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Regulative response of the cranial neural tube after neural fold ablation: spatiotemporal nature of neural crest regeneration and up-regulation of *Slug*

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SUMMARY

After unilateral ablation of the avian cranial neural folds, the remaining neuroepithelial cells are able to replace the missing neural crest population (Scherson et al., 1993). Here, we characterize the cellular and molecular nature of this regulative response by defining: (1) the time and location of neural crest cell production by the neuroepithelium; (2) rostrocaudal axial differences in the regulative response; and (3) the onset of expression of *Slug*, a transcription factor present in premigratory and migrating neural crest cells. Using DiI and HNK-1 antibody labeling techniques, we find that neural crest regeneration occurs only after apposition of the remaining neuroepithelium with the epidermis, suggesting that the developmental mechanism underlying regeneration of the neural crest may recapitulate initial generation of the neural crest. The regulative response occurs maximally at the 3-5 somite stage, and slowly declines thereafter. Surprisingly, there are profound regional differences in the regenerative ability. Whereas a robust regulation occurs in the caudal midbrain/hindbrain, the caudal forebrain/rostral midbrain regenerates neural crest to a much lesser extent. After neural fold removal in the hindbrain, regenerated neural crest cells migrate in a segmental pattern analogous to that seen in unablated embryos; a decrease in regulative response appears to occur with increasing depth of the ablation. Up-regulation of *Slug* appears to be an early response after ablation, with *Slug* transcripts detectable proximal to the ablated region 5-8 hours after surgery and prior to emergence of neural crest cells. Both bilateral and unilateral ablations yield substantial numbers of neural crest cells, though the former recover less rapidly and have greater deficits in neural crest-derived structures than the latter. These experiments demonstrate that the regulative ability of the cranial neuroepithelium to form neural crest depends on the time, location and extent of neural fold ablation.

Key words: neural crest, neural fold, *Slug*, cell migration, regeneration, chick

INTRODUCTION

The neural crest is an embryonic cell population that arises from the dorsal portion of the neural tube and undergoes extensive migrations. Neural crest cells then differentiate into autonomic and sensory ganglia, adrenal medullary cells, pigment cells and cartilage of the face (LeDouarin, 1982). Cell lineage studies have shown that single cells within the dorsal neural tube can form both neural crest and neural tube derivatives (Bronner-Fraser and Fraser, 1988; 1989). This shared neural tube/crest lineage raises the intriguing possibility that these tissues may be somewhat interchangeable. In a previous study, we explored the ability of the neural tube to compensate for the absence of neural crest cells by ablating the neural folds in the cranial region. By using focal injections of the vital dye, DiI, we examined the migratory behavior and derivatives of residual neural tube cells following unilateral neural fold ablation. We found that the neuroepithelial cells adjacent to the ablated region were able to regulate for the loss of neural tissue and reform the neural crest (Scherson et al., 1993). This regulative capacity was observed in 3-7 somite stage embryos and declined markedly thereafter.

Recent evidence suggests that interactions between the neural plate and presumptive epidermis induce formation of neural crest cells (Moury and Jacobson, 1989; Selleck and Bronner-Fraser, 1995). Similar interactions also can induce the expression of genes such as *Wnt-1*, *Wnt-3a* and *Slug* (Dickinson et al., 1995), which are characteristically localized in the dorsal neural tube. The relationship between initial generation of the neural crest and regeneration of the neural crest is unknown. An intriguing possibility is that the regulative response of the neuropithelium after ablation recapitulates the initial events in neural crest formation. The ability of the intermediate neural tube to form the neural crest, a dorsal cell type, following ablation of the dorsal neural folds may indicate that dorsoventral polarity is reestablished after ablation.

In the present study, we have further documented the regulative response of the neuroepithelium after cranial neural fold ablation by defining the time of new neural crest cell production, regional differences in the regulative response and the

temporal pattern of *Slug* expression following ablation. *Slug*, a vertebrate gene encoding a zinc finger protein of the Snail family, is expressed in the dorsal neural folds and migrating neural crest cells (Nieto et al., 1994). Furthermore, antisense oligonucleotides to chick *Slug* specifically inhibit the emergence of cranial neural crest cells. Its restricted dorsal expression pattern and apparent importance in neural crest production make *Slug* a candidate for an early response gene after cranial neural fold ablation. We find that neural crest regeneration occurs only after apposition of the neuroepithelium and epidermis. This suggests that the developmental mechanism underlying regeneration of the neural crest may recapitulate initial generation of the neural crest. Unexpectedly, we discovered marked differences in regulative ability along the rostrocaudal axis, with substantial regulation in the caudal midbrain and rostral hindbrain but only limited regulation in the caudal forebrain/rostral midbrain. *Slug* expression is an early response to ablation, becoming evident in cells proximal to the ablated region between 5-8 hours after surgery and prior to emergence of neural crest cells. Our results demonstrate that the cranial neuroepithelium has a remarkable regulative capacity that is temporally and regionally regulated.

MATERIALS AND METHODS

Neural fold ablations

White Leghorn chick embryos were incubated for 1.5 days, to the 1-8 somite stage (ss). A window was cut in the egg, India ink (Pelikan Fount) was injected underneath the blastoderm and the vitelline membrane was deflected from above the embryo. Both unilateral and bilateral ablations were performed using a fine glass knife. An incision was made between the neuroepithelium and adjacent ectoderm, followed by another longitudinal incision which bisected the neural tube dorsoventrally. These cuts were followed by two transverse incisions, one rostrally (typically at the level of the caudal forebrain) and the other caudally (at or near the middle to the caudal end of the hindbrain), after which the dorsal neural tube tissue was teased away. This surgery typically results in the removal of the dorsal half to one-third of the neuroepithelium. Some embryo received 'deep' ablations in which the dorsal 2/3 of the neural tube were removed. Most ablations were performed at the 3-7 somite stage (stage 8 according to the criteria of Hamburger and Hamilton, 1951) unless noted otherwise.

Iontophoretic injection of DiI

DiI was diluted in 100% ethanol (0.5%w/v). The DiI solution was back-loaded into micropipettes (5-10 MΩ resistance if filled with KCl), pulled from thin-walled aluminosilicate glass capillaries (with filament) using a Sutter P-80/PC Micropipette Puller. Micropipettes were held in a pipette holder with a silver wire immersed in the DiI solution. After positioning the pipette in the ablated region of the neural fold or tube, DiI was injected iontophoretically with a maximum of 90 nA for 2-20 seconds using a 9 volt battery through a 100 MΩ resistor. Injection size and location was verified under the fluorescence microscope, since it is usually invisible to the eye.

Surface labeling of the ectoderm with DiI

Whole ectoderm labeling was accomplished by a slight modification of the methods used to label cranial neural crest cells in the mouse embryo (Serbedzija et al., 1992). The entire surface ectoderm of embryos was labeled with DiI prior to neural tube closure (1-3ss) by adding a few drops of DiI stock solution, diluted 1:15 in 0.3 M sucrose, onto the surface of the embryo after tearing the vitelline membrane. The egg was returned to the incubator for 1 hour followed by washing with 3-5 drops of saline solution to remove excess dye. The embryos were reincubated until they reached the 3-6ss, by which time the rostral neural folds had approximated. Subsequently, the neural folds were ablated either unilaterally or bilaterally. Embryos were resealed with adhesive tape and returned to the incubator until the time of fixation (at 2-4 hour intervals during the first postablation day or at the third and fourth days). After dye application, the epidermis and neural tube were brightly labeled, but the only dye-labeled cells within the cranial mesenchyme were derived either from the neural crest or the ectodermal placode cells. Because the latter begin to internalize only after the 13ss, neural fold-derived cells are the only labeled cells within the cranial mesenchyme in early embryos. In a few embryos, allowed to survive for 3-4 days postablation, DiI labeling of the surface ectoderm was performed after ablation and reclosure of the neural tube in order to label placodal derivatives only.

Cryostat sectioning

Embryos were fixed in 4% paraformaldehyde overnight and were prepared for antibody staining or for whole-mount in situ hybridization using standard techniques. For cryostat sectioning, embryos were washed in PBS for 1 hour and immersed in 5% and then 15% sucrose solutions for 4-12 hours at 4°C. They were then infiltrated with a solution of 15% sucrose and 7.5% gelatin (Sigma, 300 Bloom) for 4 hours at 37°C, and embedded in fresh gelatin. Embryos were quick-frozen in liquid nitrogen and allowed to equilibrate to −30°C prior to transversely sectioning them on a Zeiss Microm cryostat at a thickness of 10 μm for DiI-labeled embryos and 30-40 μm for *Slug* hybridized embryos. Sections were mounted on subbed slides. DiI-labeled sections were examined without a coverslip. Slides to be stained with antibodies were stored at −20°C until the time of staining.

In situ hybridization

Digoxigenin-labeled probes corresponding to nucleotides 1-358 from the chick *Slug* sequences (Nieto et al., 1994) were used for whole-mount in situ hybridization, essentially as described by Wilkinson and Nieto (1993). Embryos up to stage 10 were treated with proteinase K for 5 minutes. After photographing the whole-mounts, selected embryos were cryosectioned as described above to examine in detail the cellular patterns of gene expression.

Immunocytochemistry

HNK-1 antibody (American Type Culture) was applied to sections as undiluted supernatant whereas anti-neurofilament antibody (RMO 270.3; Lee et al., 1987) was diluted 1:300 in phosphate-buffered saline (PBS) with 0.1% bovine serum albumin. Sections were typically incubated in primary antibodies overnight in a humidified chamber at 25°C, followed by washes in PBS. FITC-conjugated secondary antibodies against mouse IgM (for HNK-1) or IgG (for anti-NF-M) were applied to sections for 1.5 hours at room temperature. Sections were washed in PBS and coverslipped with gelmount (Biomedia).

RESULTS

Analysis of the regulative response after ablation using whole ectoderm/neural plate labeling with DiI

Our previous studies demonstrated that cranial neuroepithelial cells regulate to reform the of the neural crest after ablation (Scherson et al., 1993). Open questions include when and where the regulative response occurs. One possibility is that neural crest cells delaminate from the cut edge of the neuroepithelium shortly after ablation. Alternatively, the regeneration of new neural crest cells may require contact between the

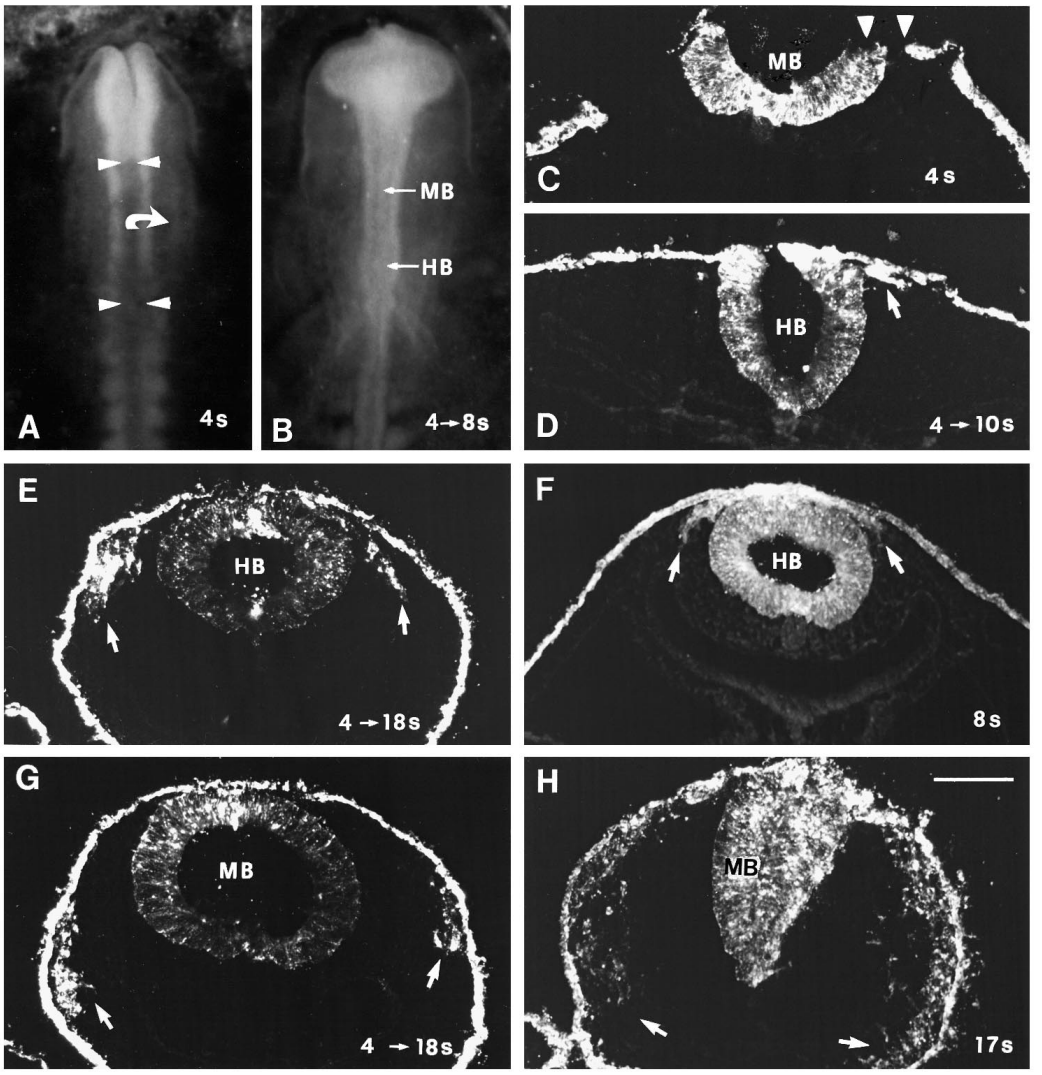

Fig. 1. (A,B) Stereoscopic images of the same living embryo viewed (A) immediately after bilateral ablation of the midbrain and rostral hindbrain neural folds at the 4ss and (B) again at the 8ss. The curved arrow indicates that the neural folds were discarded. Except for the fact that the onset of cranial neural crest migration was delayed by several hours (beginning close to the 13ss rather than the 7ss), the embryo developed relatively normally. (C-E,G) Transverse sections through embryos in which the midbrain and rostral hindbrain neural folds were ablated bilaterally after the ectoderm and neuroepithelium were labeled prior to neural tube closure, with the lipophilic dye, DiI. This technique labels the surface ectoderm, neural tube, neural crest cells and placodal cells. Neural crest cells are the only internalized cells with DiI label until about the 13-15ss, when some placodal cells also invaginate. (C) A 4ss embryo immediately after bilateral ablation of the neural folds at the level of the midbrain/rostral hindbrain. The double arrowhead indicates the position of the remaining neural tube and surface ectoderm at the midbrain level prior to reapposition and healing. (D) An embryo similar to that pictured in C, which was ablated at the 4ss and fixed at the 10ss. At the level of the hindbrain, a few DiI-labeled neural crest cells (arrow) are present in the cranial mesenchyme, having emigrated from the neural tube after reapposition with the surface ectoderm; no migrating neural crest cells were visible at the midbrain level. (E,G) Another embryo with bilateral neural fold ablation at the 4ss and fixed at the 18ss. At the level of the hindbrain (E), neural crest cells (arrow) clearly emerge from the neural tube after reapposition with the surface ectoderm. At the level of the midbrain (G), a few DiI-labeled cells (arrows) are observed along normal neural crest pathways, but their numbers are greatly reduced compared with those observed in the unablated embryo (compare with H). These could either arise from the dorsal neural tube or the diffuse dorsolateral epidermal placodes. (F,H) Transverse sections through unablated embryos after surface labeling of the ectoderm with DiI prior to neural tube closure. Normal embryos fixed at the 8ss (F) and 17ss (H) showing the normal pattern of neural crest migration. Neural crest cells (arrows) first emigrate after neural tube closure at the 6-7ss. By the 11ss, they have extended midway around the dorsoventral extent of the embryo and have reached the ventral-most position by the 17 somite stage. MB, midbrain; HB, hindbrain. Bar, 300 μm in A,B; 100 μm in C,D; 124 μm in E,G; and 112 μm in F,H.

epidermis and the neuroepithelial cells. To distinguish between these possibilities, we have labeled neuroepithelial cells without marking the cranial mesenchyme cells through which they migrate.

To test the site of neural crest cell emergence after ablation and characterize neural crest regeneration in detail, we have labeled the entire surface ectoderm, including the neural plate and neural folds, with DiI prior to neural tube closure (Serbedzija et al., 1989). Embryos then were reincubated until they reached the 3-6ss, at which time ablations were performed. Initially, the only DiI-labeled cells within the cranial mesenchyme are neural crest cells until the time that placodal cells begin to invaginate (>13ss). In contrast to previous experiments in which most embryos received only a unilateral neural fold ablation (Scherson et al., 1993), we ablated the neural folds bilaterally to avoid possible complications due to the presence of the contralateral neural folds/crest. The results of bilateral and unilateral ablations were then compared.

Examination of embryos immediately after bilateral ablation demonstrated that the neural folds were clearly removed after the operation (Fig. 1A,C). By the 8ss, the neural tube reapproximated in some embryos (Fig. 1B) and by the 13ss, groups of migrating neural crest cells could be seen with a stereomicroscope. This represented an approx. 7 hour delay with

respect to neural crest migration in unoperated embryos. In embryos having bilateral neural fold ablations (*n*=20) performed after DiI labeling of the entire surface ectoderm, DiI-labeled neural crest cells were only observed in the cranial mesenchyme after the neural tube reestablishes contact with the surface ectoderm (Fig. 1D). The time at which these regenerated cells were detected varied somewhat from embryo to embryo, but appeared to correlate with the depth of the ablation, with shallow ablations generating neural crest cells more rapidly than deeper ablations. In addition, the time of ablation was important. Those embryos operated on at the 3-4ss regenerated DiI-labeled neural crest cells within 7-12 hours, whereas older embryos took longer or failed to regenerate neural crest cells. Thus, the regulative response after bilateral ablation has a rather narrow time window, occurring best between the 2-5ss and rapidly declining thereafter. This contrasts with that previously reported for unilateral ablations (Scherson et al., 1993), in which the ability to regulate remains robust until the 7ss.

For many of the experiments described above, extensive ablations were performed that typically extended from the caudal forebrain through several levels of the hindbrain. Because the entire ectodermal primordium was DiI-labeled prior to ablation, we were able to visualize and compare the regulative response at multiple rostrocaudal levels within the same embryo. Unexpectedly, we observed region-specific characteristics of the regulative response that were highly reproducible. Serial sections through 7 embryos fixed at the 8-18ss and examined in detail showed conspicuous regeneration of neural crest cells in the middle to caudal midbrain and hindbrain (Fig. 1E,G). New production of neural crest cells at these levels was evident as early as the 10ss in some embryos (Fig. 1D). In contrast, the regeneration of neural crest cells from the neural tube was far less obvious in the rostral midbrain and caudal forebrain regions, where only a few labeled cells were evident in the cranial mesenchyme after neural fold ablation. Thus, regeneration appeared to occur earlier and produced larger numbers of neural crest cells at the caudal midbrain/hindbrain region than in more rostral regions of the embryo.

The distribution of DiI-labeled cells in ablated embryos was compared with similarly labeled normal embryos. In 6-20ss embryos (*n*=6), DiI-labeled cells were detected in the cranial mesenchyme by the 8ss adjacent to the midbrain/rostral hindbrain (Fig. 1F). This suggests that initiation of neural crest migration starts at the 6-7ss, confirming the conclusions of other labeling techniques (Smith et al., 1979; Vincent et al., 1983; Nieto et al., 1994) and definitively showing that there is no cranial neural crest migration prior to neural tube closure in avian embryos. From the 9-17ss, DiI-labeled cells migrate progressively more ventrally (Fig. 1H) toward the foregut.

To confirm the results of DiI surface labeling, some bilaterally ablated embryos (*n*=16) were stained with the HNK-1 antibody, which recognizes migrating neural crest cells (Tucker et al., 1984). Similar to the results of DiI-labeling, HNK-1 immunoreactive cells were observed in the cranial mesenchyme only after apposition of the neuroepithelium and epidermis (approx. 10-13ss), whether or not the neural tube had closed (Fig. 2A). By the 18ss, numerous HNK-1 immunoreactive cells were observed along cranial neural crest migratory pathways (Fig. 2B), particularly at the midbrain/rostral hindbrain level, but their numbers and ventral extent of migration appeared reduced in comparison to normal embryos.

In order to compare the results of bilateral and unilateral neural fold ablations, we examined the regeneration of neural crest cells when the neural folds were ablated on one side only after DiI labeling (*n*=5) or by HNK-1 immunoreactivity. When the neural tube failed to close in the midline after unilateral ablation, the results were essentially identical to those observed after bilateral ablations. There were fewer neural crest cells on the ablated side relative to the control side (Fig. 2D) and labeled neural crest cells were observed only after reapproximation of the remaining neuroepithelium with the surface ectoderm. In contrast, when the neural tube closed within a few hours after unilateral ablation, there tended to be larger numbers of neural crest cells on the ablated side (Fig. 2C,E,F). The numbers and extent of migration of neural crest cells was greater after unilateral than bilateral ablation, and the former embryos were able to regenerate neural crest cells for somewhat longer time periods than those ablated bilaterally. These results suggest that the contralateral neural tube either contributes substantial numbers of neural crest cells to the ablated side after unilateral neural fold removal or provides an important support system which facilitates neural crest production. However, it is clear from focal DiI-labeling experiments (Scherson et al., 1993) that the ipsilateral side also produces numerous neural crest cells after unilateral ablation.

Temporal analysis of the regulative response

The rostrocaudal difference in the extent of regulation could reflect a prepattern in the regenerative ability of the neural tube. Alternatively, there may be a temporal wave of regulative ability that travels rostrocaudally. Since the ablations described above were typically performed at the 4-6ss, similar experiments were repeated at the 1-3ss, reflecting the earliest timepoints at which the cranial neural folds could be easily manipulated and removed. Ablations were performed following DiI surface labeling. Embryos were examined for the presence of DiI-labeled cells in the cranial mesenchyme and/or HNK-1 immunoreactivity.

For ablations performed at these earlier stages (*n*=8), we observed a spatial pattern to the regulative ability such that there were consistently more neural crest cells observed at the caudal midbrain/rostral hindbrain than at more rostral levels (data not shown). This favors the idea that rostrocaudal differences are established prior to the 1 somite stage. However, the differences in degree of regulative response between the forebrain/rostral midbrain and caudal midbrain/hindbrain were not as notable for ablations performed at the 1-3ss than those done at the 4-6ss, suggesting that there also may be temporal component to the regenerative ability that regresses rostrocaudally.

Analysis of the regulative response after ablation using iontophoretic injections of DiI into the neuroepithelium

The above results suggest that those neural crest cells emerging from the neural tube after neural fold ablation do so only after reapposition of the remaining neural tube with the surface ectoderm. Using whole neural fold and ectoderm labeling, however, we cannot analyze the precise source of the internalized neural crest cells at a particular axial level. To do so

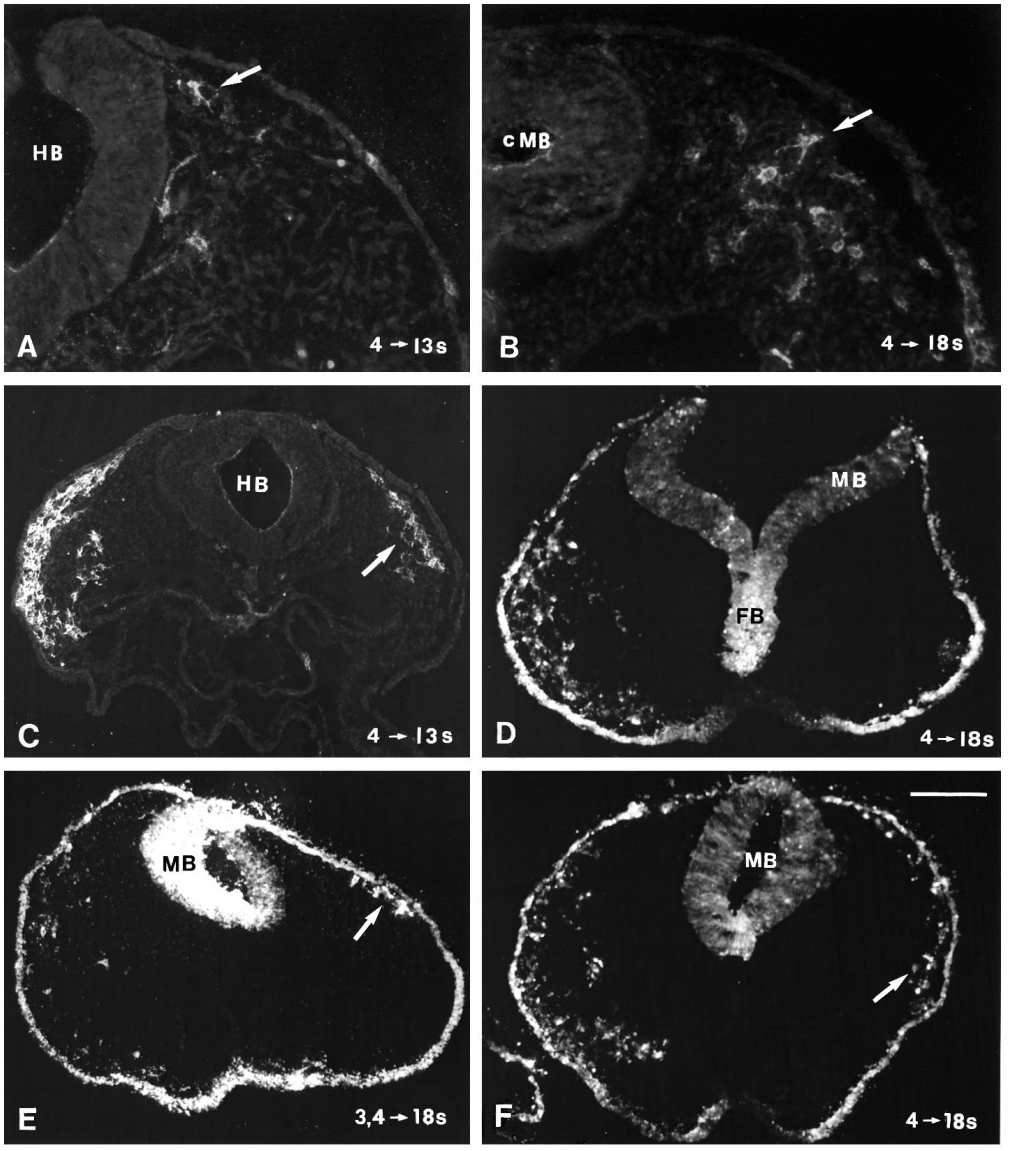

Fig. 2. Embryos after bilateral (A,B) or unilateral (C-F) neural fold ablation. (A) HNK-1 immunostaining of a transverse section through the embryo pictured in Fig. 1A,B after fixation at the 13ss. A few HNK-1 immunoreactive neural crest cells (arrow) are evident in the hindbrain and midbrain. (B) HNK-1 immunostaining of a transverse section at the level of the caudal midbrain of another embryo which received a bilateral neural fold ablation at the 4ss and was fixed at the 18ss. The numbers of neural crest cells (arrow) have increased and are present along normal pathways, but are markedly lower than in unablated embryos (compare with Fig. 1H). (C) HNK-1 immunostaining of an embryo after unilateral ablation at the 4ss and fixation at the 13ss. Neural crest cells (arrow) are present but reduced in number on the ablated compared with the unablated side. The numbers of neural crest cells appear higher after unilateral compared with bilateral ablations (compare with A). (D,F) An embryo with DiI-labeling of the surface ectoderm followed by unilateral neural fold ablation at the 4ss and fixation at the 18ss. (D) At the level of the caudal forebrain/rostral midbrain, no neural crest cells were noted on the ablated side (right), though the normal complement was present on the unablated side. Note that the neural tube did not close at this axial level. (F) At the level of the caudal midbrain/rostral hindbrain in the same embryo, DiI-labeled neural crest cells (arrow) were observed on the ablated side (right), though the numbers were decreased compared with those on the unablated side. (E) Another DiI-labeled embryo unilaterally ablated at the 3-4ss and fixed at the 18ss viewed at the level of the midbrain. Note that the neural tube appears reduced in size on the ablated side. DiI-labeled cells are evident on the ablated side (arrow), though their extent of migration is reduced compared with the unablated side. cMB, caudal midbrain; MB, midbrain; HB, hindbrain. Bar, 54 μm in A,B; 130 μm in C; 120 μm in D-F.

requires focal injections of DiI. In our original study, we observed some DiI-labeled cells emerging from the ventral cut edge of the neural tube within a few hours after unilateral ablation and prior to closure of the neural tube (Scherson et al., 1993). However, these previous injections were relatively large, labeling on the order of 20-30 cells. Because it was possible that we might have labeled adjacent cranial mesenchyme cells in addition to neural tube cells, this issue needed to be examined using more refined techniques including smaller, focal dye injections.

For these experiments, we performed bilateral removals of the neural folds (n=12) at the level of the midbrain and hindbrain in 4-6ss embryos. Then, DiI was applied adjacent to the cut edge using small, iontophoretic injections; controlled amounts of dye were released that labeled only 5-10 cells (Fig. 3A,B). In most cases, injections were made in the caudal half of the midbrain. Embryos were allowed to develop until the 8-12ss and were then fixed and examined by whole-mount imaging followed by cryostat sectioning (Fig. 3C-E). One embryo collected at the 8ss had DiI confined to the neural tube, suggesting that neural crest cells had not left the neural tube at this stage of the regeneration process. The lack of DiI staining in the adjacent mesenchyme illustrates the specificity of the injection. We observed migrating DiI-labeled cells that had emigrated from the neural tube only after the regenerating neural tube established contact with the presumptive epidermis in 6 embryos. Although the youngest embryo, ablated at the 4ss and

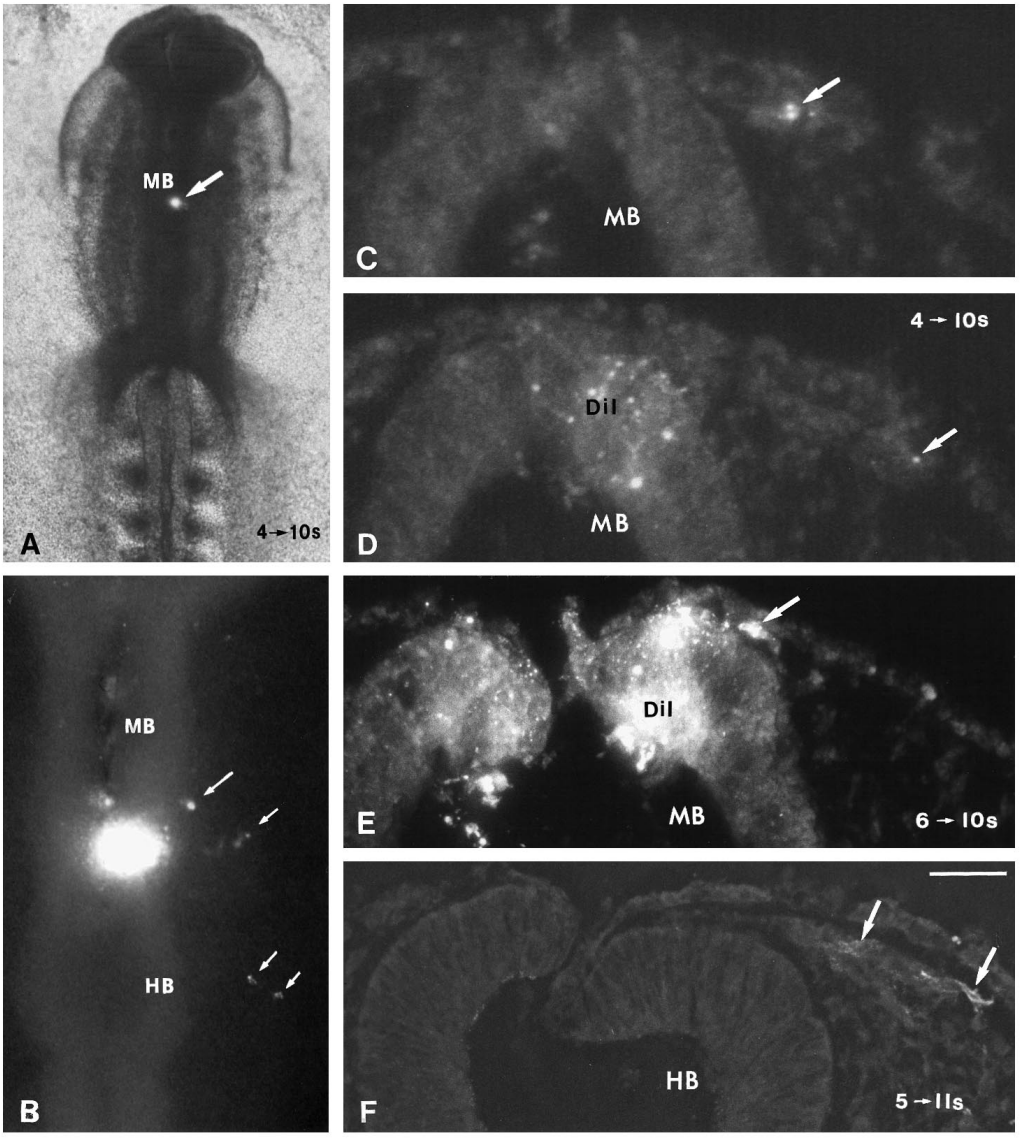

Fig. 3. Iontophoretic, focal injections of DiI into remaining neural tube cells after bilateral neural fold ablation. (A-D) An embryo ablated and labeled at the 4ss and fixed at the 10ss. (A) Whole-mount bright field superimposed with epifluorescence view soon after fixation illustrates the position of the labeled cells (arrow) in the midbrain. (B) At higher magnification of the fluorescence image, a plume of DiI label remains within the neural tube. In addition, a number of individual labeled cells (arrows) have migrated away from the injection site. The two caudolaterally migrating cells are moving toward the region where the trigeminal ganglion will form. (C) A transverse section through the same embryo at the level of the most rostral arrow illustrates that the labeled cell (arrow) is localized between the ectoderm and neural tube, a position characteristic of a newly emigrated neural crest cells. (D) Another section through the same embryo at the level of the second-most rostral arrow shows labeling of DiI within the neural tube as well as a DiI-labeled neural crest cell (arrow) below the surface ectoderm. (E) A transverse section through a different embryo after bilateral ablation at the 6ss and fixation at the 10ss. This embryo received a focal injection in both the left and right residual neural tube walls. The arrow indicates a neural crest cell emigrating after reapposition of the ectoderm and neural fold at midbrain level. (F) For comparison with C-E, a transverse section at the level of the rostral hindbrain through a comparable embryo stained with HNK-1 antibody after ablation at the 5ss and fixation at the 11ss. Note that migrating neural crest cells (arrows) are below the surface ectoderm. MB, midbrain; HB, hindbrain; Bar, 250 μm in A; 140 μm in B; 30 μm in C-F.

injected near the midbrain/hindbrain border (Fig. 3A-D), appeared to recover most rapidly, 2 embryos ablated at the 6ss also produced neural crest cells (Fig. 3E). Another 5 embryos did not regenerate labeled neural crest cells at the times examined, but may have done so if allowed to recover beyond the 8-12ss. The results of our iontophoretic focal injections at cranial levels confirms that production of neural crest cells after ablation primarily occurs after reapproximation of the cut neuroepithelium with the presumptive epidermis whether or not the neural tube has closed. This is consistent with the possibility that interactions between the presumptive epidermis and neural tube may be important for neural crest cell generation.

Regulative ability of the hindbrain neural crest

The experiments described above examined the regenerative ability of the neural tube primarily in the midbrain and rostral hindbrain. To extend this study further caudad along the neural axis, we performed long bilateral ablations extending from the caudal midbrain past the level of presumptive rhombomere (r) 6 in 2-7ss embryos (n=13) to emphasize the effects of ablations on hindbrain neural crest cells. The results are summarized in Table 1 and Fig. 4.

When ablations were performed prior to the 6ss, there was a marked production of neural crest cells in the caudal midbrain and throughout the hindbrain, with best regulation occurring after ablations performed at early stages. The segmental pattern of neural crest migration was maintained with prominent streams of neural crest cells adjacent to r2, r4 and r6 analogous to that seen in unablated embryos (Fig. 4). Although neural crest cell migration was delayed compared to normal embryos, the segmental pattern of migration was

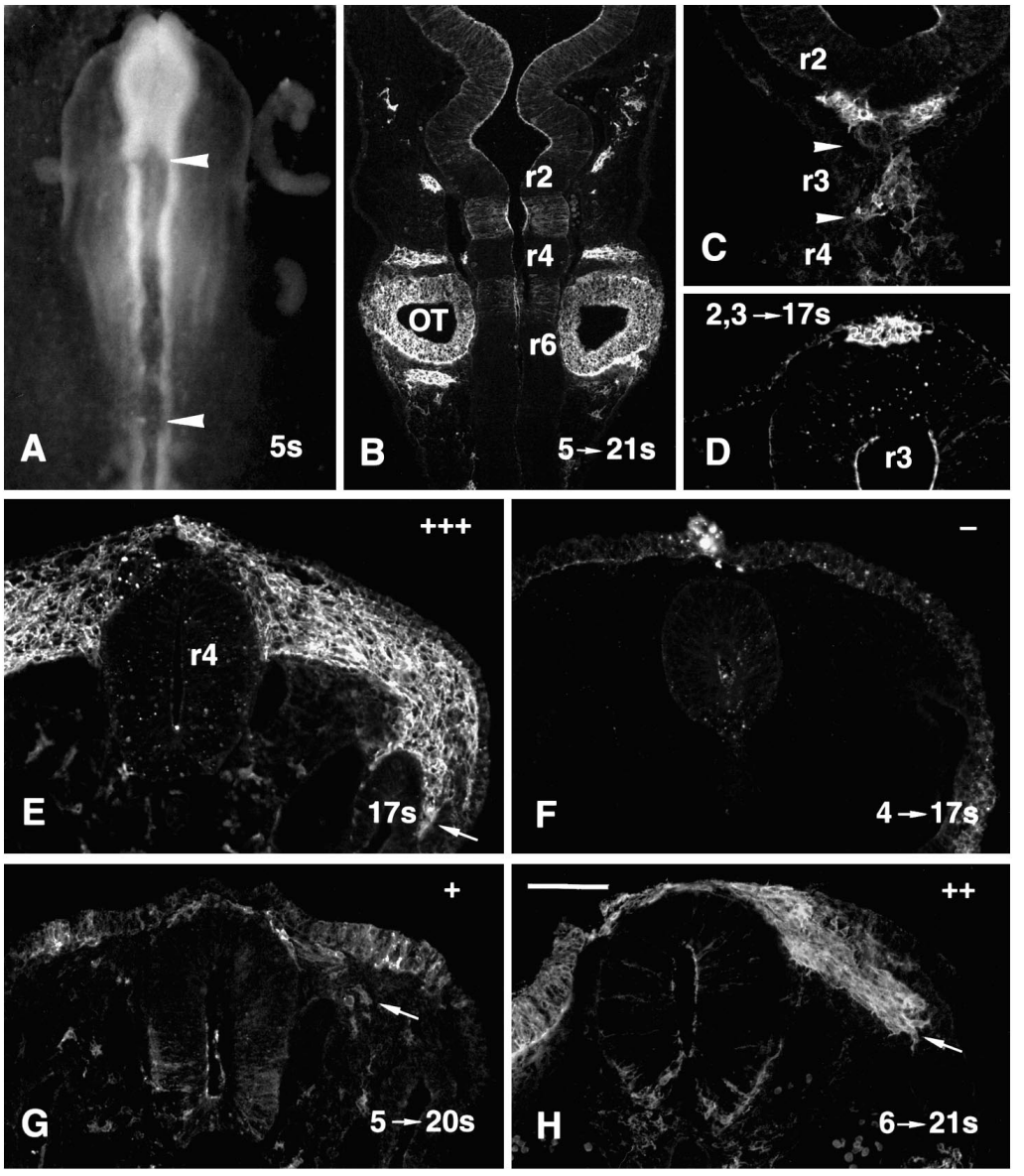

Fig. 4. Effects of long bilateral ablations that extend from the midbrain through the hindbrain and often to the level of the first somite. (A) A stereoscopic image of a 5ss embryo immediately after ablation. Arrowheads denote the rostral and caudal borders of the ablation, from the rostral midbrain to the first somite. Remnants of the dorsal neural tube (after removal) are visible to the left and right of the head. (B) A longitudinal section through the embryo shown in (A) after fixation at the 21ss and staining with the HNK-1 antibody. Streams of neural crest cells are observed adjacent to rhombomeres (r) r2, r4 and r6, displaying a normal migratory pattern but reduced in size compared with control embryos. Note that r3 and r5 have faint HNK-1 immunoreactivity. The otic vesicle (OT) is also HNK-1 immunoreactive. (C). A grazing dorsal, longitudinal section just below the ectoderm through the same embryo shown in B demonstrates that there is a continuous array of HNK-1 immunoreactive cells on the *dorsal* aspects of r2-4. (D) A transverse section extending to the level of r3 demonstrating an excessive number of HNK-1 immunoreactive cells on the dorsal aspect of r3. (E) Transverse section through the r4 level of the 17ss embryo shown in D in which r4 was not ablated. (F-H) Transverse sections through the r4 level in embryos with various depths of ablations and associated degrees of recovery (see Table 1), with no neural crest (–), few neural crest (+) and reduced neural crest (++) in the hindbrain; embryos were ablated at the 4ss, 5ss and 6ss and fixed at the 17ss, 20ss and 21ss, respectively, in F-H. Bar, 230 μm in A; 150 μm in B; 60 μm D; and 75 μm in C,E-H.

unchanged. Neural crest cells were seen on the dorsal surface of r2 through r6, consistent with the idea that there is uniform generation of neural crest cells by all rhombomeres (Sechrist et al., 1993; Birgbauer et al., 1995). By analyzing the number and distribution of the labeled neural crest cells (Table 1), we observed that the regulative response was maintained through the 7ss. The numbers of regenerated cells appeared to reflect both the time and depth of ablation. For example, there were somewhat fewer neural crest cells in the regenerated hindbrain of progressively older embryos. However, in four embryos in which particularly deep ablations were performed at the level of the hindbrain (see Table 1), few or no neural crest cells were observed at the level of the ablation despite the fact that ablations were performed prior to the 7ss. Because these embryo had the majority of the hindbrain neural tube removed, it is possible that they would have required additional time for recovery; alternatively, perhaps neural tube cells close to the midline are no longer able to regulate. Embryos allowed to survive for several days post-ablation (see below) provide evidence that all neural crest derivatives form after deep bilateral ablations but are permanently reduced in size.

Long-term effects of neural fold ablations

In our previous studies, embryos appeared remarkably normal by 36-48 hours after unilateral ablation of the neural folds and had the normal complement of neural crest derivatives by 5-7 days post-ablation (Scherson et al., 1993). To compare the long-term effects of unilateral and bilateral ablations, we allowed embryos to survive for 3-4 days following ablation

Table 1. Regulation of neural tube to form neural crest after bilateral ablation in the hindbrain

	cmb	r2	r4	r6
Standard ablations				
3 s → 18 s			+/++	+/++
4 s → 16^+ s	++	++	++	+++
4,5 s → 18 s	+/++	+	+/–	++
5 s → 15^+ s	+/–	+/–	+	+
5 s → 20 s	+/++	++	+	++/+++
5 s → 21 s	++	++	++	++/+++
6 s → 20 s	++	++	++	+++
6 s → 21 s	+/–	+	++	++/+++
7 s → 23 s	+	+/–	+	+/++
Deep ablations				
2,3 s → 17,18 s	+/–	+/–	+	++
4 s → 17 s	–	–	–	++
5 s → 17,18 s	+	+	+	+
5^+ s → 20 s	–/+	–/+	+	+

Extent of neural crest recovery: +++, relatively normal; ++, reduced; +, few; –, none.

(reaching stages 22-26 by the criteria of Hamburger and Hamilton, 1951). In addition, one embryo was allowed to survive 8 days following ablation (stage 36-37). In some embryos, DiI was applied to the surface ectoderm after neural tube closure, thus selectively labeling the placodal but not the neural crest population. Using this approach, we confirmed the substantial contribution of the ectodermal placodes to the trigeminal ganglion. We subsequently used neurofilament immunoreactivity to analyze the neural crest-derived ciliary ganglion as well as the trigeminal ganglion (Fig. 5), which has a dual contribution from the neural crest and the ectodermal placodes (LeDouarin, 1982).

When bilateral ablations were performed at the 4-7ss, some abnormalities were evident. In whole-mount views of these embryos, the head and first branchial arch appeared somewhat reduced in size and the mouth was enlarged. In sections through these embryos (n=8), both the ciliary and trigeminal ganglia were present. However, the ganglia and the brainstem often appeared greatly reduced in size (Fig. 5A,B) in comparison to these structures in embryos after unilateral ablation (n=3; data not shown) or in the unperturbed embryo (Fig. 5C). Concomitant with the decreased size of the trigeminal ganglion, DiI labeling of the placodes revealed an apparent increase in the percentage of placodally derived, relative to neural crest-derived, ganglion cells. In one embryo, there were abnormal nerve connections such that the proximal nerve fibers of the trigeminal nerve joined the facial nerve rather than its usual brainstem entry point (See also Hammond and Yntema, 1958). Interestingly, differences were noted in both the location and size of the ganglion on the right and left sides of bilaterally ablated embryos, suggesting differences in response that may correlate with some variability in depth of ablation and the numbers of regenerated neural crest cells. The fact that the size of the ciliary and trigeminal ganglia remained reduced in an embryo fixed 8 days after a deep ablation, performed at the 3-4ss, favors the idea that deep bilateral ablations lead to a permanent deficit in neural crest-derived structures. Thus, the neural tube can form all neural crest derivatives, but cannot fully compensate for the loss in cell number.

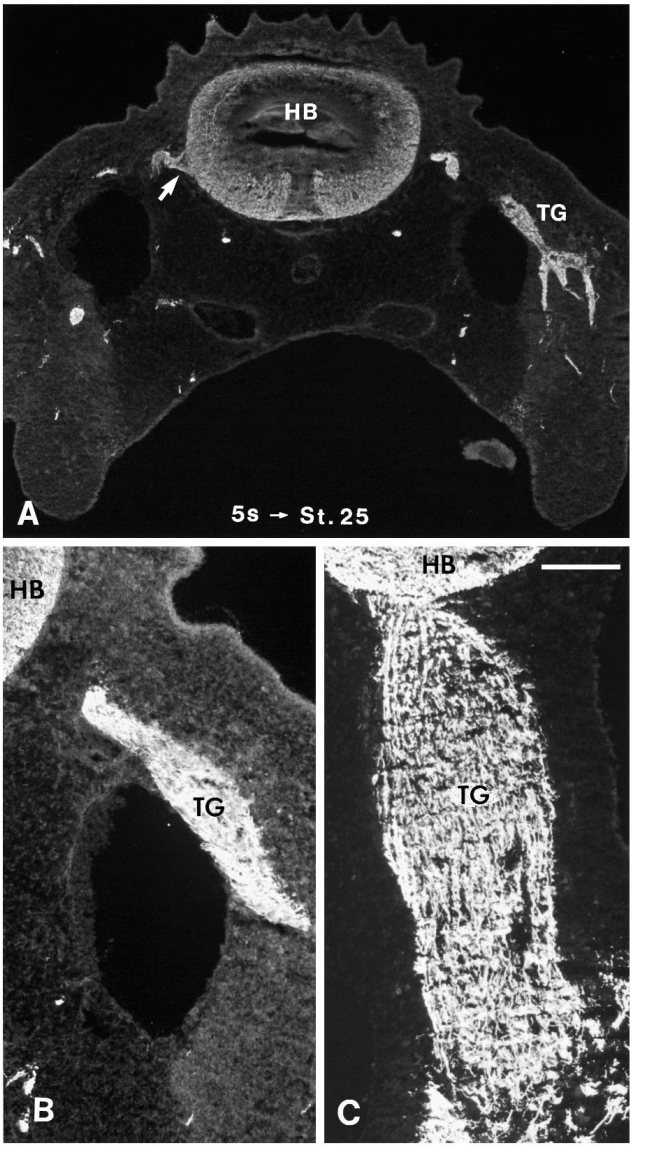

Fig. 5. Comparison of the trigeminal ganglion in an embryo 3.5 days after bilateral ablation (A,B) relative to a normal embryo (C). Transverse sections were stained with an antibody that recognizes a neurofilament protein. (A,B) An embryo that was ablated at the 5ss and fixed at stage 25 viewed at low (A) and higher (B) magnification. The trigeminal ganglion was present but greatly reduced in size compared with that in a slightly younger (stage 24) normal embryo (B compared with C). The arrow in A indicates the position of the nerve roots, which emanates from the midlateral portion of the neural tube rather than the ventrolateral position they occupy in unablated embryos. The hindbrain also was reduced in size, and the development of the head was generally delayed. HB, hindbrain; TG, trigeminal ganglion. Bar, 250 μm in A; 112 μm in B,C.

Analysis of *Slug* expression in ventrolateral neural tube cells following neural fold ablation

The finding that cells normally destined to form the ventrolateral neural tube in the caudal midbrain/rostral hindbrain have the capacity to form neural crest cells if the endogenous neural folds are removed suggests a shift from their normal developmental program. Presumably, this change in prospective fate

is accompanied by alterations in gene expression, since a different set of genes is expressed in the dorsal versus ventral portions of the unperturbed neural tube (Jessell and Dodd, 1992). Genes expressed in the dorsal neural tube might be expected to be up-regulated after ablation. One of the earliest genes known to be expressed in the neural folds is *Slug*, a vertebrate gene encoding a zinc finger protein of the Snail family (Nieto et al., 1994). Thus, *Slug* serves as an excellent marker for both presumptive and early-migrating neural crest cells. We examined whether *Slug* is an early response gene after cranial neural fold ablation.

In normal embryos during neurulation, *Slug* expression is found within the dorsal cranial neural folds by the 4-5ss as well as in the primitive streak in caudal portions of the embryo (Nieto et al., 1994). The presence of *Slug* transcripts in early migrating cranial neural crest cells and more caudal neural folds in a 9ss embryo is illustrated in Fig. 6. *Slu*g mRNA is found in both premigratory neural crest cells in the dorsal neural tube and in migrating cells.

Embryos were examined for the presence of *Slug* transcripts after either unilateral (n=12) or bilateral (n=41) removal of the neural folds. Immediately after ablation of the neural folds (n=5), *Slug* expression is absent from the ablated regions, indicating that the neural crest-forming region has been removed (Fig. 7A,C). When comparing control and operated sides after unilateral neural fold removal (Fig. 7A), the unablated side shows intense *Slug* expression along the entire length of the neural folds, whereas the ablated region is clearly devoid of *Slug* transcripts at the level of the removal, though *Slug* expression is present rostral and caudal to the ablated region. We examined embryos at the 7-21ss after either unilateral (Figs 7B, 8F) or bilateral (Figs 7D, 8A-E) ablation and found that *Slug* expression is up-regulated in the ventrolateral neural tube cells prior to re-emergence of the cranial neural crest. *Slug* is initially expressed approximately 5-8 hours after ablation and its expression remains in migrating neural crest cells.

The onset of *Slug* expression was dependent upon the stage of ablation (Fig. 8), with those ablations performed at early stages (3-4ss) having some *Slug*-positive cells within neural folds as early as 5-6 hours post-ablation. In contrast, ablations at the 5-6ss typically expressed *Slug* at the level of the ablation delayed by 9-10 hours post-ablation. However, considerable variability was observed from embryo to embryo; for example, the embryos illustrated in Fig. 8A,B were both ablated at the 5ss, though the latter showed more up-regulation of *Slug* transcripts than the former. Up to 24 hours after unilateral ablation, the numbers of *Slug*-positive neural crest cells and their extent of migration are usually lower on the ablated side than the control side (Fig. 8F). After 36 hours, the ablated side has substantial recovery. In embryos in which ablations were done at the 6-7ss and allowed to survive for 1 day (n=6), the caudal forebrain/rostral midbrain level appeared to have little or no *Slug* expression (Fig. 8E); occasionally, a deficit in numbers of *Slug* expressing cells at the caudal forebrain/rostral midbrain level was observed in ablations performed at earlier stages as well. In embryos with >7 somites at the time of ablation, regulation of *Slug* expression was usually absent at both midbrain and hindbrain levels, supporting the idea that the regulative process is temporally limited (Scherson et al., 1993).

The appearance of *Slug* expression adjacent to the ablated neural fold region seemed to vary with axial level, reflecting the rostrocaudal variations in regulative ability described above with whole ectoderm DiI-labeling. Accordingly, up-regulation of *Slug* expression preferentially occurred at the level of the caudal midbrain and rostral hindbrain. In contrast, far less *Slug* expression was apparent at the level of the rostral midbrain (Figs 7B, 8A-E). The depth of the ablation also contributed to the extent of *Slug* expression.

DISCUSSION

Our previous study demonstrated that, after ablation of the endogenous neural folds, the residual intermediate neural tube cells regenerate cranial neural crest cells (Scherson et al., 1993). Here, we extend this work to examine the temporal pattern of neural crest regeneration, regional differences in regulative ability and gene expression induced shortly after ablation. Rather than producing neural crest cells directly from the neural tube, we find that production of neural crest cells after ablation occurs after apposition of the neural plate and epidermis, similar to the inductive interaction that appears to be responsible for initial generation of the neural crest (Selleck and Bronner-Fraser, 1995). This suggests a common developmental mechanism for generation and regeneration of the neural crest. Transcripts encoding the zinc finger protein *Slug* were found to be up-regulated shortly after ablation, suggesting that it is an early-response gene. Interestingly, we find that neural tube cells in the caudal forebrain and rostral midbrain exhibit a much less robust regulative response than those at the caudal midbrain/rostral hindbrain. Thus, the regulative response appears to be position as well as stage dependent. This regional difference in regulative ability occurs regardless of the stage at which the ablation is performed, suggesting that the rostrocaudal pattern of regulative response is established prior to neural tube closure.

The regulative response of neuroepithelium raises the intriguing possibility that a larger region of the neural plate may possess the capacity to form neural crest cells than that predicted by the normal fate map (Rosenquist, 1981). Previous data have shown that the neural tube, neural folds and epidermis share a lineage up to the time of neural tube closure, but that the epidermal lineage diverges thereafter (Selleck and Bronner-Fraser, 1995). Even after neural tube closure, neural crest and neural tube cells share a lineage (Bronner-Fraser and Fraser, 1989; Frank and Sanes, 1991; Artinger et al., 1995; Bronner-Fraser, 1995). Furthermore, dorsal spinal cord cells maintain the ability to produce neural crest-like derivatives through avian embryonic day 5 (Sharma et al., 1995). These data suggest an intimate interrelationship between neural crest and neural tube cells. Thus, much of the neuroepithelium may represent a equivalency group with respect to developmental potential. In support of this idea, single cell lineage analysis of embryos receiving a notochord graft adjacent to the dorsal neural tube has revealed that the progeny of individual neural tube cells assume a wide variety of fates, as diverse as sensory ganglion cells, commissural neurons, motor neurons and floor plate cells (Artinger et al., 1995). As such, it may not be surprising that early cranial neural tube cells normally destined to form intermediate cell types can adopt a ‘dorsal’ neural crest fate.

In the present study, we show that *Slug* is potentially

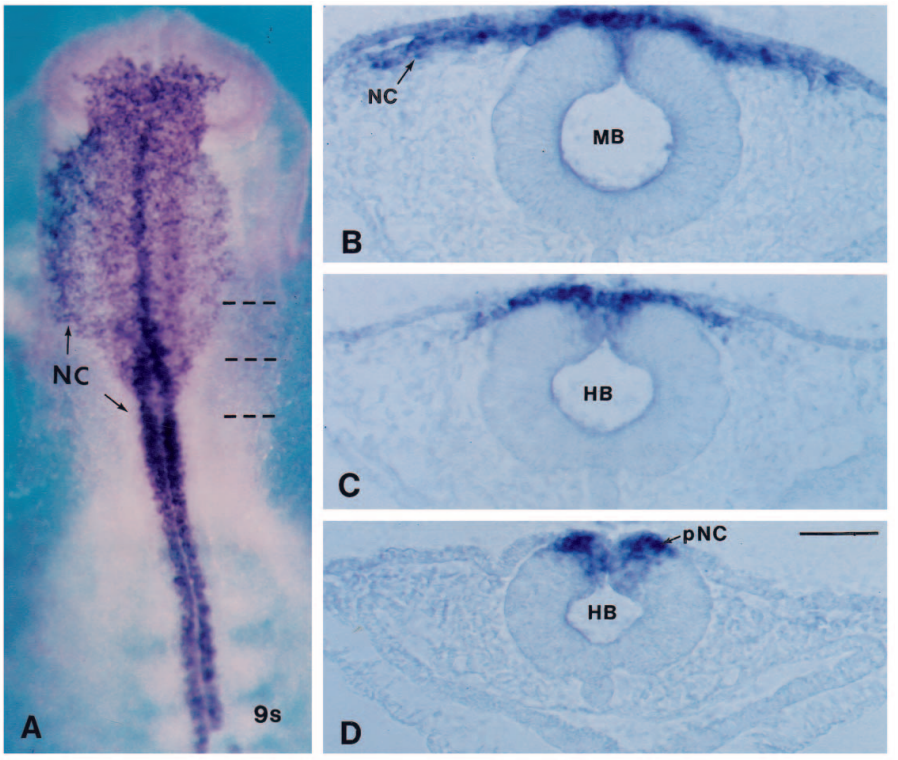

Fig. 6. Whole-mount in situ hybridization of a normal 9ss embryo with a *Slug* probe. (A) In whole-mount view, *Slug* is evident in migrating neural crest cells adjacent to the caudal forebrain, midbrain and rostral hindbrain, as well as premigratory neural crest cells further caudally. Dashed lines indicate the levels of sections in B-D. (B) At the level of the midbrain, *Slug* expression is detected in migrating neural crest cells beneath the surface ectoderm. Note that staining is minimal in the dorsal neural tube. (C) In the rostral hindbrain, neural crest migration has just begun and some *Slug* is still present in the dorsal midline of the neural tube. (D) Further caudally in the hindbrain, neural crest migration has not yet begun and *Slug* is prominent in premigratory neural crest cells in the dorsal neural tube. MB, midbrain; HB, hindbrain; NC, neural crest; pNC, premigratory neural crest. Bar, 210 μm in A; 60 μm in B-D.

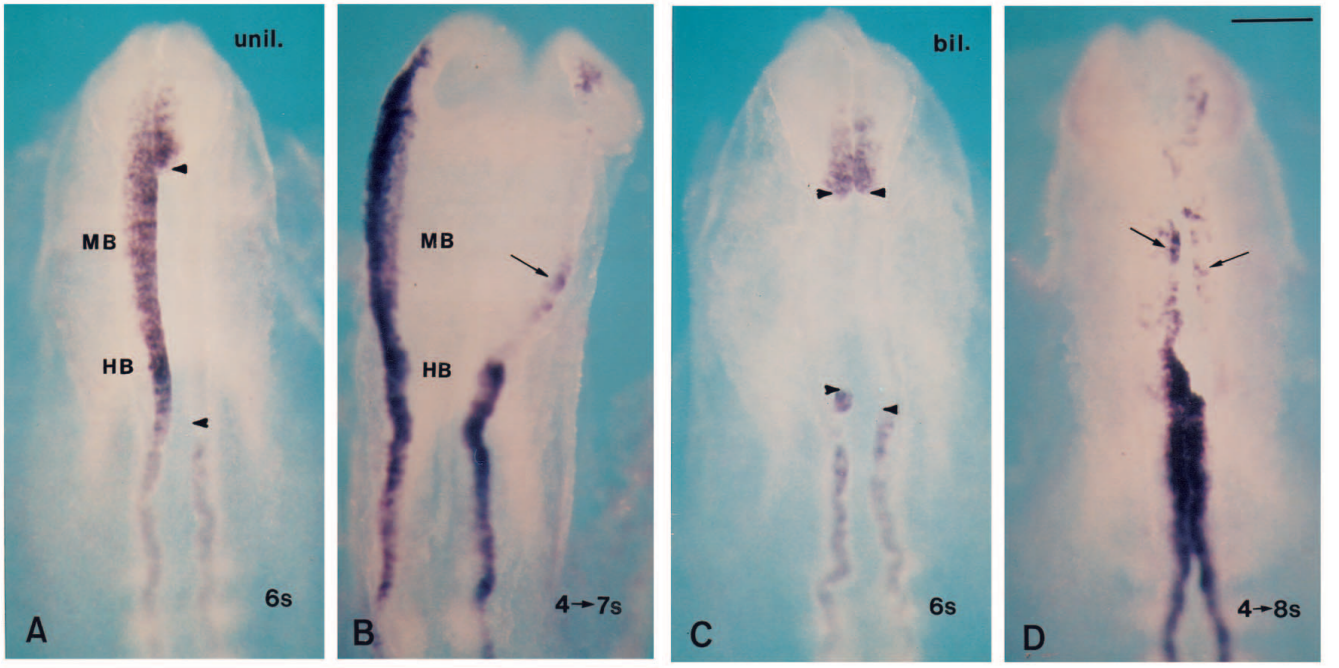

Fig. 7. Whole-mount in situ hybridization with a *Slug* probe of embryos after unilateral and bilateral neural fold ablations. (A) Immediately after unilateral ablation of the right neural fold of a 6ss embryo at the level of the midbrain and hindbrain, the right side is devoid of *Slug* expressing cells between the arrowheads. (B) An embryo ablated unilaterally at the 4ss and fixed 6-7 hours later by which time it has reached the 7ss; some *Slug*-positive cells (arrow) are detected on the ablated side at the level of the caudal midbrain/rostral hindbrain. (C) Immediately after bilateral ablation at the 6ss, *Slug* expression is absent from the neural folds on both sides. At the rostral border of the ablation, *Slug* expression is evident in premigratory neural crest cells. In contrast, *Slug* is absent in the mid-region of the ablation and present only on the left side at the caudal margin of the ablation in this embryo. (D) A 4ss embryo fixed 6-7 hours after bilateral ablation by which time it has reached the 8ss. *Slug* transcripts (arrows) are observed in the midbrain and hindbrain. MB, midbrain; HB, hindbrain; unil., unilateral; bil., bilateral. Bar, 210 μm.

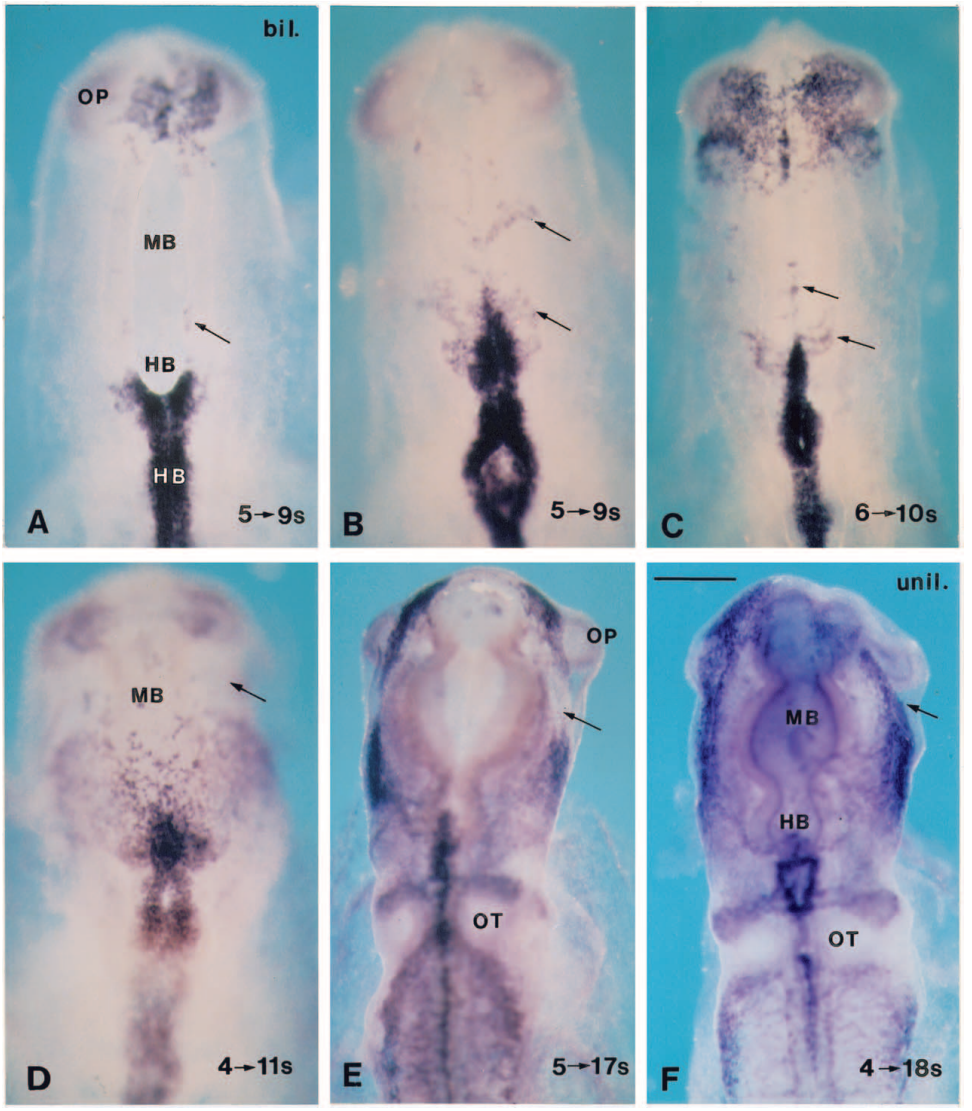

Fig. 8. Whole-mount in situ hybridization of *Slug* expression at progressive stages after bilateral (A-E) or unilateral (F) neural fold ablation. (A) An embryo after a deep bilateral ablation performed at the 5ss and fixed 6-7 hours later at the 9ss; *Slug*-positive cells are present at the rostral hindbrain level (arrow). (B) Another embryo after bilateral ablation at the 5ss and fixed 6-7 hours later at the 9ss with extensive *Slug* expression in the ablated region; *Slug*-positive cells are present both in the neural tube and in migrating neural crest cells at the level of the midbrain and hindbrain (arrows). (C) An embryo ablated at the 6ss and fixed 6-7 hours later at the 10ss; migrating *Slug*-positive cells are present at the level of the rostral hindbrain (lower arrow), but are detected only at the dorsal midline in the midbrain, indicating slower recovery at this axial level. (D) An embryo bilaterally ablated at the 4ss and fixed 12 hours later at the 11ss. Migrating *Slug*-positive cells are prevalent at the caudal midbrain/hindbrain level, but are sparse in the caudal forebrain/rostral midbrain region (arrow). (E) An embryo bilaterally ablated at the 5ss and fixed 18 hours later at the 17ss. Migrating *Slug*-positive cells are prevalent at the level of the caudal midbrain/hindbrain, but remain sparse at the caudal forebrain/rostral midbrain (arrow). (F) An embryo after unilateral ablation at the 4ss and fixation 22 hours later at the 18ss. In contrast to the paucity of neural crest cells in the caudal forebrain/rostral midbrain level noted after bilateral ablation, *Slug*-positive cells (arrow) are present at this level after unilateral ablation but do appear to be reduced in number. OP, optic cup; OT, otic cup; MB, midbrain; HB, hindbrain; bil., bilateral; unil., unilateral. Bar, 180 μm.

involved in the regulative process. It is up-regulated in the neural tube 5-8 hours after initial ablation. Its expression precedes neural crest migration and remains present in migrating cranial neural crest cells. *Slug* has been previously shown (Nieto et al., 1994) to be required for the emigration of cranial neural crest cells from the neural tube. The present experiments demonstrate that *Slug* expression represents an early response to the ablation. It may be one of a series of genes whose transcription is initiated in residual neural tube cells after ablation. Within the nervous system, *Slug* is normally only expressed in the dorsal neural tube and neural crest. Thus, its expression in regulating cells of the intermediate portions of the neural tube supports the idea that these cells retain the ability to form neural crest derivatives.

The developmental mechanism underlying regeneration of the neural crest may recapitulate initial formation of the neural crest. Regenerated neural crest cells emerge from the residual neural tube after contact with the overlying ectoderm. One possibility is that new neural crest cells are generated via an inductive interaction between the neuroepithelium and the presumptive epidermis. In gastrulating or neurulating embryos, neural crest cells form after experimental juxtaposition of neural tissue with non-neural ectoderm (Moury and Jacobson, 1989; Selleck and Bronner-Fraser, 1995; Dickinson et al., 1995). The finding that *Slug* expression is induced by neural plate/ectoderm interactions (Dickinson et al., 1995) provides further support for the idea that interactions between these two tissues are necessary for the regulative response of the neural tube to form neural crest. Neural plate/ectoderm interactions are sufficient for the induction of *Wnt-1* and *Wnt-3a* in neural plate explants (Dickinson et al., 1995), suggesting that non-neural ectoderm induces dorsal properties in the developing neural tube. This raises the intriguing possibility that in addition to generating the neural crest, the non-neural ectoderm respecifies dorsal properties in the intermediate neural tube. It is interesting to note that the ventral-most region of the neural tube that is in contact with the notochord does not become 'dorsalized' even after contact with the ectoderm (Dickinson et al.,

1995). This suggests that there may be an interplay between dorsalizing signals from the ectoderm and ventralizing signals from the notochord (Jessell and Dodd, 1991) that determines cell fates along the dorsoventral axis.

Our results and those of the previous study (Scherson et al., 1993) demonstrate that the regulative ability of the cranial neural tube to form neural crest is transient, declining after the 7 somite stage. Because these regenerative events appear to involve an interaction between the neural tube and ectoderm, a salient question is whether this change reflects a decrease in the competence of the neural tube itself or the ectoderm to produce an inductive signal. In previous studies in which neural plate or intermediate neural tube was recombined with ectoderm, we found that ectoderm from a wide variety of stages, ranging from stages 4 to 20, had the capacity to produce neural crest cells when combined with neural plate (Dickinson et al., 1995). This suggests that neural plate, rather than the non-neural ectoderm, changes as a function of time which in turn may restrict the regulative ability of the cranial neural tube. A definitive test of this idea will require heterchronic grafting of the neural tissue and/or surface ectoderm.

We noted profound rostrocaudal differences in regulative capacity between caudal forebrain/rostral midbrain and caudal midbrain/hindbrain. What accounts for such rostrocaudal variation? Because development occurs in a rostrocaudal progression, the caudal forebrain/rostral midbrain is comparatively more mature than the caudal midbrain/hindbrain at the time of ablation. This may result in a decreased plasticity of the remaining neural tube cells at the former axial level. Accordingly, one might predict that ablations performed at earlier times may have greater regulative ability. Surprisingly, when ablations were performed at ealier stages (1-3ss), rostrocaudal differences between the degree of regulative response were still observed in most cases. This suggests that rostrocaudal variations in the the regulative ability along the neural axis may already be present by the time the neural folds have formed. Other evidence that there may be a prepattern along the neural axis with respect to regulative response comes from experiments indicating that trunk and vagal neural tube cannot regenerate the neural crest after ablation (Kirby et al., 1983; Kirby, 1989). In addition, notable differences exist between rostral and more caudal cranial neural crest cells in normal embryos; the former are generated over a shorter period of time than those in more caudal regions (Lumsden et al., 1991; Sechrist et al., 1993), suggesting that a larger precursor pool may exist at the caudal midbrain/rostral hindbrain level. However, the observation of a prepattern cannot rule out the possibility that the regulative ability follows a temporal wave which regresses rostrocaudally. In fact, we did observe a better regulative response at the forebrain/rostral midbrain at earlier stages than later stages. Furthermore, there is a clear decrease in the regulative response at the 6-7ss, following the decline of nervous system induction after stage 4-5 (Storey et al., 1992).

Embryos with unilateral ablations appear remarkably normal a few days after ablation, whereas those with bilateral ablations have deficits in neural crest-derived structures. There are several possible explanations for the seemingly normal complement of neural crest cells and their derivatives after unilateral removal of the neural folds. First, the contralateral neural tube may contribute neural crest cells after unilateral neural fold ablation. Although our previous experiments with focal injections of DiI into the contralateral neural folds demonstrated that some cells migrated contralaterally (Scherson et al., 1993), our whole embryo DiI-labeling suggests that this may represent a larger population than originally thought. This is not surprising given that focal injections label only a small percentage of migrating cells whereas whole-ectoderm labeling would be expected to label all ectodermal cells including neural crest cells. Second, there may be some compensation from existing neural crest cell populations either rostral or caudal to the ablated region at these axial levels. Focal injections reveal a small amount of rostrocaudal movement of regenerated neural crest cells, making such a compensation at least partially feasible. In addition to a contribution from the contralateral side, it is possible that other ectodermal populations, such as the diffuse rostral ectodermal placodes, may produce some neural crest-like cells at this axial level. Preliminary evidence suggests that there are more placodal cells entering the head, up to the mid-optic vesicle level than previously anticipated; these may increase after neural fold ablation (Sechrist, Zamanian and Bronner-Fraser, unpublished observation). In addition, we cannot rule out the possibility that the proximal ectoderm lateral to the closing neural folds contributes some neural crest cells after ablation as occurs in the initial formation of neural crest cells (Selleck and Bronner-Fraser, 1995).

We observed some interesting similarities and differences between unilateral and bilateral neural fold ablations. In both cases, there was substantial compensation at the level of the caudal midbrain/rostral hindbrain, but much less regulation at more rostral axial levels. This was most obvious in bilateral ablations, in which neural crest migration was delayed, particularly at the level just caudal to the optic vesicles. An important difference between unilaterally and bilaterally ablated embryos was that the former appeared relatively normal (Scherson et al., 1993) whereas some of the latter had delays in craniofacial development and numerous abnormalities. These included increased vessel size and blood pooling in the rostral head, some deficiencies in crest-derived cranial ganglia and reductions in the first branchial arch. The extent of abnormalities with bilateral ablations appears to be age-dependent with operations performed during the early half of neurulation (3-4ss) exhibiting more recovery than those done at slightly later stages (5-7ss). Because Hammond and Yntema (1958) observed reduced ganglia in embryos observed at late stages (7-8 days post-ablation) following similar neural fold ablations, these deficits are likely to be permanent, reflecting a smaller complement of neural crest cells. Similarly, Hamburger (1961) performed whole neural tube removals at the level of the rostral hindbrain and observed abnormal cell aggregates in the placodal component of the trigeminal ganglion, probably reflecting a diminution or absence of neural crest cells. Interestingly, bilateral ablations performed at the hindbrain level (Hunt et al., 1995) lead to restoration of a normal Hox code. This supports the idea that more caudal levels may have a more robust regulative response than more rostral levels. In contrast to the present findings, Hunt et al. (1995) found no abnormalities in craniofacial development after hindbrain ablation. A possible explanation for the failure of Hunt and colleagues to find craniofacial defects after bilateral ablation is that their removals may have been more superficial that those performed in our study; furthermore,

some embryos appeared to have been ablated after the onset of neural crest migration. Despite the differences between unilateral and bilateral ablations found in the present study, the data clearly show that the neural tube has the capacity to form substantial numbers of neural crest cells after neural fold ablation. These results highlight the surprising plasticity and regulative ability of the cranial neuroepithelium, which should serve as an excellent model system for understanding the molecular mechanisms underlying formation of the neural crest.

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