

# Origins of the avian neural crest: the role of neural plate-epidermal interactions

Mark A. J. Selleck\* and Marianne Bronner-Fraser

Developmental Biology Center, University of California at Irvine, Irvine, California 92717, USA

\*Author for correspondence

## SUMMARY

We have investigated the lineage and tissue interactions that result in avian neural crest cell formation from the ectoderm. Presumptive neural plate was grafted adjacent to non-neural ectoderm in whole embryo culture to examine the role of tissue interactions in ontogeny of the neural crest. Our results show that juxtaposition of non-neural ectoderm and presumptive neural plate induces the formation of neural crest cells. Quail/chick recombinations demonstrate that both the prospective neural plate and the prospective epidermis can contribute to the neural crest. When similar neural plate/epidermal confrontations are performed in tissue culture to look at the formation of neural crest derivatives, juxtaposition of epidermis with either early (stages 4-5) or later (stages 6-10) neural plate results in the generation of both melanocytes and sympa-

thoadrenal cells. Interestingly, neural plates isolated from early stages form no neural crest cells, whereas those isolated later give rise to melanocytes but not crest-derived sympathoadrenal cells. Single cell lineage analysis was performed to determine the time at which the neural crest lineage diverges from the epidermal lineage and to elucidate the timing of neural plate/epidermis interactions during normal development. Our results from stage 8 to 10+ embryos show that the neural plate/neural crest lineage segregates from the epidermis around the time of neural tube closure, suggesting that neural induction is still underway at open neural plate stages.

Key words: cell lineage, chick embryo, ectoderm, induction, neural tube, neurulation, peripheral nervous system, quail embryo

## INTRODUCTION

During neurulation in vertebrate embryos, the ectoderm becomes subdivided into three embryonic tissue types: the neural tube, the neural crest and the epidermis. The epidermis will eventually cover the entire surface of the embryo and contribute to the skin, while the neural tube and neural crest together give rise to most of the nervous system. Cells of the neural tube develop into the neurons and glia of the central nervous system (CNS), whereas neural crest cells differentiate into the sensory neurons, postganglionic autonomic neurons and Schwann cells of the peripheral nervous system (PNS), as well as pigment cells, catecholamine-secreting cells of the adrenal medulla and some cranial cartilage (reviewed by Le Douarin, 1982).

Fate mapping experiments performed on chick embryos at the definitive streak stage (stage 4; Hamburger and Hamilton, 1951) locate the prospective neural plate immediately rostral to Hensen's node (Rudnick, 1935; Spratt, 1952; Rosenquist, 1981; Bortier and Vakaet, 1992), with presumptive neural crest cells lying at its border with future epidermis. As neurulation proceeds, the edges of the neural plate (i.e. the neural folds) approximate and fuse, and the neural tube separates from the overlying epidermis with which it was initially contiguous. Neural crest cells have been shown to arise from the neural folds and subsequently exit from the dorsal neural tube

(Serbedzija et al., 1989; Bronner-Fraser and Fraser, 1988, 1989), from which they migrate along characteristic pathways towards their target sites.

What mechanisms are involved in the generation of neural crest cells from the ectoderm? Most studies on the origin of neural crest cells have been conducted using amphibian embryos. Rollhäuser-ter-Horst (1979) and more recently Moury and Jacobson (1989) experimentally juxtaposed epidermis and neural plate and found that neural crest cells are generated at the junction of the two tissues. Additional experiments, in which epidermis and neural plate are taken from different pigmented species of Urodele, reveal that neural crest cells arise from both the epidermis and neural plate (Moury and Jacobson, 1990). Interestingly, melanocytes arise primarily from the neural plate whereas the epidermis gives rise to spinal and cranial ganglia, suggesting that neural crest cells also share a lineage with epidermis. At least in the Urodele, it would seem that neural crest cells arise as a result of interactions between the epidermis and neural plate.

The times at which ectodermal cell lineages segregate may give insights into the timing of such interactions in the embryo. To date, cell lineage experiments investigating neural crest formation in the chick embryo have been conducted after neural tube closure (Bronner-Fraser and Fraser, 1988, 1989; Frank and Sanes, 1991; Fraser and Bronner-Fraser, 1991). These studies have shown that the progeny of single cells

within the dorsal neural tube can form both neural tube and neural crest derivatives, suggesting a common progenitor for some CNS and PNS derived cells. However, these studies have not addressed the question of when the neural tube/neural crest lineage segregates from the epidermal lineage. It is possible that these lineages separate prior to neural tube closure, even as early as the time of neural induction (stage 4–6, Hamburger and Hamilton), in which case inductive interactions that generate neural crest cells could be early events based on interactions between these non-equivalent populations. Alternatively, the interactions that generate neural crest cells may continue until later stages of neural tube closure. If single ectodermal cells in the closing neural folds can contribute progeny to all three ectodermal derivatives, then this would indicate that the mechanisms generating neural crest cells from the ectoderm are still ongoing at this stage. Because previous lineage experiments in avian embryos focused on the lineages of the neuroepithelium, there is no information regarding a shared lineage between the neural crest and epidermis, as seems to be the case in the Urodele.

Here, we present three main experiments to study the origins of neural crest cells in the avian embryo. To address the question of whether neural crest cells arise as a result of inductive interactions, we have grafted prospective neural plate adjacent to presumptive epidermis both in whole embryo culture and in tissue culture, and have used a variety of cellular markers to identify trunk neural crest derivatives. To determine the 'crestogenic' potential of neural tissue, we have cultured neuroepithelium from different developmental stages and used markers to assess the formation of neural crest cells. To investigate lineage relationships of the ectoderm, we have performed a series of fate mapping and cell lineage experiments on cells at the neuroepithelium/epidermis boundary at different developmental stages. We provide evidence that (i) epidermal/neural plate interactions are sufficient to generate a range of neural crest derivatives in the avian embryo, (ii) the ability of the neural plate to generate some neural crest derivatives changes between stages 4 and 6, and (iii) the commitment of ectodermal cells to a neural/neural crest fate, thought to commence at primitive streak stages, is still in progress at relatively late stages of development, at levels of the open neural plate. Our results are also consistent with the idea that the epidermis and neural tube/neural crest lineages segregate around the time of tube closure.

## MATERIALS AND METHODS

### Grafting and tissue culture experiments

Recent fate mapping experiments (Bortier and Vakaet, 1992) have shown that ectoderm lying immediately rostral to Hensen's node will contribute to ventral spinal cord and hindbrain. Accordingly, recent studies showed that neural induction is already in progress by this stage (Roberts et al., 1991). Therefore, we have used ectoderm rostral to Hensen's node as 'early' (prospective) neural plates for our experiments. Since one cannot be sure that the medial ectoderm of a stage 4 embryo is committed to a neural fate, we have used more developmentally 'mature' neural plates, isolated from stage 6 to 10 embryos, for some of the tissue culture experiments.

#### In ovo grafting experiments

To determine whether neural plate/epidermal interactions can

generate neural crest cells in the avian embryo, prospective neural plates were grafted adjacent to presumptive epidermis in whole embryo culture. Fertile chicken and quail eggs (White leghorn) were incubated for 18 to 20 hours to give definitive-streak stage embryos (stage 4). In some experiments, chick tissue was grafted into chick hosts, while in others quail tissue was transplanted into chick hosts.

#### Isolation of graft tissues

To isolate prospective neural plates and epidermis, embryos were explanted into PBS and transferred to Sylgard- (Dow Corning) coated dishes containing calcium- and magnesium-free Tyrode's saline (CMF). Trypsin (0.1% w/v) was added to the saline to aid the dissection and ensure that the graft was not contaminated with other cell types. Using a mounted glass microelectrode, prospective neural plates were isolated from 120  $\mu\text{m}$  squares of ectoderm lying immediately rostral to Hensen's node. Prospective epidermis was isolated from pieces of ectoderm, approximately 120  $\mu\text{m}$  square, lying at the area pellucida/area opaca boundary (Bortier and Vakaet, 1992; see Fig. 1A). Following isolation, the graft was allowed to recover in complete culture medium (CCM; 15% horse serum, 10% chick embryo extract in Eagle's minimum essential medium) for approximately 60 minutes, prior to transfer to host embryos.

#### New culture and grafting

Host embryos were grown in modified New culture (New, 1955; Stern and Ireland, 1981). Briefly, embryos were explanted into Pannett and Compton saline (Pannett and Compton, 1924) and the vitelline membrane cut around the yolk equator. The membrane, with adhering blastoderm, was wrapped around a plexiglass ring on a watch-glass such that the embryo lay ventral-side uppermost in the culture. Using a tungsten needle, a 'pocket' was made between the yolky hypoblast and ectoderm at, or lateral to, the area pellucida/area opaca boundary (at the same rostrocaudal level as Hensen's node), into which grafts were placed (see Fig. 1B). After grafting, saline was removed from inside the culture ring and the embryo placed into a 35 mm culture dish (Nunc) containing a pool of albumin. Embryos were cultured at 38°C in a humidified incubator for 24 hours, after which they were fixed in Zenker's or 4% paraformaldehyde for 1–2 hours.

#### Immunocytochemistry in the whole mount

Antibodies to the carbohydrate antigen HNK-1 recognize a number of different cell types, including neural crest cells. On account of this, the HNK-1 antibody was used to assess whether neural crest cells were present in the grafts, by whole mount antibody staining (as described in Selleck and Stern, 1992). Embryos were rinsed in PBS (pH 7.4) and the endogenous peroxidase activity blocked with hydrogen peroxide (0.25% in PBS) for 2–3 hours. Specimens were then rinsed further in PBS, PBT (PBS with 0.2% bovine serum albumin, 1% Triton X-100 and 0.01% thimerosal) and then PBT containing 5% heat-inactivated goat serum. Following an overnight incubation in 1:1 HNK-1 supernatant with PBT/goat serum, embryos were rinsed in PBT and subsequently incubated overnight with peroxidase-conjugated goat anti-mouse IgM (Jackson Immunochemicals) in PBT/5% goat serum at a dilution of 1:150. After several rinses with PBS, peroxidase activity was revealed by placing embryos into a 1 mg/ml solution of 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma) containing 0.001% hydrogen peroxide. Embryos were subsequently dehydrated, cleared in histosol and mounted in Permount (Fisher Scientific). Whole-mount embryos were examined and photographed with an Edge high definition, high magnification stereo light microscope. In a few cases, stained embryos were subsequently embedded in paraplast and sectioned.

#### Immunocytochemistry on sections

In some experiments, quail neural plates were grafted adjacent to chick epidermis to investigate the tissue origin of neural crest cells. QCPN is an antibody that recognizes the perinuclear membrane of

quail cells and was therefore used to distinguish chick from quail in these grafting experiments. QCPN culture supernatant was obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences at John Hopkins University School of Medicine, Baltimore, MD, and the Department of Biology at the University of Iowa, Iowa City, IA, under contract number NOI-HD-2-3144 from the NICHD.

The double antibody labeling was performed as follows. Embryos were fixed in Zenker's solution, embedded in paraplast and sectioned at 10  $\mu$ m. After hydrating, they were rinsed in PBS and incubated in 3% bovine serum albumin (BSA) in PBS for 30 minutes at room temperature. The BSA solution was drained from the slides and the sections incubated for 1 hour in HNK-1 supernatant at room temperature. Following brief rinses in PBS, sections were incubated with FITC-conjugated goat anti-mouse IgM (Zymed) for an additional hour. Sections were subsequently labeled with the QCPN antibody in a similar way, using a TRITC-conjugated goat anti-mouse IgG (Zymed) secondary antibody. After staining, sections were coverslipped with Gel Mount (Biomed), examined and photographed as described below.

### Tissue culture experiments

Our tissue culture experiments were used to (i) determine whether neural crest cells are formed as a result of neural plate/epidermal interactions, and (ii) assess the 'crestogenic' potential of neural plates at different stages of development. Fertile quail and chicken eggs were incubated for 18-24, 27-33 or 48 hours until embryos had reached definitive streak to head fold stages (stages 4-6), stages 9-10 and stages 12-13 respectively. Prospective neural plates taken from definitive streak- to head fold-stage embryos or neural plates from stage 9-10 embryos (7 to 10 somite pairs) were cultured either alone or with epidermis taken from stage 4 embryos. Controls consisted of culturing epidermis alone, or neural crest cells derived from closed neural tubes (stages 12-13). In a few experiments, we co-cultured stage 4 presumptive neural plates with paraxial mesoderm isolated from a stage 10 embryo.

### Isolation of tissues

Definitive primitive streak-stage prospective neural plate and epidermis explants were isolated as described above for New culture grafting experiments. For older neural plates, stage 6 embryos or embryos with 7-10 somite pairs were explanted into PBS and pinned out in dishes containing CMF with 0.1% trypsin. At this stage, the neural plate surrounding Hensen's node is open and the neural folds clearly visible. Using a mounted glass microelectrode, 4 incisions were made: one through the neural plate lying medial to the neural folds, one slightly lateral to the midline and two transverse cuts rostral and caudal to Hensen's node (Fig. 1C,D). The explant was gently teased from the underlying mesoderm and allowed to recover in CCM, as described above.

Segmental plate and somites were isolated from stage 10 embryos in a similar way. Embryos were pinned out in CMF containing 0.1% trypsin and after removal of overlying ectoderm, the rostral half of segmental plates and the three most recently formed somites were dissected from adjacent mesoderm and endoderm. The explants were subsequently transferred to CCM, prior to culture.

To isolate neural tubes, stage 12-13 embryos were explanted into PBS and the axial structures adjacent to the 4 last formed somites and rostral half of the segmental plate isolated. These were treated with collagenase (1 mg/ml in Howard-Ringer's saline; Pettway et al., 1990) on ice for 15 minutes and at 38°C for a further 7 minutes, before triturating the tissues with a Pasteur pipette. Neural tube fragments were allowed to recover in CCM before transfer to culture dishes.

### Tissue culture

Tissue culture was performed as described previously (Artinger and Bronner-Fraser, 1992). Plastic tissue culture dishes (35 mm diameter

or 15 mm 4 well multidish; Nunclon) were incubated with 25  $\mu$ g/ml fibronectin (NYC blood bank) in Howard's Ringers at 38°C for 1 hour. After coating the dishes, the excess fibronectin was replaced with complete culture medium at 38°C for another hour. Subsequently, the CCM was aspirated and explants were transferred to the dishes in 5  $\mu$ l of CCM (1 graft per dish) and allowed to settle for 30 minutes prior to adding more CCM. Cultures were maintained in a gassed, humidified incubator at 38°C for 2 days or 10-12 days, the CCM being changed every two days. In the case of neural tube cultures, intact tube was scraped from the culture dish 24 hours after explantation, leaving only migratory neural crest cells.

### Detection of neural crest cells

Catecholamine synthesizing cells were detected using an antibody to the enzyme tyrosine hydroxylase (TH). Culture supernatant for anti-TH was obtained from the Developmental Studies Hybridoma Bank. Cultured cells were fixed in 4% paraformaldehyde for 1 hour, rinsed with PBS followed by PBT and incubated with anti-TH supernatant for 2 hours. Following further rinses with PBT, cultures were incubated with FITC- or TRITC-conjugated goat anti-mouse IgG antibodies (1:150 in PBT; Zymed) for a further 2 hours, prior to rinsing in PBS and examination (see below).

In addition to using an anti-TH antibody, catecholamine-containing cells also could be detected because they fluoresce under ultraviolet illumination after fixation in 4% paraformaldehyde/0.25% glutaraldehyde (Sechrist et al., 1989). The presence of neurons in some cultures was confirmed by immunostaining with an antibody to the non-phosphorylated form of the intermediate molecular mass neurofilament protein (NF-M; RMO 270.3; Lee et al., 1987), kindly provided by Dr Virginia Lee. The staining procedure is similar to that described above for anti-TH. Melanocytes, because they contain dark melanin granules, are visible with the naked eye or compound microscope.

Every culture was examined for the presence of melanocytes. Most of the cultures were subsequently stained with the anti-TH antibody, while only a few from each experiment were stained with the anti-NF-M antibody. Double antibody staining was not performed in these experiments.

### Fate mapping experiments

Fertile chicken eggs were incubated for 16-33 hours to give embryos ranging from stages 3+ to 10+ (Hamburger and Hamilton, 1951). While placed on their sides, eggs were windowed as described previously (Stern and Keynes 1987; Selleck and Stern, 1991 or Artinger and Bronner-Fraser, 1992). After windowing the shell, a few drops of ink (Pelikan Fount India diluted 1:10 in Tyrode's saline) were injected beneath the blastoderm to render the embryo more clearly visible. Using an electrolytically sharpened tungsten needle, a small hole was made in the vitelline membrane overlying the neural tube/neural plate in the region of Hensen's node.

### DiI-labeling experiments

The egg, prepared as described above, was viewed through a binocular dissecting microscope. Using a three-dimensional micromanipulator, a micropipette containing a solution of DiI (0.5% in absolute ethanol) was placed into the neural folds. By applying gentle air pressure to the microelectrode, DiI was extruded into the surrounding tissues, thereby labeling a small group of cells.

Following the injection, eggs were sealed with Scotch electrical tape and reincubated for a further 24-36 hours. Embryos then were explanted, rinsed in phosphate-buffered saline (PBS) and fixed and stored in 0.25% glutaraldehyde in 4% paraformaldehyde. Embryos were subsequently viewed in whole mount.

### Single cell injection experiments

Windowed eggs were placed onto the stage of a compound microscope fitted with extra-long working distance objectives and epifluo-

rescence optics. Single cells (one cell per embryo) lying within and around the neural folds of the neural plate were fluorescently labeled by intracellular iontophoretic injection of lysinated-rhodamine-dextran (LRD; Gimlich and Braun, 1985) in a way similar to that described previously (Wetts and Fraser, 1988; Bronner-Fraser and Fraser, 1988; 1989; Selleck and Stern, 1991; Collazo et al., 1993). Microelectrodes, with resistances around 100 M $\Omega$ , were made by pulling aluminosilicate capillary glass (1.2 mm O.D., 0.9 mm I.D.; AM Systems) in a P-87 Flaming/Brown micropipette puller (Sutter Instrument Co.), the tips of which were then backfilled with LRD (100 mg/ml in water; Molecular Probes). 1.2 M LiCl was used to fill the microelectrode and microelectrode holder (Frederick Haer & Co.). Both injection and recording were done using an intracellular recording amplifier with headstage (IR-183; Neuro Data Instruments Corp.) connected to a digital storage oscilloscope (DSO 400; Gould). In this way, both recording and injection were made through the same electrode.

Using a Huxley-style micromanipulator (Camden Instruments), the electrode was placed close to the injection site and advanced into the tissue. After 'ringing' the electrode tip into a cell (determined by the appearance of a membrane potential), current pulses of about 8 nA were used to fill the cell for a 30 second period. The injection was immediately confirmed by viewing the cell under epifluorescence optics.

After incubation for a further 36-48 hours, embryos were removed from the egg, washed in PBS and fixed in 4% paraformaldehyde overnight. The location of labeled descendants was established by viewing the embryo in whole mount and after cutting paraffin sections.

#### Analysis of fluorescently labeled cells

All fluorescently labeled cells (both immunostained cultures, DiI- and LRD- labeled cells) were examined under epifluorescence optics with either an Olympus Vanox-T microscope or a Zeiss Axiovert inverted microscope. Photographs were made with Kodak Ektachrome 400 or Tri-X pan 400 film. In the case of the lineage experiments, fluorescent and bright-field images also were collected using the VidIm software package (S. Fraser, J. Stollberg and G. Belford, unpublished). In this way, labeled cells and the background could be superimposed to generate clear, informative images. This technique also has the advantage that faint cells, that would otherwise necessitate long photographic exposure times, could be clearly detected and rendered visible. VidIm color prints were made on a Sony color video printer UP-5000.

## RESULTS

### Confrontation of neural plate and epidermis in whole embryo culture

By juxtaposing otherwise non-interacting tissues, one can explore the role of tissue interactions in neural crest formation. To examine the role of neural tube-epidermal interactions in neural crest genesis, we grafted prospective neural plates from stage 4 donor embryos adjacent to prospective epidermis in stage 4 host embryos. The border between the area pellucida and area opaca was chosen as the graft site because recent fate mapping experiments (Bortier and Vakaet, 1992) have shown that its ectoderm is non-neural, i.e. prospective epidermis. For the neural plate grafts, only the prospective ventral neural tube region of the neural plate was explanted, since these do not contribute to neural crest cells under normal circumstances. Fig. 1 illustrates the locations and stages from which the donor tissue was removed and the region of the host to which it was

transplanted. The presence of neural crest cells was assessed after 24 hours using the HNK-1 antibody. At the stages used for analysis, HNK-1 selectively recognizes migrating neural crest cells and few other cell types (Tucker et al., 1984).

### Grafts of stage 4-5 neural plate into stage 4-5 epidermis

When cells rostral to Hensen's node were grafted into the region of the prospective epidermis ( $n = 12$ ), numerous HNK-1 immunoreactive cells formed. Fig. 2A illustrates a particularly striking example, in which HNK-1 positive cells with the appearance of a 'bird's nest' were observed surrounding the periphery of the neural plate graft, with only a few stained cells in the center. In a few specimens examined after sectioning, HNK-1 immunoreactive cells were found both within the neural plate and in the overlying epidermis (Fig. 2B), suggesting that neural crest cells can arise from both of these tissues.

Because one cannot exclude the possibility that the HNK-1-labeled cells seen within the epidermis are neural plate-derived cells that have become incorporated into it, we grafted quail neural plates adjacent to chick epidermis ( $n = 3$ ). After culture, the graft was double-labeled with the HNK-1 antibody (to assay for the presence of neural crest cells) and QCPN (an antibody that labels quail cells specifically). Numerous QCPN+/HNK-1+ cells were detected in the graft but no double-labeled cells could be found in host tissue, from which we conclude that quail cells (i.e. cells of the grafted neural plate) had not become incorporated into the overlying epidermis (Fig. 2C,D).

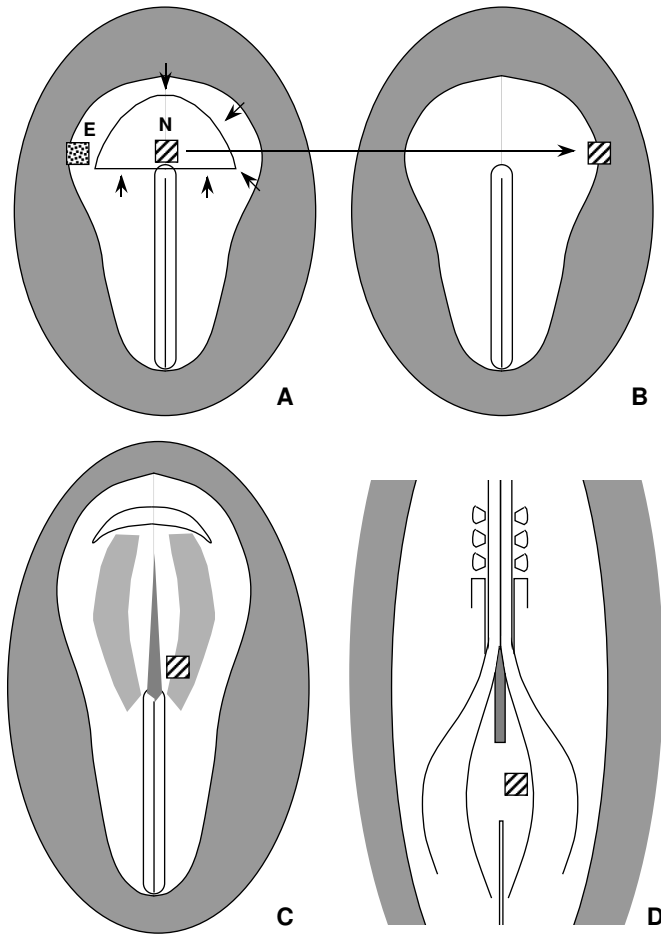
The finding the HNK-1 immunoreactive cells were present in both the epidermis and the neural plate graft suggests that neural crest cells do indeed arise from both of these tissues after their confrontation. However, we cannot rule out the possibility that the prospective neural plate 'neuralized' the overlying non-neural ectoderm of the host, which in turn generated HNK-1 positive cells at the new neural-epidermal junction. In fact, the host ectoderm overlying the graft occasionally was observed to thicken (Fig. 2E,F).

### Grafts of stage 4-5 epidermis into stage 4-5 epidermis

In control experiments, a portion of epidermis was grafted to the same region of the host. No HNK-1 immunoreactive cells were observed after juxtaposition of epidermis with epidermis ( $n = 3$ ). When the graft was labeled with DiI, the grafted cells were seen to have incorporated into the epidermis of the host (data not shown).

### Confrontation of neural plate and epidermis in tissue culture

The above experiments suggest that putative neural crest cells, assessed by HNK-1 immunoreactivity, result from the experimentally created contact of epidermis and neural plate. Although the HNK-1 antibody primarily recognizes neural crest cells at the embryonic stages examined here, it is not a specific marker. It binds an epitope on numerous cells adhesion molecules (Kruse et al., 1984) and, by later stages of neural crest migration, recognizes a number of different cell types in addition to neural crest cells. These include the notochord and some neural tube cells. Therefore, it is necessary to utilize other markers for neural crest cells to conclusively demonstrate that neural crest cells form after juxtaposition of neural plate and epidermis.



**Fig. 1.** Orientation diagram illustrating the sites from which tissues were isolated for use in the confrontation experiments and the sites at which tissues were grafted in whole embryo culture experiments. (A) At stage 4, presumptive neural plate (N) was isolated from ectoderm lying rostral to Hensen's node. Prospective epidermis (E) was taken from ectoderm lying at the area opaca/area pellucida boundary. The arrows indicate the region of ectoderm from which the neural tube arises (see Bortier and Vakaet, 1992). (B) In grafting experiments, prospective neural plates or epidermis were grafted into stage 4 host embryos, at the area pellucida/area opaca border (site indicated by the hatched square). Hatched squares in C and D indicate the sites from which neural plates were isolated in stage 6 and stage 8-10 embryos respectively.

To this end, neural plates were removed from stage 4 to 10 embryos and either grown alone or juxtaposed with epidermis from stage 4 embryos in tissue culture. Only prospective ventral neural tube regions of the neural plate were explanted, since these do not contribute to neural crest cells under normal circumstances. Some explants were grown for 2 days and stained with the HNK-1 antibody. The majority were maintained for 10 to 12 days. By these times, a number of specific derivatives differentiate in neural crest cultures grown under similar conditions (Cohen and Konigsberg, 1975; Fig. 7D) including melanocytes, which possess dark melanin granules, catecholamine-containing sympathoadrenal cells, recognized by specific catecholamine histofluorescence (Sechrist et al., 1989) and by immunocytochemical staining with tyrosine hydroxylase antibodies (anti-TH; Fauquet and Ziller, 1989;

**Table 1. Summary of tissue culture experiments**

	nc	epi	n4-6	n4-6+epi	n8-10	n8-10+epi
HNK-1	+	-	+	+	+	+
Melanocytes	+	-	-	+	+	+
Tyrosine hydroxylase	+	-	-	+	-	+
Histofluorescence	+	-	-	+	-	+
Neurofilament	+	-	-	+	+/-	+

nc, neural crest cells derived from cultures of neural tubes.

epi, epidermis isolated from stage 4 embryos.

n4-6, neural plates isolated from embryos at stages 4-6.

n8-10, neural plates isolated from embryos at stages 8-10.

Sextier-Sainte-Claire Deville et al., 1992) and other types of neurons, recognizable by neurofilament immunoreactivity. The results are summarized in Table 1 and Fig. 3.

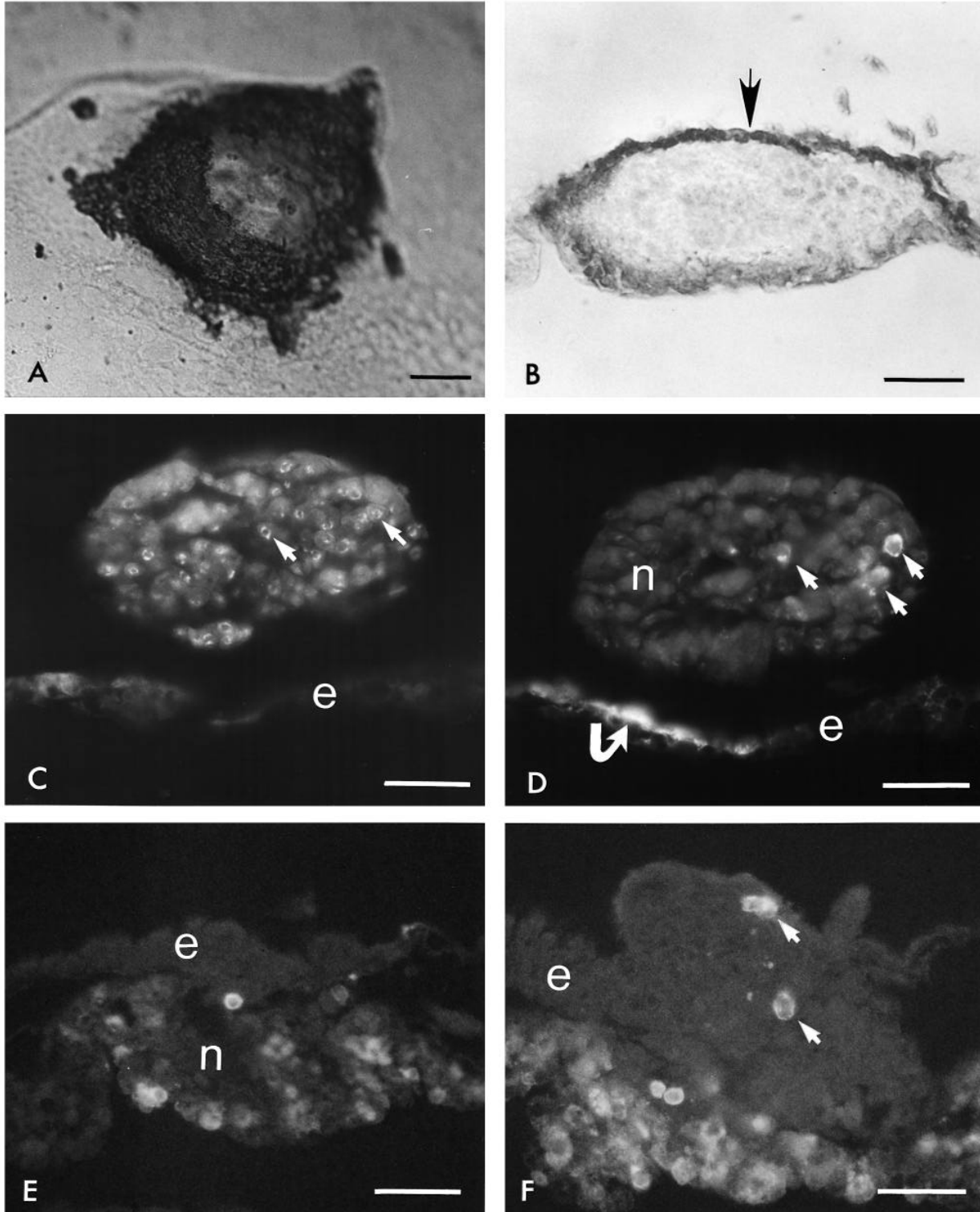
### Neural plate (stages 4-5)

Portions of the prospective neural plate were isolated from ectoderm lying immediately rostral to Hensen's node of stage 4-5 embryos and placed into culture on fibronectin-coated dishes. Neural plate tissue from this stage adhered poorly to the substrate. Therefore, for most experiments, small fragments of coverslip were placed above the explants for 12 hours to hold them to the dish, by which time they had adhered and spread on the fibronectin. Of the 13 cultures that remained adhered to the substrate, 12 were from stage 4, 1 from stage 5.

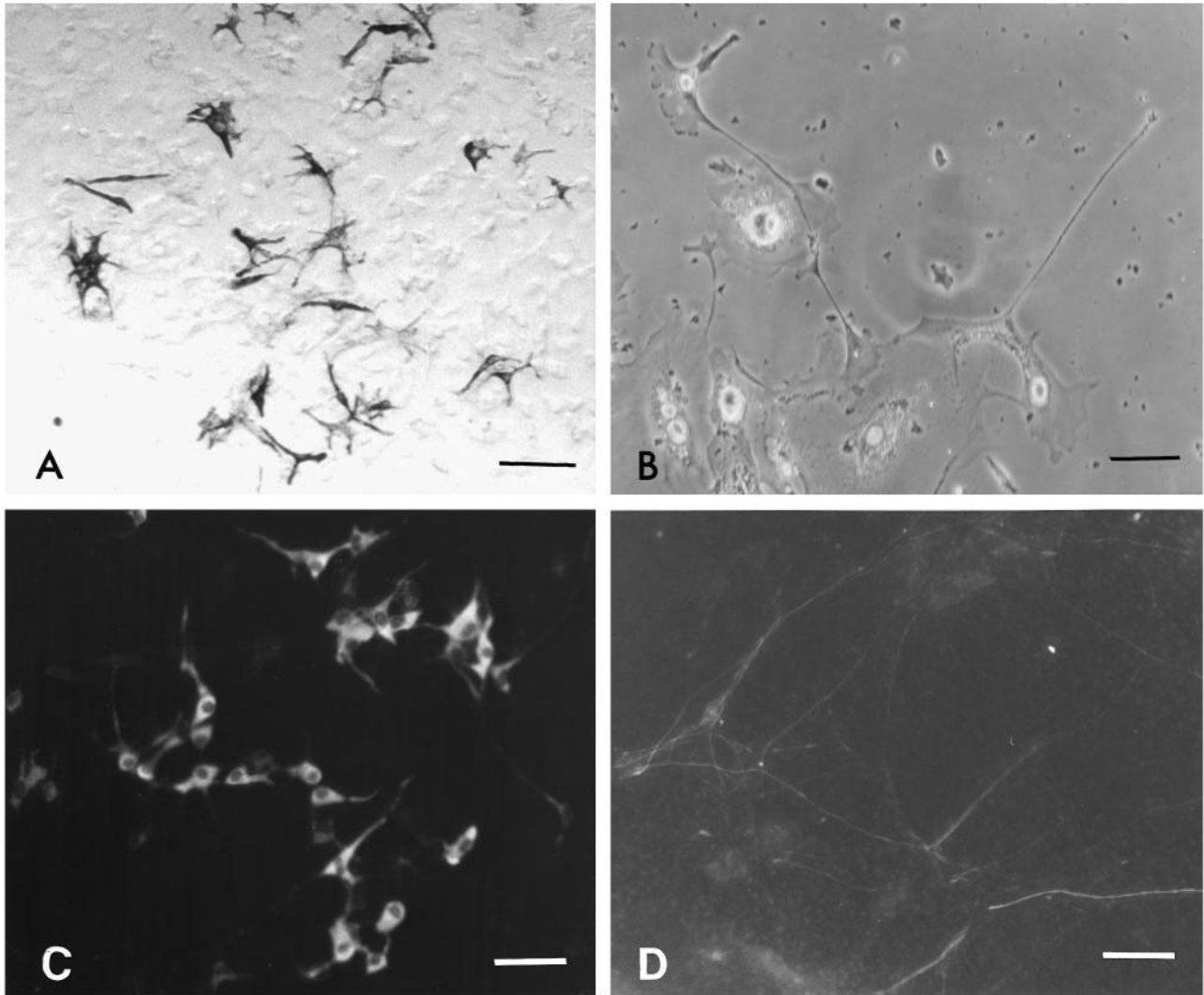
In one culture fixed after 2 days, a few HNK-1 immunoreactive cells were observed. The remaining 11 cultures were fixed after 10 to 11 days in culture. While some HNK-1 immunoreactive cells were observed, there were few neurofilament (NF) positive cells, tyrosine hydroxylase (TH) positive cells, catecholamine (CA) positive cells or melanocytes (data not shown). This suggests that early neural plates alone were unable to form the neural crest derivatives for which we assayed.

### Neural plate (stages 6-10)

Fourteen neural plate cultures from stage 6 to 10 embryos remained attached and spread on the fibronectin-coated culture dish, generally adhering better than neural plates taken from earlier stages. In 2 cultures fixed and processed for HNK-1 immunoreactivity after 2 days, numerous HNK-1 positive cells were detected. The remaining 12 cultures were fixed after 10 to 11 days. Patches of melanocytes appeared by 7 days post-explantation (Fig. 3A). However, no TH positive (TH+) or CA+ cells were noted in these explants (data not shown). In contrast to explants of ventral neural plates, similar explants that include the neural folds contain melanocytes after 4 days. Labeling with anti-neurofilament antibodies yielded a few labeled cells with short axons, which were probably neural tube rather than neural crest derived, since they had a different morphology than those observed in neural crest cultures, which appeared to have large cell bodies and long axonal processes. These results demonstrate that later neural plates can give rise to some neural crest derivatives when cultured under permissive conditions in complete media, but not to the full range of derivatives observed in neural crest cultures. Similar cultures grown in defined medium fail to produce pigment (Basler et al., 1993 and unpublished observations), indicating that the culture medium contains differentiation-promoting molecules.



**Fig. 2.** Results of grafting neural plate adjacent to prospective epidermis in New culture. (A) Whole-mount view of one grafting experiment in which the tissue has been labeled with the HNK-1 antibody. Immunoreactive cells are located principally around the periphery of the graft. (B) After sectioning another such graft, HNK-1 positive cells were found within the host epidermis (arrow) and more ventrally, within the grafted neural plate. In some experiments, quail neural plates were grafted adjacent to chick epidermis. Neural crest cells were detected using the HNK-1 antibody and QCPN, an antibody that labels quail perinuclear membranes, was used to distinguish quail from chick cells. (C) The quail-specific QCPN antibody labeled only the grafted neural plate (arrows) and demonstrated that no quail cells had become incorporated into the host epidermis (e). (D) HNK-1 immunoreactive cells were found in both the neural plate graft (n; short arrows) and within the epidermis (e; curved arrow). In some cases, the host ectoderm lying adjacent to the neural plate graft had become thickened and contained HNK-1 immunoreactive cells. (E) A section through the periphery of one of these specimens shows HNK-1+ cells in the neural plate graft (n), while no labeled cells are found in the overlying host ectoderm (e). Towards the middle of the same graft (F), the ectoderm has thickened dramatically and contains a few HNK-1-labeled cells. Scale bars, 100  $\mu$ m.



**Fig. 3.** The appearance of neural crest derivatives in tissue culture. (A) After about 7 days in culture older neural plates from stage 8-10+ embryos, start to differentiate darkly pigmented melanocytes. While these are initially dispersed, they aggregate into clumps over time. After culture of early neural plates, neurons differentiate (B), characterized by long filopodia with terminal growth cones. Only after co-culture with epidermis are tyrosine hydroxylase-immunoreactive cells seen (C), indicating the presence of catecholaminergic neural crest derivatives. These cells tend to be restricted to one region of the culture, but within this area are interspersed with many other neural crest derivatives, such as melanocytes. (D) After culture of neural crest cells derived from closed neural tubes, the processes of many neurons stain with anti-neurofilament antibody. Scale bars, 20  $\mu$ m.

### Epidermis

Six epidermal explants remained attached to the fibronectin-coated substrate. These cultures were HNK-1<sup>-</sup>, TH<sup>-</sup>, NF<sup>-</sup> and had no melanocytes at either 2 and 10 to 11 days postexplantation.

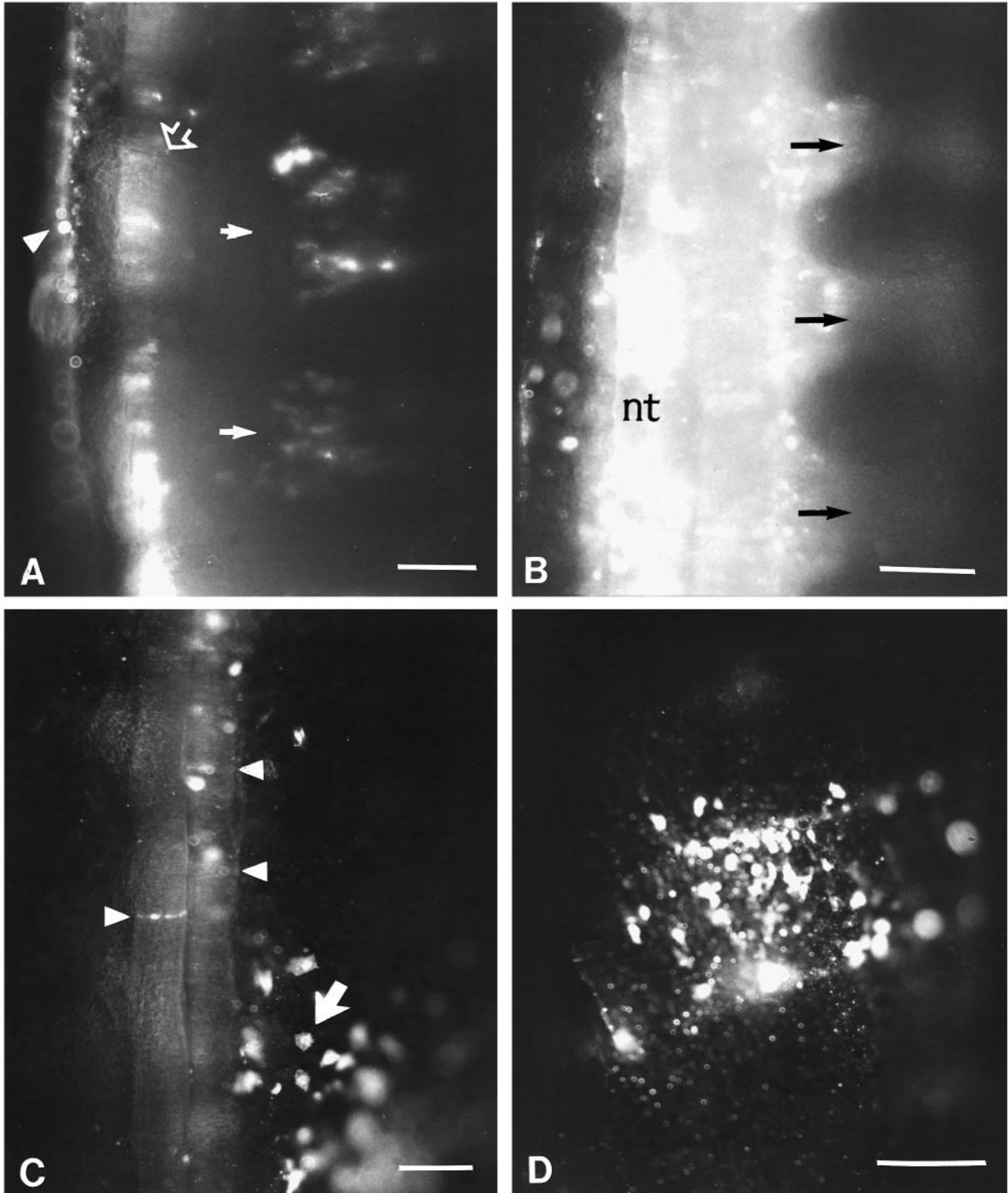
### Neural plate plus epidermis

In 15 cultures when stage 4-5 prospective neural plate was co-cultured with stage 4 presumptive epidermis, both tissues remained attached to the substrate. When the epidermis was explanted and cultured for 24 hours prior to addition of the neural plates, the latter adhered significantly better than early neural plate alone. Melanocytes developed in 9/15 of the recombinant explants (60%), generally appearing after 6 to 7 days in culture. In addition, these explants contained HNK-1<sup>+</sup>,

TH<sup>+</sup> and CA<sup>+</sup> cells. When stage 6-10 neural plate was co-cultured with epidermis, melanocytes appeared in 5/6 cases (83%). Furthermore, all cultures examined contained TH<sup>+</sup>, CA<sup>+</sup>, HNK-1<sup>+</sup> and NF<sup>+</sup> cells. This demonstrates that co-culturing either early or later neural plate plus epidermis can result in the formation of the full range of assayed neural crest derivatives.

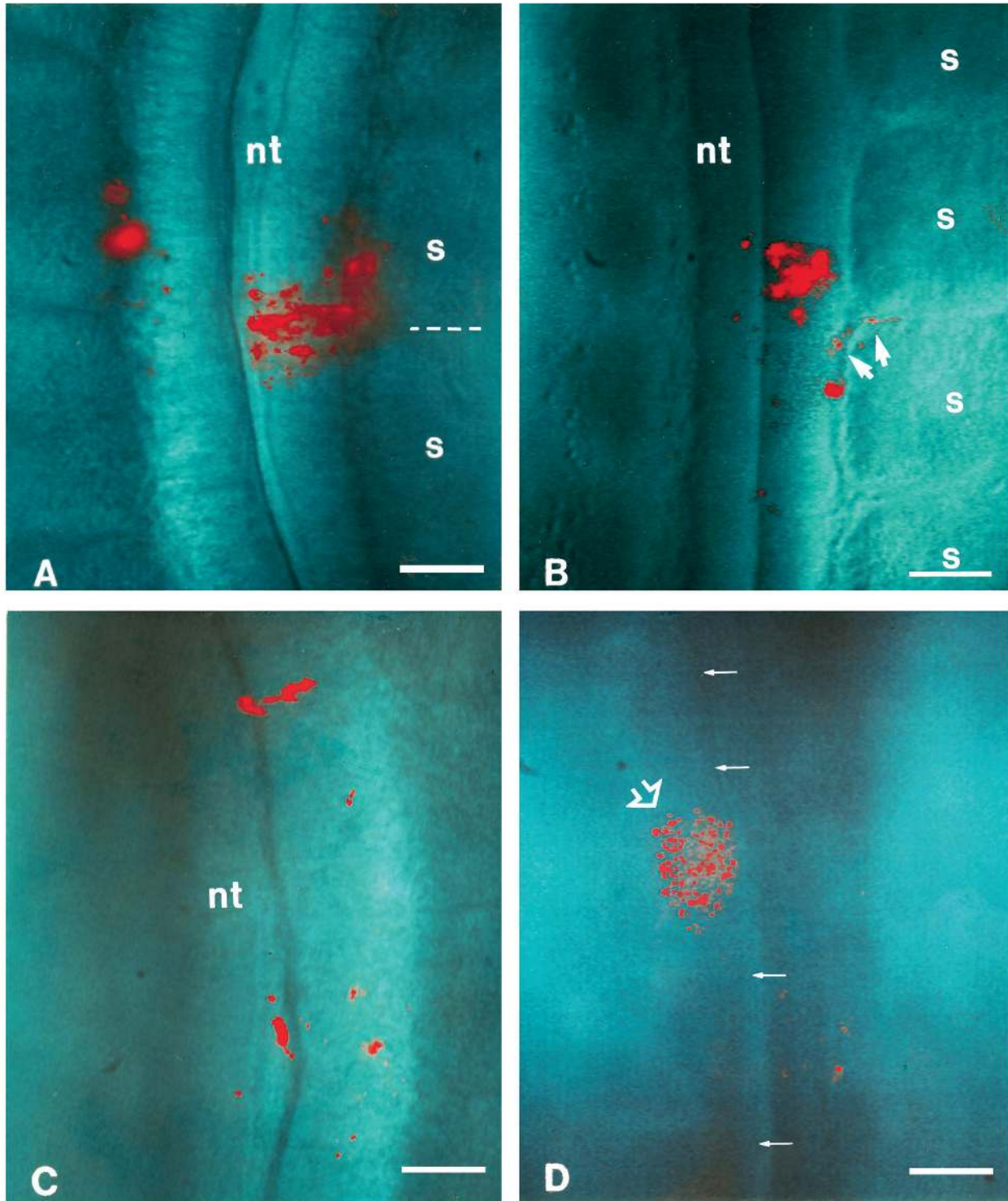
### Neural plates plus paraxial mesoderm

In 8 experiments, stage 4 presumptive neural plates were co-cultured with segmental plate or somite taken from stage 10 embryos. After 10 days in culture, melanocytes were detected in 5/8 cultures. After staining with anti-TH antibody, no TH<sup>+</sup> cells were detected. No differences were seen between the somite-neural plate and segmental plate-neural plate cultures in terms of melanocyte formation.



**Fig. 4.** Photomicrographs of embryos viewed as whole mounts under epifluorescence optics after focal injections of Dil into the neural folds of stage 8-10+ embryos. The distribution of the progeny can be determined from characteristic patterns of cell labeling. (A) In this embryo, an injection was made into the neural fold lying lateral to Hensen's node in a stage 10 embryo. All three ectodermal derivatives contain labeled progeny. Neural crest cells (solid arrows) migrate from the neural tube in a segmental pattern, while some progeny remain confined to the epidermis (solid triangle) and to the dorsal neural tube (open arrow). (B) After a more extensive injection of Dil, a reiterated stream of neural crest cells can again be seen (arrows), and the neural tube (nt) is completely labeled along an extensive length of the neuraxis. (C) Following an injection of Dil into neural folds rostral to Hensen's node in a 10 somite-pair embryo, progeny can be found restricted to the neural tube (solid triangles) and to a patch of the epidermis (arrow). No labeled cells can be found migrating through the paraxial mesoderm. In some cases where only epidermis appears labeled (D), most of the progeny are restricted to a small, well-circumscribed zone. Scale bars, 100  $\mu$ m.





**Fig. 5.** Following injection of LRD into a single cell within the neural folds of stage 8-10+ embryos and subsequent incubation, the labeled descendants of that cell can be located in whole mount using epifluorescence optics. Cells were designated as neural tube, epidermal or neural crest cells based on their positions, respectively, within the neural tube, surface ectoderm or along neural crest migratory pathways. In these specimens, images have been processed such that the fluorescence (red) has been superimposed on the bright-field view (blue). (A) After injection of a single cell rostral to Hensen's node and caudal to the region where the neural folds are approximating and fusing, the progeny are located within all three ectoderm derivatives. While most of the progeny are confined to the side at which the injection was made, some neural crest cells are found contralaterally. (B) A cell located a little more rostral also contributes to epidermis, neural tube and neural crest. In this case, a stream of neural crest cells (arrows) is seen migrating between the somites. (C) Injection of a cell a little more caudal to that in A results in more dispersed progeny, lying across about 3 'somite lengths'. Labeled cells lie within the neural tube and in the epidermis, although no progeny were found to have contributed to the neural crest. (D) The progeny of some single cells in the neural folds contribute only to epidermis. In this example, the majority of labeled descendants are restricted to one patch of epidermis (open arrow) located to one side of the neural tube (lumen indicated by arrows). More caudally, some progeny lie within the epidermis on the contralateral side. nt, neural tube, s, somite. Scale bars, 50  $\mu$ m.

### Fate mapping and cell lineage of the neural folds

To investigate the early lineage relationships between the prospective neural tube, neural crest and epidermis, we performed a series of dye-labeling and single cell lineage experiments on neurulating embryos. As a preliminary to single cell lineage analysis, we conducted a series of experiments in which focal injections of the carbocyanine dye, DiI, were made into the neural folds (Fig. 4) to label small groups of cells in embryos ranging from stages 3+ to 10+.

Individual cell lineage relationships were examined by microinjecting lysinated-rhodamine-dextran (LRD) into single neural fold cells of embryos at stages 8 to 10+ (Figs 5, 6). At these later stages, different degrees of neural tube closure exist simultaneously in a given embryo, such that the neural plate may be open caudally and fusing more rostrally. To take into account various stages of neural fold development, injections were performed at different rostrocaudal locations along the neural axis. Embryos were examined 24-48 hours after injection, by which time neural tube closure was complete, neural crest cells had left the neural tube and were migrating within the somites.

Classification of labeled cells as epidermal, neural tube or neural crest was based on their position and characteristic morphology, as analyzed both in whole mounts and, in the case of LRD injections, in transverse sections. Labeled cells within the neural tube were columnar epithelial cells, which typically extended from the apical to basal side of the neural tube. Labeled neural crest cells could be identified by their location external to the neural tube and their characteristic locations within the embryo; in addition, their segmental migratory pattern through the rostral but not caudal portion of the somites was diagnostic of this cell type. Labeled epidermal cells were small, cuboidal in shape and restricted to a small patch of the epidermis. Fig. 7 summarizes the cumulative data for both DiI (left) and LRD (right) injections into the neural folds of embryos ranging from stage 8 to 10+.

### DiI-labeling of small groups of neural fold cells

The first set of experiments was used to determine the earliest stage at which prospective trunk (but not cranial) neural crest cells are present in the open neural plate region, adjacent to the regressing Hensen's node. Therefore, focal injections of DiI were made into the neural folds, or edge of the prospective neural plate, lateral to Hensen's node, of embryos ranging from stage 3+ to 10+.

For DiI injections into cells lying at the prospective neural plate-epidermis border of stage 3+ embryos, the labeled cells were restricted to the midbrain/rostral hindbrain region ( $n = 2$ ). At stage 4, injections ( $n = 11$ ) performed at the same location with respect to Hensen's node and prospective neural plate gave rise to labeled progeny at the level of the hindbrain, with some of the fluorescent cells located within the developing heart. At later stages, the location of labeled progeny shifted caudally, so that neural crest cells labeled by injections into stage 5 embryos ( $n = 2$ ) populated caudal branchial arches and structures caudal to the anterior intestinal portal: by stage 6, injections ( $n = 2$ ) labeled neural crest cells at the level of the forelimb. Between stages 7 and 10+, cells arising from the edge of the neural plate, lateral to Hensen's node became incorporated into structures at the level of the forelimb or trunk ( $n = 6$ ): at stage 8, labeled progeny lay adjacent to the forelimb,

whereas at stage 10, injected cells were found between the forelimb and the hindlimb. Interestingly, in these later embryos, fluorescent cells were distributed widely along the rostrocaudal axis, in some cases extending along 12 somite-lengths.

Based on the information from these initial experiments, we concentrated on stage 8-10+ embryos because in such cases, labeled progeny were confined to forelimb and trunk regions of the neuraxis. Of the injections performed, 35 produced clear and interpretable results (summarized in Fig. 7). In 10 cases, labeled cells were observed in all three ectodermal derivatives, the neural tube, the neural crest and the epidermis (Fig. 4A,B). However, in other embryos, only one or two tissues contained labeled progeny cells. For example, 8 embryos contained both labeled neural tube and neural crest cells. Another 4 embryos had labeled cells in both the neural tube and epidermis (Fig. 4C) but not neural crest; 3 embryos contained label in both neural crest and epidermal cells, but not neural tube. Others contained labeled cells in a single derivative: either the neural tube ( $n = 3$ ), neural crest ( $n = 2$ ) or epidermis ( $n = 4$ ; Fig. 4D). More importantly, the results indicate that all of the neural crest precursors are located within the visible neural folds at these stages.

### LRD-labeling of individual neural fold cells

Because DiI labels a small group of cells, it cannot be used to define cell lineage relationships. Furthermore, the possibility of labeling some deep lying mesodermal cells in addition to neural folds cells cannot be ruled out. To circumvent these problems, we microinjected LRD into individual neural fold cells in chick embryos ranging from stage 8 to 10+. We visually confirmed that only single cells were labeled using the epifluorescence microscope. Of embryos receiving single cell injections, 30 contained clearly recognizable clones. In 6 cases, labeled progeny were found in all three ectodermally derived tissues (Fig. 5A,B). Although the neural tube and epidermal cells remained on the same side of the embryo that was initially injected, labeled neural crest cells often were distributed bilaterally, crossing over to the contralateral side (Fig. 5A). In 1 embryo, a clone comprised labeled cells in both the neural tube and epidermis (Fig. 5C). In another 9 embryos, the progeny of a single cell were found in both the neural tube and the neural crest (Fig. 6A,B), but not the epidermis. No clones were observed with labeled cells in the neural crest and epidermis only. Several clones contributed to single lineages such as neural tube only ( $n = 6$ ), neural crest only ( $n = 2$ ) and epidermis only ( $n = 6$ ). Figs 5D and 6D illustrate clones viewed in whole mount and transverse section, respectively, which contain labeled cells confined to the epidermis. These are easily distinguishable from clones that have labeled progeny only in the neural tube (Fig. 6C) or neural crest.

The point at which the neural folds approximate and fuse varies with developmental stage. At stages 9- to 9, neural tube closure occurs adjacent to the third-from-last formed somite, while at stages 9+ to 10- the folds are fusing lateral to the last formed somite. By stage 10, the neural tube is closed behind the last somite. Interestingly, clones populating both neural tube and neural crest were observed both rostral and caudal to the point of neural tube closure. In contrast, all clones that contributed to the neural tube, neural crest and epidermis arose from neural fold precursor cells lying caudal to the point at which the folds meet. No such pluripotent ectoderm cells were

found in regions where the neural tube had closed, consistent with the idea that the epidermal lineage segregates from the neural tube-neural crest lineage around the time of neural tube closure. Fig. 7 summarizes the results of injections made into embryos at all stages studied.

We observed a greater incidence of cells contributing to a single ectodermal derivative in open neural plate, compared with regions where the neural tube is closing. This is likely to be due to the difference in geometry of the neural folds at the time of injection: as the neural plate folds into a tube, the folds become more vertically oriented and so injections made at this stage are more likely to label dorsal neural tube cells and less likely to label ventral neural tube and epidermis, both of which contribute to a single ectodermal structure. One potential limitation with LRD labeling is that rapid mitosis can dilute the dye beyond levels of detection. Accordingly, some of the LRD-labeled cells were quite faint by the time of fixation. Therefore, we cannot rule out the possibility that some unlabeled cells are clonally related to the labeled cells observed in our study, making the numbers of labeled derivatives arising from a given clone an underestimate.

## DISCUSSION

Here, we investigate the origins of neural crest cells in the avian embryo. The mechanisms underlying the formation of neural crest cells were examined by juxtaposing ectodermal tissues in both whole embryo culture and tissue culture; DiI fate mapping and single cell injection were used to elucidate the time at which ectodermal lineages segregate. Our results indicate that: (i) epidermal-neural plate interactions can generate a range of neural crest derivatives, (ii) the ability of the neural plate to generate some neural crest derivatives changes between stages 4 and 6, (iii) the interactions involved in induction of ectodermal cells to assume a neural-neural crest fate may still be in progress at levels of the open neural plate, and (iv) the epidermis and neural tube/neural crest lineages segregate around the time of tube closure.

Our tissue culture experiments demonstrate that older neural plates (from stages 6-10) can give rise to some crest derivatives (most notably pigment cells) when cultured alone. In contrast, young neural plates alone (taken from definitive streak-stage embryos) cannot form the neural crest derivatives for which we assayed. There are two possibilities to account for the noted differences between older and younger neural plate. First, it is possible that precommitted neural crest cells were included inadvertently in the older explants. This is unlikely given that we purposely dissected ventral neural plate. In addition, the timing of melanocyte appearance was delayed by 3 days over that observed when neural folds were purposely included in explants of older neural plates. The second possibility is that the characteristics of late neural plate are markedly different from early neural plate such that the older tissue has acquired the ability to form melanocytes under permissive culture conditions. This scenario suggests a change in the developmental potential of the neuroepithelium. Because both young and old neural plates were cultured under identical experimental conditions, this difference is likely to reflect an *in vivo* difference between the neuroepithelium of stage 4 and 6 embryos.

What mechanisms might account for the differences

between young and old neural plates? Because simple 'maturation' of young neural plates in culture does not result in the generation melanocytes, it is unlikely that mechanisms intrinsic to the neuroepithelium can account for the change in developmental potential. Rather, interactions with other tissues must play a role in making the neural plate able to form neural crest derivatives. Our results indicate that contact with epidermis or paraxial mesoderm enable stage 4 prospective neural plate to generate at least some neural crest derivatives. The ability of late neural plate to generate pigment cells may depend on an earlier interaction between the neural plate and the underlying mesodermal precursors.

The results of our experiments suggest that reciprocal interactions between the neural plate and epidermis play a role in neural crest formation. Experiments conducted in whole embryo culture indicate that neural crest cells are generated at neural plate-epidermis boundaries. While our data show that putative neural crest cells arise from both the presumptive epidermis and neural plate, we cannot rule out the possibility that those found within the epidermis have arisen as a consequence of neuralization of the host non-neural ectoderm by the grafted tissue and were not generated directly. Thus, there may be two or three inductions involved in this interaction. First, as a result of contact with the epidermis, neural plate tissue may give rise to neural crest cells (Fig. 8A). A second possible interaction is that the neural tissue may induce adjacent epidermis to become neuroepithelium, from which neural crest cells are subsequently generated (Fig. 8B). A third possibility is that the epidermis may cause some cells within the neural plate explants to themselves become sources of inductive signals for the neural crest.

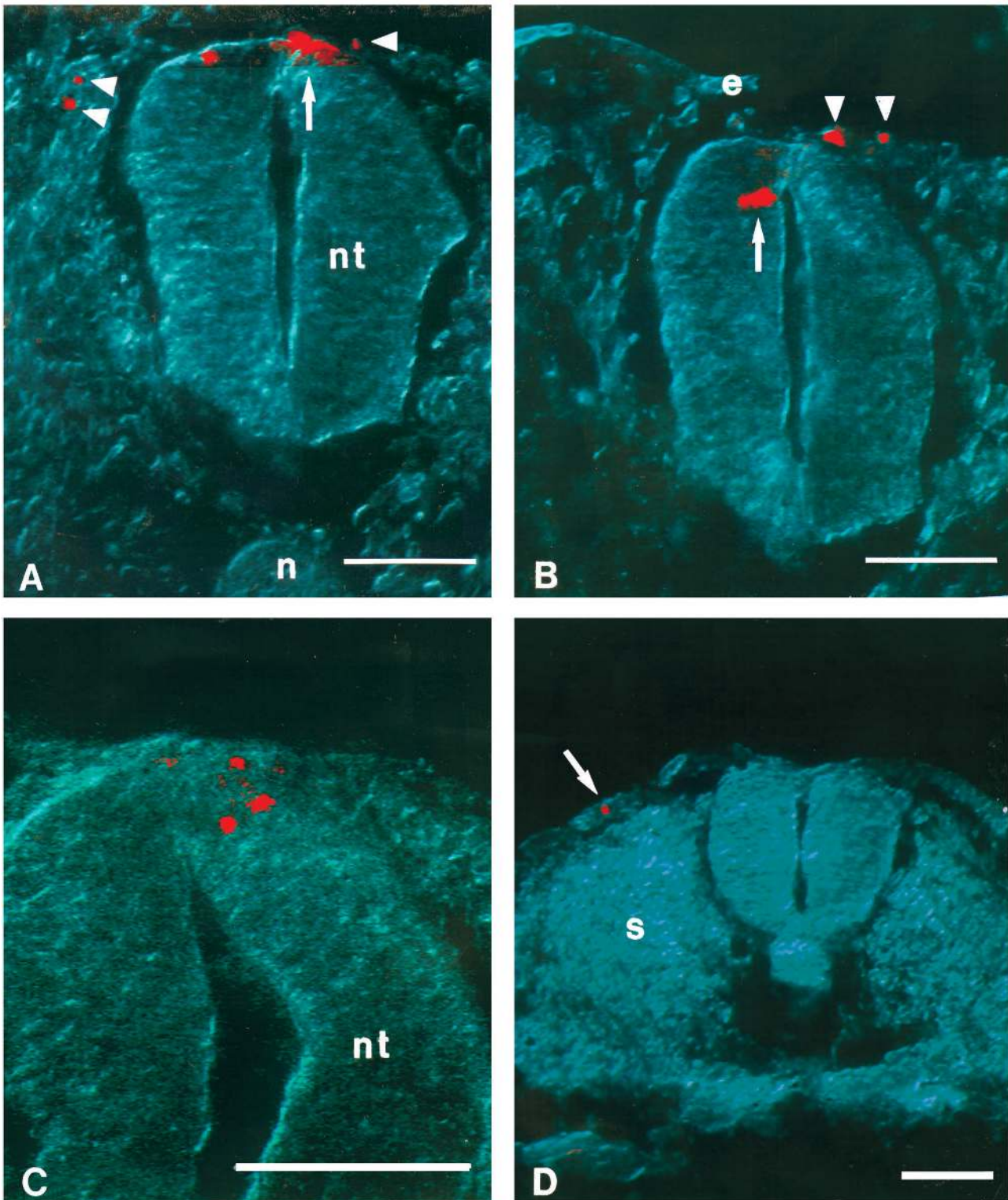
The findings from our tissue culture experiments further support the hypothesis that epidermis plays a role in neural crest formation. Young neural plates in culture generate neural crest cells when co-cultured with epidermis, and late neural plates can generate catecholaminergic cells when cultured with epidermis. What function does the epidermis have in neural crest genesis? One possibility is that the epidermis plays a direct role in the generation of neural crest cells from competent neural tube cells. A second, but not mutually exclusive, possibility is that, as a result of interactions with adjacent epidermis, a subset of neural plate cells acquire the ability to form neural crest derivatives. Once in the dorsal neural tube, other mechanisms direct some of these cells towards a neural crest fate.

The classical view of neurulation, inferred from previous fate mapping experiments, is that the neural plate becomes defined between stages 4-6, after which time its lateral margins represent a strict boundary between neural and epidermal tissues. Importantly, the present cell lineage analysis suggests that this is not the case: single cells within the neural folds of stage 8 to stage 10+ embryos can contribute progeny to all three ectodermal derivatives. These results can be explained by assuming that at this stage, (i) lateral ectodermal cells are multipotent, (ii) some of these cells continue to be neuralized by more medial neural plate, and/or (iii) some of these cells continue to be epidermalized by more lateral ectoderm. Therefore, single cells lying immediately lateral to pre-existing neural plate can contribute to all three ectodermal derivatives if some of their progeny become neuralized; others escape the influence of the neural tissue or become epidermalized to contribute to epidermis (Fig. 8C). These observations suggest that

the inductive effect of neural plate on the epidermis, and vice versa, has not ended by stage 10.

The results further indicate that the neural tube-neural crest lineage and the epidermal lineage diverge after the neural folds

approximate and the neural tube separates from the epidermis. Previous results from this laboratory have shown that single cells within the dorsal neural tube populate both the neural tube and the neural crest, (Bronner-Fraser and Fraser, 1988, 1989), demon-

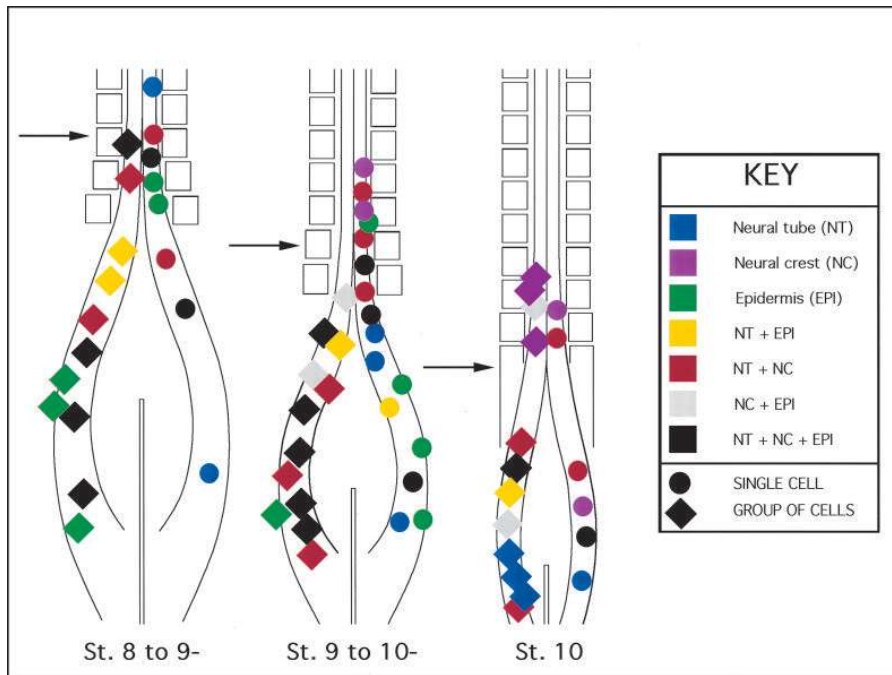


**Fig. 6.** Processed images of sectioned embryos. (A,B) LRD-labeled progeny are located within the dorsal neural tube (arrow) and have contributed to the neural crest migrating from the dorsal neural tube (solid triangles). (C) In some cases, the labeled progeny are restricted to the dorsal neural tube (the epidermis has separated from the underlying neural tube in this specimen and is not visible in this field). (D) Example of a labeled cell lying within the epidermis. nt, neural tube; s, somite; epi, epidermis; n, notochord. Scale bars, 50  $\mu$ m.

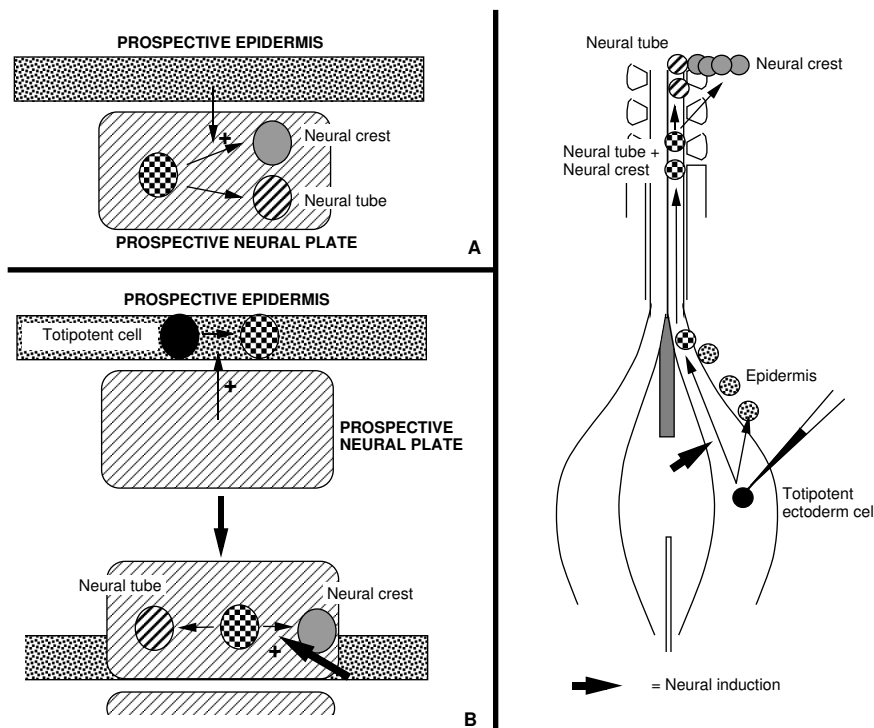
strating that at the time of injection, neural crest cells are not committed to their fate. Results from the present experiments confirm this finding. Furthermore, we failed to detect a shared epidermal/neural crest lineage, although we cannot exclude the possibility that too few injections were performed to detect such neural crest/epidermal precursors. These results may indicate that the inductive effect of epidermis on the neural plate occurs after neural tube closure, although there is no reason to exclude the

possibility that such an interaction occurs earlier and involves planar signaling between the epidermis and neural plate.

What are the molecular bases of the interactions between neural plate and epidermis? Recently, a number of groups have isolated genes whose transcripts appear within regions of the embryo that contain prospective neural crest cells such as the dorsal neural tube. These include *Pax-3* (Goulding et al., 1991), *dorsalin-1* (Basler et al., 1993), *Slug* (Nieto et al.,



**Fig. 7.** Diagram summarizing the results from both the DiI- and LRD-injection experiments. The three line diagrams represent the closed neural tube and open neural plate at the caudal end of embryos at stages 8 to 9-, 9 to 10- and 10. In each, the transition from open neural plate to closed neural tube is indicated by an arrow. Each colored block represents the tissue contributions made by labeled cells after a single injection into one embryo at that position. Diamond-shaped blocks on the left side of each diagram indicate the results of labeling small groups of cells with DiI, and circular blocks on the right side show the results from the injection of a single cell with LRD. The coloring indicates the tissues in which labeled descendants were found.



**Fig. 8.** (A,B) A model providing a possible explanation for the interactions between neural plate and epidermis, inferred from the grafting experiments. (A) As a result of interactions with non-neural ectoderm (prospective epidermis), some cells within the neuroepithelium become neural crest cells. (B) Neural plate may induce pluripotent ectoderm cells to become neural, thereby restricting their potential, and concomitantly causing that ectoderm to thicken. Planar interactions between the uninduced prospective epidermis and induced neural plate, may result in the generation of neural crest cells from the latter. (C) Our single cell lineage experiments indicate that the effect of the neural plate on pluripotent ectoderm cells has not ceased in open neural plate regions of stage 8-10+ embryos. A single pluripotent ectoderm cell within the neural folds (black) undergoes several mitotic divisions. Some of the progeny may become 'neuralized' by the neural plate and give rise to neural tube/neural crest precursors, while other cells either may

escape the influence of the adjacent neural plate or may become 'epidermalized', contributing to epidermis. Subsequently, neural tube/neural crest precursors within the dorsal aspect of the closed neural tube divide to give progeny contributing to both tissue types.

1994), *Wnt-1* and *Wnt-3a* (Dickinson and McMahon, 1992; Parr et al., 1993; Wolda et al., 1993). Experiments are in progress to relate the inductive events described above to the expression of such genes.

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