some NGN-expressing crest cells produce derivatives other than sensory neurons will require methods to map the fate of transiently NGN-expressing neural crest cells (Zinyk et al., 1998). However, the fact that ectopic NGN expression biases crest

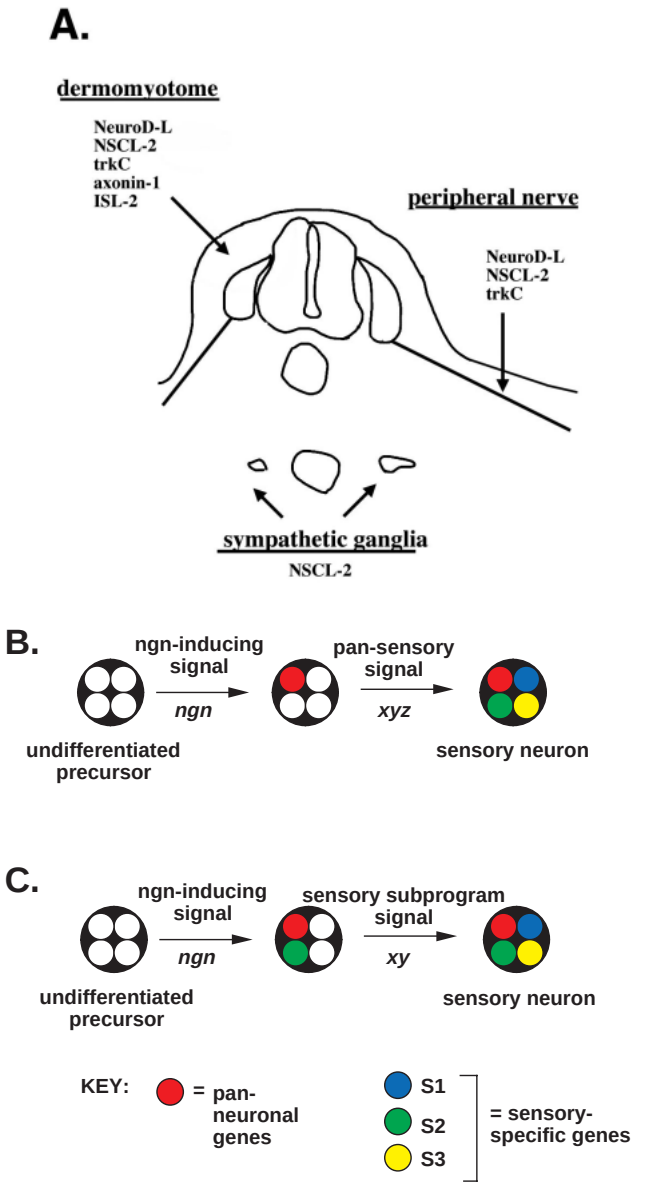

Fig. 8. (A) Ectopic induction of sensory neuron markers varies with dorso-ventral location of infected cells. The most extensive induction of sensory neuron markers in response to NGN misexpression occurs adjacent to the DRG in the dermomyotome. In more lateral or ventral locations, the peripheral nerve and the sympathetic ganglia, fewer sensory markers are induced. Note that it was not possible to score for *trkC* in sympathetic ganglia since this gene is already expressed by immature sympathetic neurons (Birren et al., 1993; DiCicco-Bloom et al., 1993). Nor was it possible to score for TAG-1/axonin-1 in peripheral nerve or in sympathetic ganglia, because spinal axons in this region normally express this protein (Furley et al., 1990; Wolfer et al., 1994). (B,C) Two alternative views of the relationship between NGN activity and sensory identity. Each colored circle represents a subset of the terminal differentiation genes expressed in sensory neurons, that are coordinately controlled by a common transcriptional regulatory program (see Key). (B) NGN only controls expression of pan-neuronal properties (red circle) and all sensory-specific properties are induced by other signals acting through a different transcriptional regulatory program(s) (*xyz*). NGN may, however, be necessary for cells to respond to these signals. (C) NGN autonomously controls expression of both pan-neuronal genes and a subset of sensory-specific genes (green circle), such as *NSCL-2*. Other sensory-specific genes (blue and yellow circles), such as *Isl2*, would require additional inducing signals to be expressed.

distribution towards the DRG, and can induce some aspects of sensory neurogenesis in both crest and non-crest cells, strongly suggests that NGN may normally restrict crest cells to a sensory fate. If so, then the early expression of NGNs in a subset of neural crest cells would indicate that this restriction occurs early in migration.

Does neurogenin encode a sensory neuron identity?

An important issue in the molecular control of neurogenesis is the extent to which generic aspects of neuronal phenotype are coordinately versus separately regulated from subtype-specific aspects of phenotype (Anderson and Jan, 1997; Edlund and Jessell, 1999). NGNs have been shown to activate generic neuronal markers in the ectoderm (Blader et al., 1997; Ma et al., 1996; Olson et al., 1998), and we have shown that they can induce at least five different pan-neuronal markers in the mesoderm. These data are consistent with the idea that NGNs can activate a ‘core’ program of neurogenesis leading to expression of generic neuronal properties. But whether NGNs additionally specify a particular neuronal subtype has not been addressed in detail. In this context, our observation that expression of NGNs can induce several sensory-specific (in addition to pan-neuronal) genes in a mesodermal derivative is striking.

In *Drosophila*, misexpression experiments have shown that proneural genes such as *ac-sc* and *atonal* contribute to the specification of neuronal subtype in both the embryonic PNS and CNS (Jarman and Ahmed, 1998; Jarman et al., 1993; Skeath and Doe, 1996). Furthermore, two different vertebrate members of the atonal family have been implicated in the generation of different types of retinal neurons (Kanekar et al., 1997; Yan and Wang, 1998). These data suggest that such bHLH factors can contribute to some aspects of neuronal subtype. Such a contribution must, however, be context-dependent, since (for example) ATONAL specifies both chordotonal organs in the PNS (Jarman et al., 1993) and photoreceptors in the eye (Jarman et al., 1994). Similarly, *ngns* are expressed in the ventral neural tube (as well as in other regions of the CNS), where they may be involved in motorneuron differentiation, for example (Sommer et al., 1996; Gradhwohl et al., 1996). On the one hand, proneural genes could autonomously specify both a core program of neurogenesis and a ‘ground’ state neuronal subtype, which can be modified by different environments, and indeed, it has been suggested that the ground state for NGN-expressing cells may be sensory, although this has been based on a single sensory marker (Blader et al., 1997) or a lack of motorneuron markers (Olson et al., 1998). On the other hand, proneural genes might specify only a core program of neurogenesis, and all aspects of subtype identity would be specified independently by environmental signals acting via other transcription factors (Groves et al., 1995; Lo et al., 1998). In the spinal cord, for example, Sonic Hedgehog induces a homeodomain transcription factor called MNR2, which coordinately specifies multiple aspects of motorneuron identity (Tanabe et al., 1998).

The use of five different sensory-specific markers has

allowed us to examine the contribution of NGNs to sensory neuron identity in more detail. The most extensive induction of sensory markers was observed in a dorso-lateral location, within the dermomyotome adjacent to the DRG (Fig. 8). Mesodermal cells in this location are in the correct position to receive most of the environmental cues that would be received by neuronal precursors in the DRG. Ectopic expression of NGN in areas ventral to the DRG – the peripheral nerves and the SG – resulted in the induction of fewer sensory markers (Fig. 8). This apparent positional restriction in the ectopic expression of sensory markers could reflect either an increased distance from dorsal sensory-inducing cues, or a decreased distance from ventral cues inducing non-sensory fates. The fact, however, that some sensory markers were detected in all ectopic locations suggests that NGNs induce at least a subset of sensory-specific properties. The notion that different components of a given neuronal identity may be controlled by distinct genetic subprograms (Anderson and Jan, 1997) has received experimental support from studies of motor neuron (Lin et al., 1998; Sockanathan and Jessell, 1998) and autonomic neuron (Groves et al., 1995; Lo et al., 1998; Morin et al., 1997) subtype specification. In this case, NGNs might be sufficient to induce some components of sensory phenotype, while conferring competence to respond to local cues in the DRG environment that control other components.

Induction of neurogenesis within the mesoderm

All prior studies of NGN misexpresion have shown that NGN can induce neurogenesis in non-neurogenic ectoderm (Blader et al., 1997; Ma et al., 1996; Olson et al., 1998). However, as neurons are ectodermally derived and have been suggested to represent a 'default' pathway of differentiation for this germ layer (Kelly and Melton, 1995), these studies did not distinguish whether *ngns* can simply trigger neurogenesis in a tissue already competent for this fate, or rather can divert tissues of more distant embryonic lineages to a neuronal fate. The present data provide the first demonstration that NGNs can ectopically induce neuronal markers in the mesoderm. We have also found that NGNs can induce some neuronal markers in cultured embryo fibroblasts (S. Perez and D. J. Anderson, unpublished). Nevertheless, the ability of NGNs to promote neurogenesis in heterologous cell types appears to be stage- or context-dependent, since ectopic expression of NGN-1 in differentiated glial or muscle cells in vitro does not induce neuronal differentiation (L. Lo and D. J. Anderson, unpublished observations). In the same way, ectopic expression of *ac-sc* in *Drosophila*-induced ectopic sense organ differentiation in a highly spatio-temporally restricted manner (Rodriguez et al., 1990). These data for proneural genes stand in contrast to those obtained for the myogenic bHLH protein, MyoD, which has been demonstrated to induce myogenesis in a wide variety of cell types at different stages of development (Weintraub et al., 1989). This apparent difference in the competence of heterologous cell types to respond to myogenic versus neurogenic determination factors could reflect differences in the tissue distribution of essential cofactors, or of counteracting negative regulators (Chen et al., 1998). Alternatively, it could reflect a fundamental difference in the chromatin accessibility of muscle-specific and neuron-specific target genes, or in the ability of myogenic versus neurogenic determination genes to 'open' transcriptionally inaccessible chromatin (Gerber et al., 1997).

Conclusion

The data presented here demonstrate that the NGNs are expressed in a subset of migrating neural crest cells, and that NGN is likely to specify at least some aspects of sensory neuron differentiation within the neural crest. Taken together with loss-of-function data from the mouse (Ma et al., 1998; Fode et al., 1998; Q. Ma et al., unpublished), these gain-of-function and expression studies strongly suggest that most if not all NGN-expressing crest cells are fated to give rise to sensory neurons. Our data are therefore consistent with a growing body of evidence (Erickson and Goins, 1995; Henion and Weston, 1997; Raible and Eisen, 1994) suggesting that there is a greater degree of early fate specification among some neural crest cells than has previously been appreciated. They also identify a transcription factor that may be causally involved in such early fate specification.

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