

# Fate determination of neural crest cells by NOTCH-mediated lateral inhibition and asymmetrical cell division during gangliogenesis

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## SUMMARY

Avian trunk neural crest cells give rise to a variety of cell types including neurons and satellite glial cells in peripheral ganglia. It is widely assumed that crest cell fate is regulated by environmental cues from surrounding embryonic tissues. However, it is not clear how such environmental cues could cause both neurons and glial cells to differentiate from crest-derived precursors in the same ganglionic locations. To elucidate this issue, we have examined expression and function of components of the NOTCH signaling pathway in early crest cells and in avian dorsal root ganglia. We have found that *Delta1*, which encodes a NOTCH ligand, is expressed in early crest-

derived neuronal cells, and that NOTCH1 activation in crest cells prevents neuronal differentiation and permits glial differentiation in vitro. We also found that NUMB, a NOTCH antagonist, is asymmetrically segregated when some undifferentiated crest-derived cells in nascent dorsal root ganglia undergo mitosis. We conclude that neuron-glia fate determination of crest cells is regulated, at least in part, by NOTCH-mediated lateral inhibition among crest-derived cells, and by asymmetric cell division.

Key words: Neural crest, Neuron, Glia, *Notch*, *Delta*, *Numb*, Lateral inhibition, Asymmetric cell division, Chick

## INTRODUCTION

During vertebrate embryonic development, neural crest cells emerge from the dorsal neural tube and migrate on distinct pathways to give rise to a wide variety of cell types including melanocytes in the skin, and neurons and glial cells in the peripheral nervous system (PNS). Although fate-restricted precursors have been recognized within the migrating crest cell population, it is generally assumed that the fates of crest cells are gradually determined under the influence of environmental cues from surrounding tissues. As a well-accepted example of such an environmental factor, BMP2/4 from dorsal aorta has been shown to induce the sympathetic neuron phenotype in crest-derived cells (Shah et al., 1996; Reissmann et al., 1996; Varley and Maxwell, 1996). Other soluble factors and extracellular matrices may also affect fate determination (see reviews by Stemple and Anderson, 1993; Anderson, 1997). Emerging evidence suggests, however, that interaction among crest cells themselves may also function as a fate-restricting mechanism. In zebrafish, for example, it has been suggested that the interactions between crest populations that migrate at early and late times on the same pathway prevent the late population from taking the neurogenic fate (Raible and Eisen, 1996). Likewise, in vitro clonal analysis has revealed that, under conditions in which crest cells can interact with each other, the vast majority of individual clonal progenitors were developmentally restricted, and that fate-restricted neurogenic

and melanogenic precursors segregate soon after emergence from the neural tube explant (Henion and Weston, 1997). Although, in these studies, it is impossible to conclude that such precursors are irreversibly committed to certain fates, the fact that fate restrictions appear under culture conditions known to permit the differentiation of other major crest-derived phenotypes in closely contiguous cells, supports the inference that crest cell-autonomous fate restricting mechanisms exist.

It is well known that NOTCH signaling plays a key role in tissue-autonomous fate determination referred to as a lateral inhibition both in fruit fly (*Drosophila*) neurogenesis and in neurogenesis of the vertebrate central nervous system (CNS) (reviewed in Artavanis-Tsakonas et al., 1999). NOTCH and its ligands are membrane-anchored proteins, and NOTCH appears to be activated through binding of ligands expressed in neighboring cells. Thus, cells that express higher levels of a NOTCH ligand (e.g. DELTA), take a primary fate, representing a 'default regulatory pathway', and activate the NOTCH signaling pathway in neighbors to prevent them from taking the same fate. Activating NOTCH signaling in the vertebrate CNS has been shown to inhibit neuronal differentiation, whereas repressing NOTCH activation increases neuronal differentiation (Coffman et al., 1993; Chitnis et al., 1995; Austin et al., 1995; Dorsky et al., 1995; Dornseifer et al., 1997; Henrique et al., 1997; Haddon et al., 1998; Appel and Eisen, 1998; Wakamatsu et al., 1999). Based on these observations, it has been proposed that, in the ventricular zone of the

developing spinal cord, activation of NOTCH signaling by NOTCH-ligand-expressing neighbors prevents neuroepithelial cells from undergoing neuronal differentiation.

NOTCH signaling is now known to be modulated in various ways in many embryonic systems. For example, *Deltex* and *Notchless* are known to bind the ankyrin/cdc10 repeat motif of the cytoplasmic domain of NOTCH, and modulate NOTCH signaling positively and negatively, respectively (Matsuno et al., 1995; Royet et al., 1998). Moreover, *Numb* gene products, which have been identified both in *Drosophila* (Rhyu et al., 1994) and vertebrates (Verdi et al., 1996; Zhong et al., 1996, 1997; Wakamatsu et al., 1999) are known to antagonize NOTCH signaling by binding to the cytoplasmic domain of NOTCH (Guo et al., 1996; Zhong et al., 1996, 1997; Wakamatsu et al., 1999). Importantly, NUMB is often asymmetrically segregated between daughter cells during mitosis. In *Drosophila* nervous system development, NUMB localizes asymmetrically in mitotic precursor cells, and is subsequently distributed to one of the daughter cells (Rhyu et al., 1994; Spana et al., 1995). As a result, NUMB creates a difference in sensitivity to NOTCH activation in the two daughter cells. In *Drosophila*, for example, when external sensory organ precursor cells divide, NUMB is inherited in only one (the IIb) precursor, whose daughter cells eventually produce glia and IIIb (a precursor for neuron and sheath cells), but not hair and socket cells (reviewed by Campos-Ortega, 1996; see also Reddy and Rodrigues, 1999). Asymmetrical cell division is also known to occur in vertebrate CNS development (Chenn and McConnell, 1995; Wakamatsu et al., 1999), and NUMB asymmetrically localizes in the basal cortex of mitotic neuroepithelial cells of chicken embryos (Wakamatsu et al., 1999).

Since the expression of *Notch* genes and their ligands in the vertebrate PNS has been noted (Myat et al., 1996; Lindsell et al., 1996; Shawber et al., 1996), NOTCH signaling may possibly be involved in the fate determination and/or differentiation of crest-derived cells. Here we show that NOTCH signaling is involved in the neuron/glia cell fate decision in avian trunk crest-derived cells. We also show that NUMB is asymmetrically localized in some mitotic crest-derived cells, suggesting that its differential segregation into one of the daughter cells modulates NOTCH signaling in this system.

## MATERIALS AND METHODS

### Experimental animals

Fertilized chicken (F<sub>1</sub> of White Leghorn × New Hampshire) and Japanese quail (*Coturnix coturnix japonica*) eggs were obtained from the Oregon State University, Animal Sciences Department, Corvallis, Oregon. Embryos were staged according to Hamburger and Hamilton (1951).

### Neural crest culture

Primary culture of quail trunk neural crest cells and BrdU pulse-labeling were performed as described previously (Marusich et al., 1994; Wakamatsu et al., 1997). For the labeling studies, cultures were exposed to BrdU for 1.5 hours. NT3 and zVAD-fmk were purchased from PeproTech and Bachem, respectively, and added to some crest cultures as described by Wakamatsu and Weston (1997); Jacobsen et al. (1996).

### In ovo BrdU labeling

BrdU pulse-labeling of chicken embryos was performed as described previously (Marusich et al., 1994). In some cases, after the 2 hours of labeling with 100–200 µl of 10 µg/ml BrdU, 200 µl of thymidine (60 µg/ml) was added to the embryos to stop the incorporation of BrdU. Such embryos were then incubated to allow further development. Labeled embryos were subsequently processed for in situ hybridization on sections (see below), followed by anti-BrdU (mouse IgG1; Boehringer) immunostaining (see below).

### Molecular cloning of quail homologs

The full coding region of quail *Delta1* and a partial sequence of quail *Serrate1* were amplified from oligo(dT) primed day-3 embryo cDNA generated with Superscript II (Gibco). Primer sets were designed according to the previously published chicken genes (Henrique et al., 1995; Myat et al., 1996). Quail *Notch2* was amplified from oligo(dT) primed cDNA of E6 quail embryo dorsal root ganglia with the forward primer TG(C/T)TT(C/T)AA(C/T)GGNGGNACITG and the reverse primer A(A/G)NCC(A/G)TCCCAN(C/T)C(A/G)CA(C/T)TC, followed by PCR with the same forward primer and the nested reverse primer CC(A/G)TCCCAN(C/T)C(A/G)CA(C/T)TCI(G/T)C. The forward and reverse primers correspond respectively to the conserved amino acids CFNGGTC and (A/E)EC(E/G)WDGL that are found in the human, rat, *Xenopus* and zebrafish homologs of *Notch1* as well as the mouse homologs of *Notch2* and *Notch3*.

### Antibodies and immunological staining

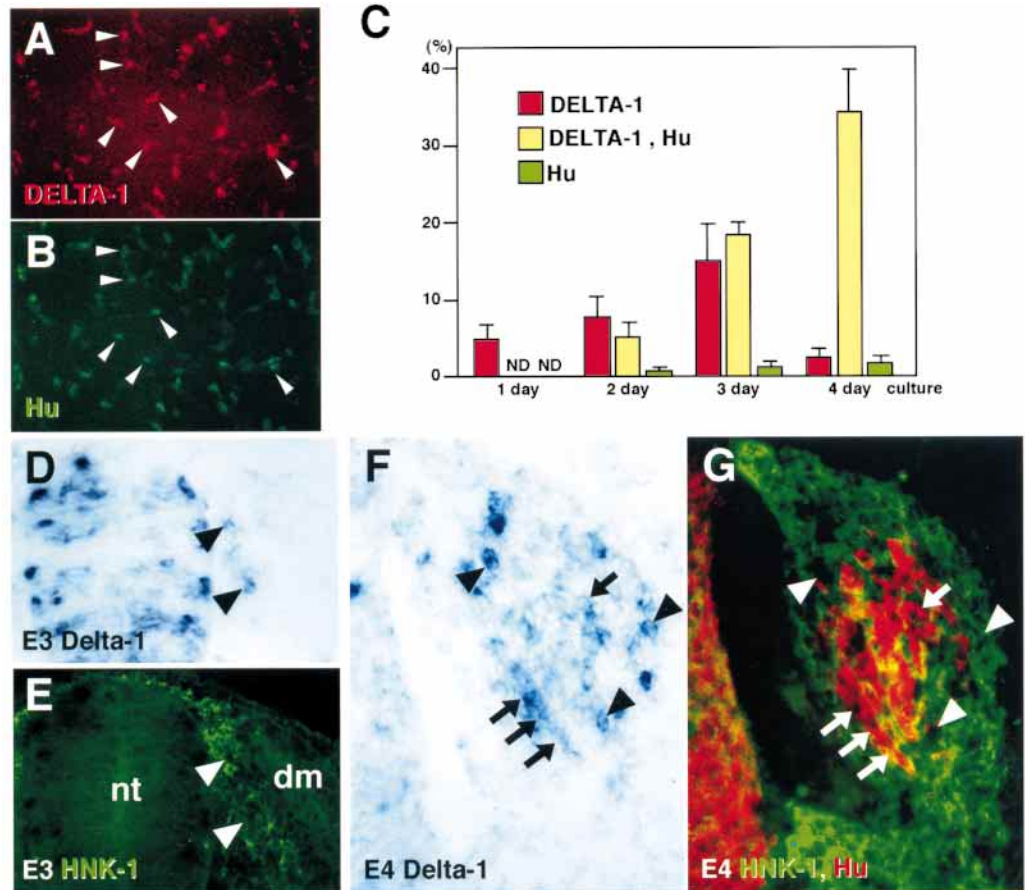
Rabbit anti-DELTA1 antiserum was raised by immunizing rabbits with a synthetic polypeptide (LKLQEFVNKKGLLSNRNC; Alpha Diagnostic International). Subsequently, antibody was purified with an antigen-coupled affinity column (Sulfolink kit; Pierce) according to the manufacturer's instructions. Specificity of the antibody was confirmed by western blotting against bacterially expressed DELTA1 protein and immunostaining of *Delta1*-transfected L cells, a mouse fibroblast cell line (data not shown). 16A11 anti-Hu, HNK1, 7B3, 1E8 anti-P0, anti-Sox2, anti-NUMB antibodies were used as described previously (Marusich et al., 1994; Tucker et al., 1988; Henion and Weston, 1997; Bhattacharyya et al., 1991; Kamachi et al., 1998; Wakamatsu et al., 1999). Anti-BrdU (mouse IgG1, Boehringer),  $\gamma$ -tubulin gtu-88 (mouse IgG1, Sigma), FLAG M2 (mouse IgG1, Sigma) and FLAG rabbit polyclonal (Zymed) antibodies were purchased from manufacturers. Fluorochrome-conjugated secondary antibodies were purchased from Southern Biotechnologies (anti-mouse IgM-FITC, and -TRITC, anti-mouse IgG1-FITC, Texas Red and biotin, anti-mouse IgG2b-FITC), Chemicon (anti-mouse IgG-cy3), Cappel (anti-rabbit IgG-cy3), Jackson (anti-rabbit IgG-FITC, anti-mouse IgG-TRITC), and Molecular Probes (NeutraAvidin-AMCA-S).

Immunological stainings were performed as described previously (Wakamatsu et al., 1993; Marusich et al., 1994), except for anti-DELTA1 and anti-P0 staining in culture. In these cases, cultured neural crest cells obtained from quail trunk neural tube or *Delta1*-transfected L-cells were both incubated with culture medium containing primary antibody at 37°C for 1 hour. After washing in culture medium 5 times, cultures were further incubated with culture medium containing either cy3-conjugated anti-rabbit IgG or cy3-conjugated anti-mouse IgG for 1 hour at 37°C. After washing with culture medium, stained cells were fixed in 4% PFA in PBS and counter-stained with the other antibody and DAPI.

### In situ hybridization

In situ hybridizations on sections and in whole-mount were performed as described previously (Wakamatsu and Weston, 1997). Chicken *SCG10* (Groves et al., 1995; Sukegawa et al., 2000), *neuregulin* (Yang et al., 1998) and *Sox2* (Kamachi et al., 1998) probes were gift from Drs Kimiko Fukuda, Lorna Role and Hisato Kondoh, respectively. In situ with chicken probes in quail tissues gave identical results with the same probes in chicken tissues.

**Fig. 1.** Crest-derived neuronal cells express *Delta1* both in culture and in vivo. (A,B) Crest cells cultured for 3 days were stained with anti-DELTA1 (cy3, red) and anti-Hu (FITC, green) antibodies. Most Hu-positive neuronal cells coexpress DELTA1 (arrowheads). Punctate staining of anti-DELTA1 antibody (A) is characteristic of the cell surface antigen. (C) Counts of DELTA1- and Hu-expressing cells in culture. Means were obtained from three independent experiments. ND, Not detected. (D,E) Transverse section of a day-3 (stage 17) embryo. Arrowheads indicate a subpopulation of HNK1-positive migrating crest cells (FITC, green) expressing *Delta1* mRNA (blue-purple). Expression of *Delta1* is also present in the neural tube. (F,G) A transverse section of a day-4 (stage 23) embryo, showing the nascent DRG. *Delta1* mRNA expression is found both in the peripheral region of DRG (arrowheads), and in Hu-positive neurons (arrows). dm, dermomyotome; nt, neural tube.



### Construction of expression vectors and transfection

FLAG epitope-tagged expression vectors of *GFP*, *CNIC<sup>4C89</sup>* and *Numb* have been described previously (Wakamatsu and Weston, 1997; Wakamatsu et al., 1999). Quail *Delta1* expression vector was constructed in pCDNA3.1 (Invitrogen). Transfection with LipofectAmine plus (Gibco) was performed on neural crest outgrowth cultured for 13 hours, and crest cells were subsequently replated into Sylgard wells as described previously (Wakamatsu and Weston, 1997; Wakamatsu et al., 1999). Detailed protocols will be provided on request. Transfection of L cells was performed with LipofectAmine plus, according to the manufacturer's instruction.

## RESULTS

### Delta1 is expressed in early crest-derived neuronal cells

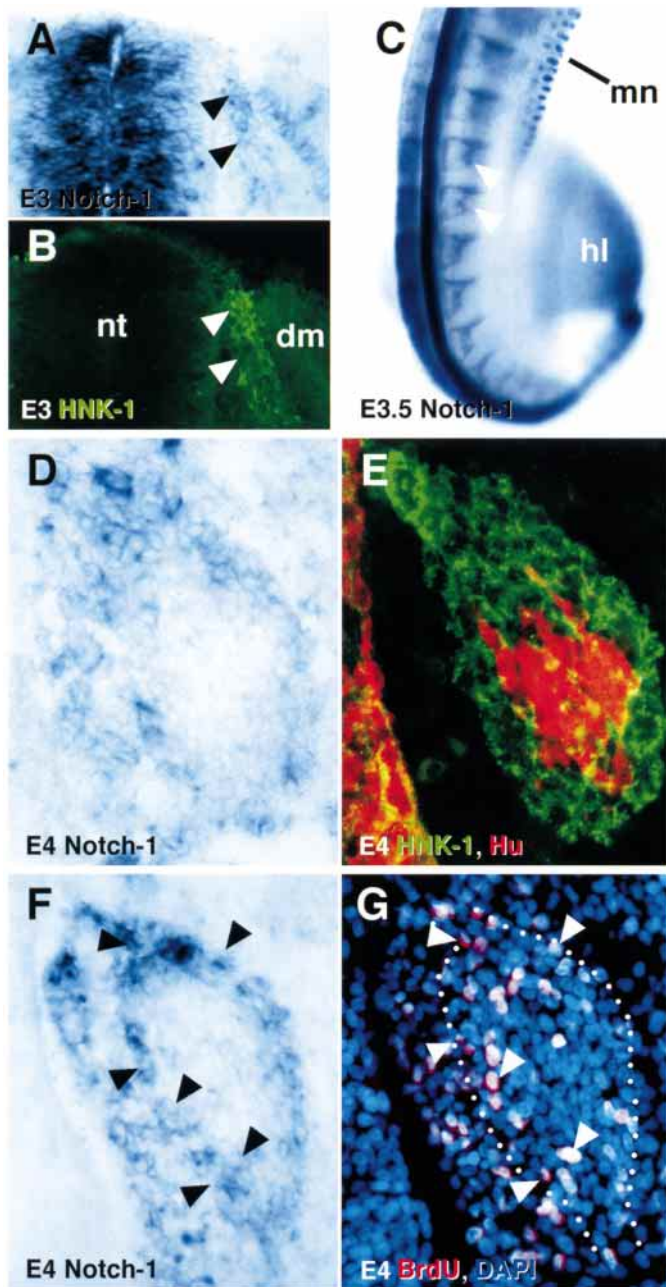
To elucidate the involvement of NOTCH-mediated lateral inhibition in neural crest cells, we first examined the expression patterns of *Notch1*, *Notch2*, *Delta1*, and *Serrate1* in neural crest-derived tissues of avian embryos. Since *Notch2* expression was low in such tissues (data not shown), and since *Serrate1* expression was only detected in maturing neurons at low levels (see also Myat et al., 1996), we focused on the expression of *Notch1* and *Delta1*.

Using anti-quail DELTA1 antibody that we developed (see Materials and Methods), we compared expression of DELTA1 protein with that of the neuron-specific RNA-binding protein Hu (Marusich et al., 1994; Wakamatsu and Weston, 1997) in outgrowth culture of quail crest cells (Fig. 1A-C). Initially,

DELTA1 protein expression was observed in a small subset of crest cells before any Hu expression was detected (Fig. 1C). Subsequently, however, most of the DELTA1-positive cells coexpressed Hu (Fig. 1A-C). No DELTA1 expression was detected in melanocytes (data not shown). These observations suggested that DELTA1-positive crest cells have neuronal identity.

Next, the embryonic expression of *Delta1* mRNA was examined in detail at the wing axial level of stage 16 to 33 (Hamburger and Hamilton, 1951) chicken embryos. *Delta1* expression was first detected in a subset of crest cells residing in the migration staging area (MSA; Weston, 1991) between the dorsal neural tube and dorsomedial edge of the somite at stage 16+/17- (data not shown). At stage 17, the *Delta1*-positive crest-derived cells, which coexpressed the neural crest marker, HNK1, were present more ventrally on the medial crest migration pathway (Fig. 1D, E). Cells on this pathway normally give rise to the components of the PNS. At stage 21-24, Hu-positive neurons appeared to form the neuronal core of nascent DRG, surrounded by HNK1-positive/Hu-negative cells (Fig. 1G). Most of the Hu-positive DRG cells coexpressed *Delta1* (Fig. 1F,G), as was also observed in vitro. A subpopulation of DRG cells in the peripheral HNK1-positive/Hu-negative domain also expressed *Delta1* (Fig. 1F,G). Based on the DELTA1 expression in culture, this expression pattern appeared to represent early neuronal cells. These observations suggested the possibility that *Delta1*-positive cells in the peripheral domain of developing DRG were the source of the Hu-positive cells in the core of the ganglion.





### Undifferentiated crest cells express *Notch1*, and produce both neurons and satellite glial cells in developing DRG

Initially, *Notch1* is expressed in most trunk crest cells both in vivo (Fig. 2A,B) and in culture (data not shown). At the onset of gangliogenesis in stage 23-24 embryos, *Notch1* expression was restricted to the periphery of the developing DRG and was absent from differentiating Hu-positive neurons in the core of the DRG (Fig. 2C-F). As development proceeded, the proportion of *Notch1*-positive cells in the peripheral domain gradually decreased. Later, when most neurogenesis in the ganglion had concluded (between stages 28-33), expression of both *Notch1* and *Delta1* was downregulated (data not shown), and Hu-negative satellite glial cells were observed intercalated between Hu-positive neurons (not shown, but see Figs 3K, 4C,D).

**Fig. 2.** *Notch1* is expressed in crest-derived cells, but subsequently is downregulated upon neuronal differentiation. (A,B) Transverse section of a day-3 (stage 17) embryo. Arrowheads indicate that *Notch1* mRNA (blue-purple) is expressed in most of the HNK1-positive migrating crest cells (FITC, green). Expression of *Notch1* is also present in the neural tube and dermomyotome. (C) Whole-mount preparation of day-3.5 embryo, showing *Notch1* mRNA expression in the periphery of nascent DRG (arrowheads). (D-G) Transverse sections of a day-4 (stage 23) embryo, showing nascent DRG. *Notch1* expression is restricted in the peripheral region of the DRG (D,F; blue-purple), and Hu-positive neurons in the core of DRG (E, TRITC, red) do not express *Notch1*. *Notch1*-positive cells often incorporate BrdU (G, TRITC, red; arrowheads). Blue in G indicates DAPI nuclear-staining. dm, dermomyotome; hl, hind limb bud; mn, mesonephros; nt, neural tube.

To examine the proliferative state of *Notch1*-positive cells in developing DRG, embryos were pulse-labeled with BrdU at stages 22-25 (Figs 2F,G, 3A-C,G,H). In stage 22 DRG (Fig. 3A-C), although many BrdU-positive cells localized in the periphery, segregation of the neuronal core and the peripheral domain of the DRG was still incomplete, and some BrdU-positive cells intermingled with Hu-positive neuronal cells (Fig. 3A and B, arrowheads). Nevertheless, as previously reported (Marusich et al., 1994), a few BrdU-labeled Hu-positive neuronal cells were observed (Fig. 3A,B, arrow). The distinction between the core and periphery of the DRG was more evident at stage 23 (Fig. 2F,G). In these embryos, although most Hu-positive cells were not BrdU positive, many of the *Notch1*-positive cells were labeled with BrdU, indicating that these cells were actively proliferating (Fig. 2F,G). However, as would be expected, neurons that coexpressed a late neuronal marker *SCG10* (Groves et al., 1995) no longer incorporated BrdU (Fig. 3C,C'). It should be noted, however, that if embryos received a thymidine 'chase' to stop further BrdU uptake after a 2-hour labeling period, and were then allowed to develop until stage 25, many BrdU-positive cells expressed *SCG10* in the DRG core (Fig. 3D-F). We conclude, therefore, that the post-mitotic *SCG10*-positive/Hu-positive neurons in the DRG core originate from proliferating undifferentiated *Notch1*-positive cells that were present in the DRG periphery.

To examine whether peripheral cells of the DRG also produce satellite glia, embryos that had been pulse-labeled with BrdU at stage 25 were allowed to develop until stage 28 (Fig. 3I-K). In these embryos, many BrdU-positive cells were found throughout the DRG (Fig. 3I). Although some Hu-positive neurons were also labeled with BrdU, most of the BrdU-positive cells did not express Hu, but were adjacent to Hu-positive neurons (Fig. 3K). These results suggested that the labeled cells were satellite glia. This inference was supported by the temporal change in the distribution of DRG cells expressing a transcription factor, *Sox2* (Uwanogho et al., 1995; Kamachi et al., 1998) (Fig. 4). Thus, at stages 22-25, the distribution of *Sox2* mRNA was restricted to the peripheral domain (Fig. 4A,B), similar to *Notch1* expression (Fig. 2D,F). At later stages, in contrast to the downregulation of *Notch1*, *Sox2*-positive cells were found throughout the DRG (Fig. 4C). Such *Sox2*-positive cells did not express the neuronal marker, Hu, and possessed small nuclei intensely labeled with DAPI, characteristic of satellite glial cells (Fig. 4D,E). *Sox2* expression was also detected in other glial lineages, such as

Schwann cell precursors along the spinal nerve (Fig. 4C). The identity of Sox2-positive cells as glia was further supported by the expression of a glial marker, P0 (Bhattacharyya et al., 1991), on the surface of some Sox2-positive crest cells cultured for 3 days (Fig. 4F,G). Taken together, these data suggest that *Delta1*-negative/*Hu*-negative/*Notch1*-positive/*Sox2*-positive cells in the periphery of developing DRG initially produce *Delta1*-positive/*Hu*-positive/*Notch1*-negative/*Sox2*-negative neurons, and later produce *Delta1*-negative/*Hu*-negative/*Notch1*-negative/*Sox2*-positive satellite glia.

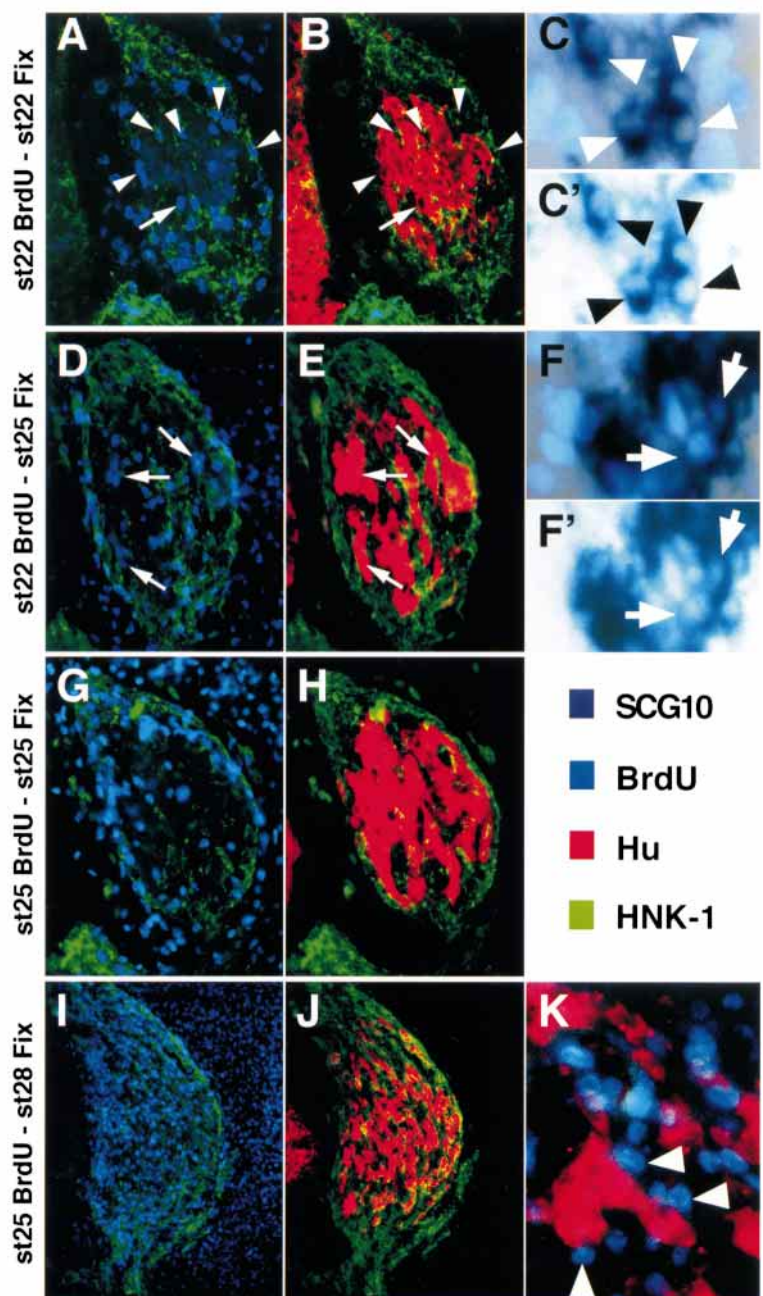
### Activation of NOTCH signaling inhibits neuronal differentiation, but not glial differentiation

The expression patterns described above revealed that *Delta1*-positive/*Hu*-negative neuronal precursors and *Notch1*-positive undifferentiated cells co-localized in the periphery of the DRG. If activation of NOTCH signaling in *Notch1*-positive cells in the peripheral domain of the DRG affects the fate of undifferentiated precursors, we reasoned that *Delta1*-positive neuronal cells might inhibit neuronal differentiation of their neighboring cells, which would remain in the proliferating population. We also predicted that at least some of these NOTCH1-activated cells would subsequently assume an alternative fate and differentiate into satellite glia. To test these predictions, we manipulated NOTCH signaling in cultured quail crest cells (Fig. 5; see Materials and Methods). To activate and inactivate NOTCH signaling, we used expression vectors containing FLAG-tagged cytoplasmic domain of chicken *Notch1* (*CNIC<sup>AC89</sup>*) and FLAG-tagged chicken *Numb*, respectively (Wakamatsu et al., 1999). FLAG-tagged *GFP* served as a negative control (Wakamatsu and Weston, 1997; Wakamatsu et al., 1999). Cultures were exposed to a 1.5-hour pulse of BrdU, 24 hours after transfection. In the control cultures, approximately 23% of the *GFP*-transfected cells incorporated BrdU (Fig. 5A). Compared to this control,

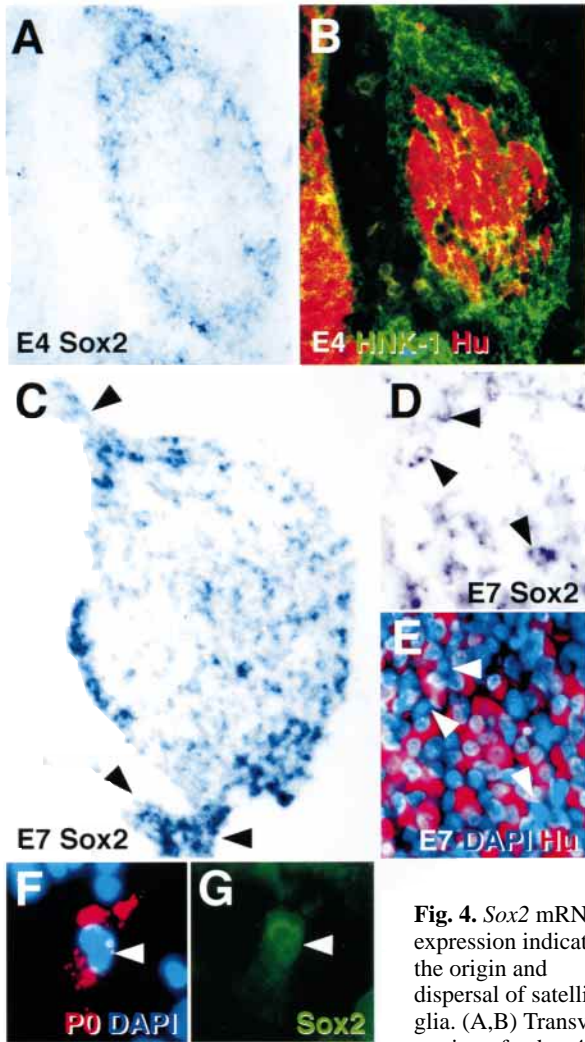
the proportion of *CNIC<sup>AC89</sup>*-transfected cells that incorporated BrdU increased moderately (37%; Fig. 5A,B), and the proportion of *Numb*-transfected cells that incorporated BrdU decreased (9%; Fig. 5A). These results suggest that NOTCH activation maintains crest cells in a proliferating, undifferentiated state.

Neuronal differentiation was assessed in cultures 72 hours after transfection (Fig. 5C-E). Activation of NOTCH signaling by *CNIC<sup>AC89</sup>* drastically reduced neuronal differentiation (5%; Fig. 5C), compared to the *GFP*-transfected control (41%; Fig. 5C). Unexpectedly, over-expression of *Numb* appeared to induce apoptosis in transfected cells, and most of the *Numb*-transfected cells disappeared within 72 hours (data not shown). When a caspase inhibitor zVAD-fmk (Fig. 5C; see also Materials and Methods) was added, however, *Numb*-

**Fig. 3.** BrdU pulse-labeling reveals rearrangement of cells during neurogenesis and gliogenesis in developing DRG. (A-C) Developing DRG, labeled with BrdU at stage 22, and fixed immediately. At this stage, *Hu*-positive neuronal cells and mitotically active undifferentiated DRG cells were partially intermingled. Most BrdU-positive cells (AMCA-S, blue in A) do not express *Hu* (TRITC, red in B; arrowheads), but a few *Hu*-positive neuronal cells incorporated BrdU (arrow; see Marusich et al., 1994). Cells strongly expressing the late neuronal marker, *SCG10* (blue-purple in C and C'; arrowheads), did not incorporate BrdU (AMCA-S, light blue in C; arrowheads). (D-F) DRG, BrdU-labeled at stage 22, chased with thymidine, and fixed at stage 25. Many BrdU-positive cells (blue in D, light blue in F) have dispersed throughout the DRG, and some of them overlap with *Hu* staining (arrows). Some *SCG10*-expressing cells (blue-purple in F and F'; arrows) incorporated BrdU (light blue in F; arrows). (G,H) DRG, labeled with BrdU at stage 25, and fixed immediately. Most BrdU-positive cells (blue in G) localize in the peripheral region of the DRG. (I-K) DRG, BrdU-labeled at stage 25, chased with thymidine, and fixed at stage 28. Many BrdU-positive cells (blue in I) have dispersed throughout the DRG. Most of them (arrowheads in K) do not express *Hu* and are intercalated with *Hu*-positive neurons (red in K), suggesting their satellite glial identity.

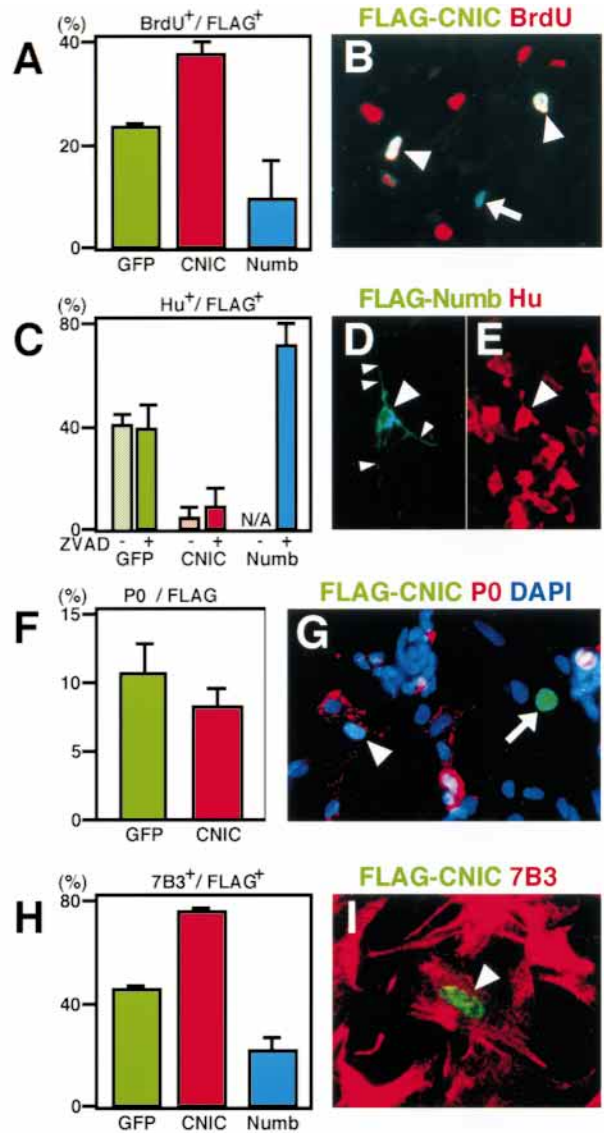






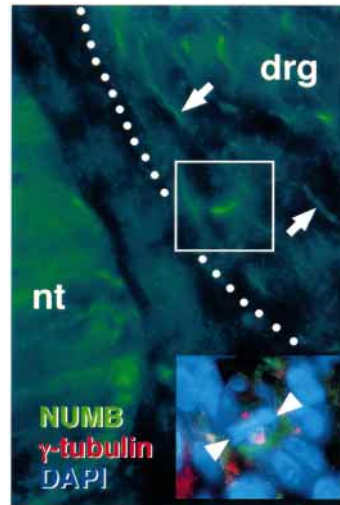
**Fig. 4.** *Sox2* mRNA expression indicates the origin and dispersal of satellite glia. (A,B) Transverse section of a day-4 (stage 22) embryo, showing the nascent DRG. *Sox2* (blue-purple in A) is expressed in the peripheral region, but not in the Hu-positive neurons (red in B) of developing DRG of a stage 22 embryo. (C) Transverse sections of a day 7 (stage 30) embryo, showing a maturing DRG. *Sox2*-positive cells are dispersed throughout the DRG (blue-purple in C). Schwann cells are also *Sox2* positive (arrowheads). (D,E) High magnification of day-7 embryonic DRG. *Sox2*-positive satellite glial cells (blue-purple in D, arrowheads) do not express Hu (red in E), and their nuclei are relatively small and intensely stained with DAPI. (F,G) A P0-positive glial cell expresses *Sox2* protein in 3-day old neural crest culture. Cell surface antigen P0 appears punctate in red, and blue DAPI staining indicates nuclei (F). *Sox2* protein is localized in the nuclei (G).

transfected cells were rescued from cell death, and neuronal differentiation was correspondingly increased (72%; Fig. 5C-E). This suggested that the dying cells were neurogenic precursors. Supplementing the culture with a neurotrophic factor, NT-3, also partially rescued *Numb*-transfected cells from cell death, and resulted in an increase of Hu-positive neurons (80%). Neither zVAD-fmk nor NT-3 altered the proportion of neurons in *GFP*- and *CNIC<sup>ΔC89</sup>*-transfected cells (Fig. 5C). At present, we do not know why *Numb* misexpression induced cell death, but this result is consistent with the increase of apoptosis reported in *Notch1* null mutant



**Fig. 5.** Manipulation of NOTCH signaling in cultured crest cells. FLAG-tagged *GFP*, activated form of *Notch1* (*CNIC<sup>ΔC89</sup>*) and *Numb* expression vectors were transfected. Each experiment was repeated 3-6 times. Error bars indicate standard deviation. (A) An effect on cell proliferation. 24 hours after transfection, BrdU pulse-labeling was performed. (B) An example of *CNIC<sup>ΔC89</sup>*-transfected cells (anti-FLAG, FITC in blue-green). *CNIC<sup>ΔC89</sup>* localizes in the nuclei as normally seen with the cytoplasmic domain of NOTCH (see Wakamatsu et al., 1999). Both BrdU-negative (arrow) and BrdU-positive cells (TRITC in red, arrowheads) are shown. Overlapping BrdU and *CNIC<sup>ΔC89</sup>* staining appears white. (C) An effect on neuronal differentiation. 72 hours after transfection, Hu expression in transfected cells was examined. No *Numb*-transfected cells were observed in the absence of caspase inhibitor zVAD-fmk (N/A, not achieved). (D,E) An example of a *Numb*-transfected neuronal cell, expressing Hu (large arrowhead). This cell possess neurite-like processes (small arrowheads). (F) An effect on glial differentiation. 72 hours after transfection, glial marker P0 expression was examined. (G) Examples of *CNIC<sup>ΔC89</sup>*-transfected P0-positive cell (arrowhead) and P0-negative cell (arrow). (H) The effect on 7B3 expression. 72 hours after transfection, 7B3 immunoreactivity was examined in the presence of zVAD-fmk. (I) An example of *CNIC<sup>ΔC89</sup>*-transfected 7B3-positive cell.

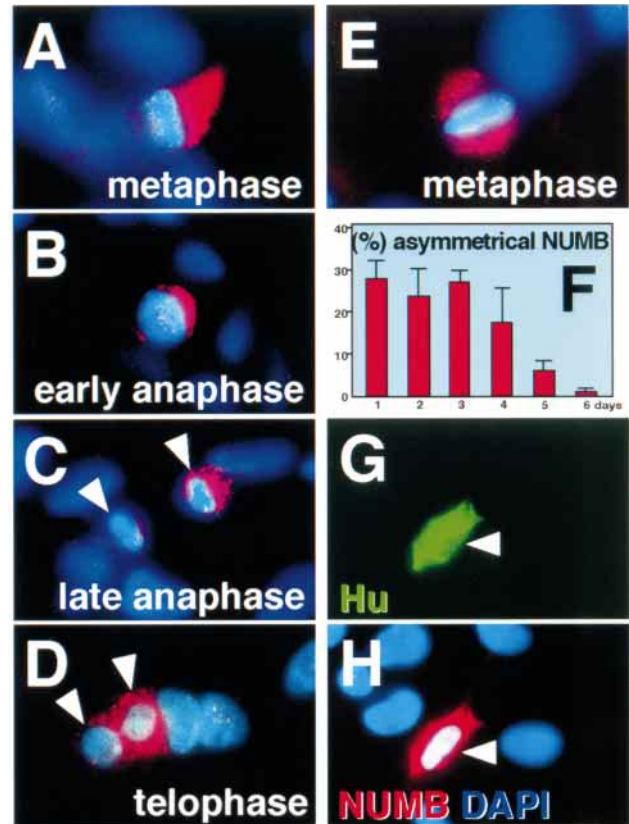
**Fig. 6.** NUMB localizes asymmetrically in mitotic DRG cells. An example of a stage 22 developing DRG, stained with anti-NUMB antibody (FITC in green). Arrows indicate cell processes of non-mitotic DRG cells stained with anti-NUMB. Dotted line indicates the boundary between the DRG and mesenchyme. Inset is a high magnification of the boxed region, showing a metaphase mitotic cell. Chromosomes are revealed by DAPI in blue, centrosomes are revealed by anti- $\gamma$ -tubulin staining in red (cy3), and NUMB is localized asymmetrically, relative to the prospective cell cleavage plane (arrowheads). nt, neural tube.



mouse embryos (Swiatek et al., 1994). Taken together, however, our results suggest that activation of NOTCH signaling prevents crest cells from taking a neuronal fate, and such activation may be the result of interaction with the neighboring neuronal cells that express *Delta1*.

It should be noted, however, that *CNIC<sup>AC89</sup>*-transfected cells after 72 hours contained a smaller proportion of BrdU-positive cells (9%), compared to the younger cultures (37%, see above). The *GFP*-transfected control culture, 72 hours after transfection, also contained only 9% of BrdU-positive cells. These results indicate that activation of NOTCH signaling with *CNIC<sup>AC89</sup>* may not simply preserve crest cells in an undifferentiated state.

To assess whether the presence of *CNIC<sup>AC89</sup>* resulted in increased glial cell differentiation, therefore, we examined the effect of NOTCH activation on differentiation of glial cells by using the glial-specific membrane protein, P0 (Bhattacharyya et al., 1991), as a marker. There was no obvious change in the proportion of P0-positive cells by NOTCH activation with *CNIC<sup>AC89</sup>*, compared to that of *GFP*-transfected control (7.9% versus 10.8%; Fig. 5F,G). However, as described above, 41% of *GFP*-transfected population were neurons, whereas only 5% of the *CNIC<sup>AC89</sup>*-transfected population were neurons. Considering this difference, among the non-neuronal cells, P0-positive cells in *CNIC<sup>AC89</sup>*-transfected population showed a moderate decrease, compared to *GFP*-transfected population (8.1% vs 18.1%). We also compared immunoreactivity in the two populations with the monoclonal antibody 7B3, which recognizes a nestin/transitin-like protein present in both undifferentiated precursors and crest-derived glial cells, but not in differentiated neurons or melanocytes (Henion and Weston, 1997; Henion et al., 2000). After transfection with *CNIC<sup>AC89</sup>*, the proportion of 7B3-positive cells increased, compared to the *GFP*-transfected control (75% versus 45%; Fig. 5H,I). In contrast, *Numb*-transfected cells in the presence of zVAD-fmk (see above) showed a decrease of 7B3-positive cells (22%; Fig. 5H). Taken together, these results indicate that NOTCH activation inhibits crest differentiation into neurons. Subsequently, at least part of this population can undergo glial differentiation.



**Fig. 7.** NUMB localizes asymmetrically in cultured crest cells. (A-D) Asymmetrically localized NUMB in mitotic crest cells, after 2 day cultures. Based on the chromosome orientation revealed by DAPI staining (blue), higher concentration of NUMB (cy3 in red) will be segregated in one of the daughter cells in A and B. Arrowheads indicate daughter cells in C and D. (E) In this case, NUMB appears to be segregated symmetrically. (F) The proportion of mitotic crest cells, in which the chromosomes are oriented to segregate NUMB unevenly, progressively declines as neurogenesis proceeds in vitro. (G,H) 3-day culture, stained with anti- Hu (FITC in green) and anti-NUMB (cy3 in red) antibodies, showing uniform distribution of NUMB-IR.

### NUMB is asymmetrically segregated in some crest-derived cells undergoing mitosis in developing DRG and in culture

Expression of NUMB protein, a NOTCH antagonist, was observed in nascent DRGs of stage 22 chicken embryos. NUMB immunoreactivity was present in many but not all the mitotic cells in the periphery of nascent DRGs, as well as in the processes of non-mitotic cells (Fig. 6). Importantly, in stage 22 DRGs, nearly 40% of mitotic cells had asymmetrically localized NUMB, in which chromosome orientation would cause NUMB to be inherited unevenly in daughter cells after cytokinesis (Fig. 6). In contrast to the basal localization in neuroepithelial cells (Wakamatsu et al., 1999), however, asymmetry of NUMB localization could not be oriented with respect to any known anatomical landmark within the nascent DRG. At later stages of development, such as stages 25-27, only a few mitotic figures were observed. In those mitotic cells, NUMB localized diffusely and symmetrically.

When crest cells were cultured free from surrounding



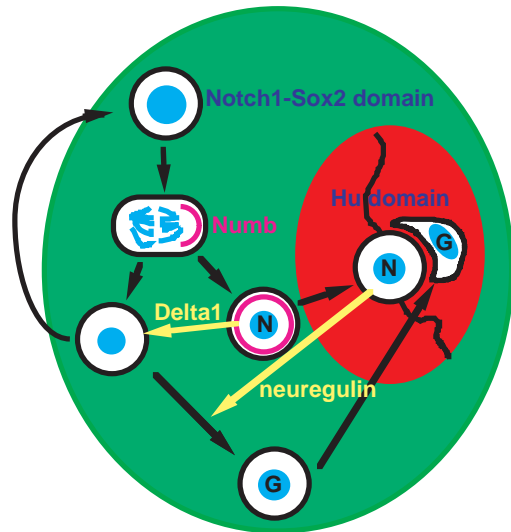
tissues, NUMB was also seen to be localized asymmetrically in mitotic cells (Fig. 7A-D), suggesting that some cell-intrinsic mechanism effects the intracellular localization of NUMB in crest cells. Thus, NUMB was asymmetrically localized, with respect to the cleavage plane in approximately 20-30% of mitotic cells (Fig. 7F). In these cells, NUMB would be inherited in high concentration by only one of the daughter cells. The remaining mitotic cells either lacked detectable NUMB expression, or appeared to segregate NUMB symmetrically (Fig. 7E). Under our culture conditions, neurogenesis was almost complete by 5 days (not shown, but see Fig. 1A), and the number of mitotic cells that possessed NUMB asymmetrically declined rapidly (Fig. 7F). In all stages examined, however, NUMB was symmetrically distributed throughout the cytoplasm of mitotic Hu-positive neuronal cells (data not shown), suggesting the machinery regulating asymmetrical NUMB segregation no longer functions in fate-restricted neuronal cells. NUMB immunoreactivity was enriched in the processes of non-mitotic Hu-negative cells, and consequently sequestered away from the cell body (data not shown), as also observed in vivo (Fig. 6; see also Wakamatsu et al., 1999). In non-mitotic Hu-positive neuronal cells, NUMB was observed throughout the cell body and their processes (Fig. 7G,H), so that activation of residual NOTCH molecules might be prevented, as suggested previously (Zhong et al., 1997).

## DISCUSSION

### Cell-proliferation, differentiation and migration in developing DRG

DRGs contain sensory neurons and satellite glia. In developing DRGs, clusters of Hu-positive differentiating neurons appear to form a neuronal core surrounded by Hu-negative crest-derived cells (Wakamatsu and Weston, 1997; this study). A similar distinction between the DRG core and what was referred to as a 'marginal zone' has also been noted by Hamburger et al., (1981). Although Hu-positive cells can undergo limited cell division (Marusich et al., 1994), possibly as neuronally fate-restricted intermediates, these cells subsequently start to express *SCG10*, characteristic of older neurons, and do not divide further. BrdU pulse-labeling indicates that Hu-negative cells surrounding the core of neuronal cells in the developing DRG actively proliferate and that some of these peripheral cells subsequently undergo neuronal differentiation. Thus, we propose that between stage 18-25, neuronal cells segregate from the peripheral region of the ganglion and are incorporated into the DRG core, so that the number of neurons in the core gradually increases (see Fig. 8). Later, between stage 26-28, proliferating cells surrounding the neuronal core of the DRG give rise to satellite glia, as indicated by BrdU labeling and *Sox2* expression (Fig. 8), although limited neurogenesis may also take place, as described previously (Carr and Simpson, 1978). These newly formed glial cells appear to move from the peripheral region and intercalate with neurons in the DRG core, where they may continue to proliferate at later stages of development (Carr and Simpson, 1978). Accordingly, the peripheral region of the DRG appears to resemble the ventricular zone of the developing spinal cord (see Wakamatsu et al., 1999).

Spatial rearrangements of neuronal and glial cells are also observed in crest cultures. Thus, although neuronal cells are



**Fig. 8.** A model of DRG development. Undifferentiated *Notch1*-positive/*Sox2*-positive cells in the peripheral region of the DRG undergo asymmetric cell division. One of the daughter cells, which received NUMB, will differentiate into neuron (N) and express *Delta1*, and subsequently coexpress Hu. Hu-positive cells form a neuronal core in the DRG. *Delta1*-positive neuronal cells activate NOTCH1 in neighboring cells, preventing neuronal differentiation. NOTCH-activated cells in the peripheral region will either repeat the cycle, or under the influence of neuron-derived NEUREGULIN, differentiate into satellite glia (G). Satellite glia will later intercalate with Hu-positive neurons.

initially uniformly distributed in such cultures, they later form aggregates (see also Henion and Weston, 1994). Glial cells that were initially distributed as a monolayer on the culture substratum, later intercalate into the neuronal aggregates to form 'pseudo-ganglia' (data not shown). It is not clear how this redistribution is regulated. Since NEUREGULIN/GGF has been suggested to promote motility of cultured glial cells (Mahanthappa et al., 1996), neuron-derived NEUREGULIN might promote the movement of satellite glia precursors from the periphery into the core of the DRG.

### Delta1 expression reveals early crest-derived neuronal cells

As suggested previously, in the vertebrate CNS post-mitotic prospective neurons, which migrate radially from ventricular zone to cortex, express high level of either *Delta1* or *Serrate1* depending on the location in the ventricle (Henrique et al., 1995; Myat et al., 1996). In this study, we showed that some avian trunk crest cells express *Delta1*. It has been demonstrated that *Delta* expression is regulated by proneural basic-helix-loop-helix transcription factors both in *Drosophila* and vertebrates. For example, in mouse otic placodes, *neurogenin2* is required for *delta-like1* expression (Fode et al., 1998). Chicken *neurogenin1* and *2* have been recently described, are expressed in early crest-derived neurogenic cells, and promote sensory neuron differentiation (Perez et al., 1999). Chicken *neurogenin* genes are initially expressed in a subpopulation of migrating crest cells, probably slightly earlier than *Delta1*. Therefore, it is plausible that *neurogenin* gene function triggers *Delta1* expression in crest cells. *Delta1* expression itself



precedes Hu expression, but *Delta1*-positive cells later coexpress Hu-positive (Fig. 1). Taken together, these expression patterns suggest that *Delta1*-positive crest-derived cells represent early neuronal cells.

### How is NOTCH signaling involved in fate determination of DRG cells?

*Notch1* is expressed in undifferentiated crest cell populations, and is down-regulated upon neuronal and glial differentiation in vivo. We have shown that NOTCH activation inhibits neuronal differentiation, and transiently increases cell proliferation in cultured crest cells. Our results also indicate that inhibition of NOTCH activation leads to neuronal differentiation in cultured crest cells. These results, along with *Delta1* expression in neuronal cells in developing DRG, suggest that NOTCH-mediated lateral inhibition is involved in the control of neuronal differentiation in the nascent DRG (see Fig. 8).

In our transfection experiments, continuous activation of NOTCH signaling with *CNIC<sup>AC89</sup>* also moderately inhibited glial differentiation, based on P0 expression. Thus, NOTCH activation might inhibit general differentiation. Alternatively, NOTCH activation might trigger glial specification, but terminal differentiation of glial cells might require subsequent inactivation of NOTCH signaling. This model is consistent with the previous reports that NOTCH activation inhibits neuronal differentiation, but permits glial differentiation in the P19 cell line system (Nye et al., 1994), and that NOTCH activation inhibits terminal differentiation of oligodendrocyte precursors in the optic stalk (Wang et al., 1998). It should be noted that cells expressing glial marker(s) may still possess multipotency or plasticity (Morrison et al., 1999; Hagedern et al., 1999). In any case, a series of early glia-specific markers will be required to study glial differentiation of neural crest cells further.

Recently, it was reported that a cyclin-dependent kinase inhibitor, *p27<sup>Xic1</sup>* was involved in glial differentiation in *Xenopus* retina (Ohnuma et al., 1999). Since *p27<sup>Xic1</sup>* promotes glial differentiation in concert with NOTCH signaling, glial differentiation from crest-derived cells may be regulated by other signaling pathway(s), in combination with the NOTCH signaling. Previously, *NEUREGULIN/GGF* was described as an instructive differentiation factor for the glial lineage (Shah et al., 1994), while another study revealed that *neuregulin* function might promote survival and proliferation of glial cells (Dong et al., 1995). *Neuregulin* is known to be expressed in neurons of DRGs (Meyer et al., 1997 and references therein), and we confirmed *neuregulin* expression in Hu-positive neurons in the developing chicken DRG (Y. W., unpublished observation). It is conceivable, therefore, that NOTCH activation, in concert with neuron-derived NEUREGULIN, may lead the multipotent or neuro-glial precursors (see Henion and Weston, 1997) to take a glial fate (Fig. 8). Alternatively, NOTCH activation might direct undifferentiated crest cells to take a glial fate, and NEUREGULIN might induce terminal differentiation, proliferation and survival of glial cells. Regardless of the specific regulatory pathway, it must be emphasized that neuron-satellite glia fate determination can be achieved autonomously within a neural crest cell population. This notion does not exclude the possible involvement of as yet unknown environmental factor(s) from surrounding tissues for glial differentiation.

In this study, we have shown that *Sox2* is expressed in glial sublineages, such as Schwann cells and satellite glia of the PNS of avian embryo. Furthermore, prospective oligodendrocyte precursors in the CNS also appear to express *Sox2* (data not shown). Among the *Sox* family of transcription factors, *Drosophila dichaeete* has been reported to be involved in midline glia differentiation (Soriano and Russell, 1998). In vertebrates, *Sox10* has been shown to be expressed in crest-derived lineages (Bondurand et al., 1998; Southard-Smith et al., 1998), and *Sox10* mutations in both mouse and human revealed a variety of defects in neural crest derivatives (Southard-Smith et al., 1998 and references therein). The involvement of *Sox10* in glial differentiation has also been suggested (Kuhlbrodt et al., 1998a, b). How *Sox2* is involved in neural crest differentiation remains to be examined. Since *Sox2* is also expressed in undifferentiated precursors in the peripheral region of developing DRG, however, *Sox2* expression is probably not sufficient for glial differentiation.

### Asymmetric cell division may explain initial segregation of neuronal and glial cells in developing DRG

As discussed above, NOTCH signaling (possibly in combination with NEUREGULIN) may promote satellite glial differentiation by crest-derived cells, whereas *neurogenin* function will promote sensory neuron differentiation (Perez et al., 1999; Ma et al., 1999). However, these mechanisms do not address the important issue of how initial differences between neuronal and glial cell precursors are established in the first place. As previously suggested, a NOTCH-DELTA feedback loop may create differences in an originally homogeneous cell population (Collier et al., 1996). We have now shown that when crest-derived cells in developing DRG undergo mitosis, the NOTCH antagonist, NUMB, is often asymmetrically inherited by one of the daughter cells (Figs 6, 7). It is possible, therefore, that initial differences among the crest-derived cells might be established by asymmetric localization of NUMB in mitotic cells. Thus, the NOTCH signaling pathway would not be activated in cells that received NUMB, and such cells would take a neuronal fate and upregulate *Delta1* (Fig. 8). Conversely, when NOTCH is activated by DELTA1 expressed by neuronal neighbors, NOTCH signaling would suppress neurogenesis in cells that do not receive NUMB (Fig. 8).

Based on this model, we predicted that overexpression of *Numb* would promote neuronal differentiation. We have found that although overexpression of *Numb* in crest cell cultures primarily induces cell death, increased neurogenesis was observed if dying cells were rescued with the caspase inhibitor zVAD-fmk. Although this observation is consistent with the increased apoptosis in targeted *Notch1* mutant mice (Swiatek et al., 1994), and is also consistent with the involvement of NOTCH activation on survival of maturing thymocytes (Defetos et al., 1998), it could also be a consequence of excessive NUMB activity, which might not be physiologically relevant. It is important to note, however, that *Numb* misexpression does lead to neuronal differentiation if exogenous neurotrophic factor is present in the culture environment. Our result, therefore, is consistent with the report that *Numb* misexpression promotes neurogenesis in an 'immortalized' mammalian crest-derived cell line (Verdi et al., 1996).

We do not yet know how asymmetry is established in crest-

derived DRG cells undergoing mitosis. Unlike neuroepithelial cells of the CNS, which have clear apical-basal polarity, crest cells are mesenchymal so that such apical-basal polarity would not be evident. Since disruption of tissue organization in culture did not affect the asymmetrical localization of NUMB in crest cells, moreover, it seems likely that asymmetry of the crest cells is cell-intrinsically inherited. In any case, our observations reveal that neural crest-autonomous mechanisms can function, in concert with generally accepted environmental cues, to determine alternative fates of neural crest-derived cells during development of DRG.

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