Migration and Differentiation of Neural Crest and Ventral Neural Tube Cells *in vitro*: Implications for *in vitro* and *in vivo* Studies of the Neural Crest

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During vertebrate development, neural crest cells migrate from the dorsal neural tube and give rise to pigment cells and most peripheral ganglia. To study these complex processes it is helpful to make use of *in vitro* techniques, but the transient and morphologically ill-defined nature of neural crest cells makes it difficult to isolate a pure population of undifferentiated cells. We have used several established techniques to obtain neural crest-containing cultures from quail embryos and have compared their subsequent differentiation. We confirm earlier reports of neural crest cell differentiation *in vitro* into pigment cells and catecholamine-containing neurons. However, our results strongly suggest that the 5-HT-containing cells that develop in outgrowths from thoracic neural tube explants are not neural crest cells. Instead, these cells arise from ventral neural tube precursors that normally give rise to a population of serotonergic neurons in the spinal cord and, *in vitro*, migrate from the neural tube. Therefore, results based on previously accepted operational definitions of neural crest cells may not be valid and should be reexamined. Furthermore, the demonstration that cells from the ventral (non-neural crest) part of the neural tube migrate *in vitro* suggests that the same phenomenon may occur *in vivo*. We propose that the embryonic "neural trough," as well as the neural crest, may contribute to the PNS of vertebrates.

In vertebrates, the neural crest is the embryonic source of a diverse array of adult cell types, including pigment cells in the skin and neurons of the peripheral ganglia. In most vertebrate embryos, neural crest cells briefly occupy the dorsal midline of the neural tube (the precursor of the spinal cord), and migrate extensively before they differentiate. The study of vertebrate neural crest cell migration and differentiation has been greatly facilitated by analysis of this behavior in culture. Such studies have reported that avian neural crest cells differentiate *in vitro* into a variety of cell types, including pigment cells (Cohen and Konigsberg, 1975) and many neuronal derivatives. In avian

trunk neural crest cell cultures, a number of classical neurotransmitters have been reported, including catecholamines (Cohen, 1977; Loring et al., 1982; Maxwell et al., 1982), ACh (Kahn et al., 1980; Maxwell et al., 1982), GABA (Maxwell et al., 1982), and 5-HT (Sieber-Blum et al., 1983). In addition, neuroactive peptides, such as somatostatin (Maxwell et al., 1984) have been reported in neural crest cell culture.

In spite of the advantages of an *in vitro* approach to analysis of neural crest differentiation, it is clear that a fundamental problem arises when attempts are made to isolate these cells in culture. Neural crest cells are operationally defined *in vivo* as those cells that emigrate from the dorsal neural tube during embryonic development. Therefore, those cells that migrate from the dorsal neural tube are, by definition, neural crest cells, and those that remain are non-neural crest cells. A similar operational definition of neural crest cells is commonly employed *in vitro*. Neural tubes containing the neural crest are removed from embryos and placed on a planar substratum. The population of cells that emerges from the explants and moves out onto the culture dish within a day or two—the neural tube outgrowth—is assumed to be the neural crest (e.g., Cohen and Konigsberg, 1975). There is no proof that this cell population consists entirely of neural crest cells. In fact on the basis of cellular differentiation and morphology, it has been suggested that non-neural crest cells occur in outgrowths from neural tubes (Loring et al., 1981).

We sought to characterize the cells that emigrate from quail embryo neural tubes *in vitro*, and ask whether this population is the neural crest and can therefore be legitimately used for studies of neural crest differentiation, or whether other, non-neural crest, cells are present. To accomplish this, we compared the differentiation of neural tube outgrowth cells with neural crest clusters, a population of cells thought to consist exclusively of neural crest cells (Glimelius and Weston, 1981; Loring et al., 1981). We analyzed and compared the 2 types of cultures by 2 methods: (1) We used high-performance liquid chromatography (HPLC) to quantify the endogenous content of catecholamines and 5-HT, and (2) we used immunohistochemistry to visualize 5-HT-containing cells.

Our results suggest that 5-HT-containing cells in neural tube outgrowths are derived from the ventral part of the neural tube, not the dorsal part, and are therefore not neural crest cells. This means that non-neural crest cells, as well as neural crest cells, migrate onto culture substrate from explanted neural tubes and that therefore migration from neural tubes *in vitro* is not a valid operational definition for neural crest cells. Our results also

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support the idea that non-neural crest cells may migrate *in vivo* and thus contribute to the thoracic PNS.

Materials and Methods

Cell culture for HPLC. Two types of cultures containing neural crest cells were analyzed for amines by HPLC. Neural tube outgrowths were produced by a method described previously (Loring et al., 1981). Briefly, trunks (including the last 8 somites and segmental plate) of 22–25-somite Japanese quail (*Coturnix coturnix japonica*) embryos were dissected and incubated in Pancreatin (Gibco; diluted 1:4 in Locke's saline) to free neural tubes from associated tissues. Ten to 12 isolated neural tubes were placed in a 35 mm tissue culture dish (Lux) in 3 ml of culture medium [90% Ham's F12 (Flow), 7% fetal bovine serum, and 3% 10 d chick embryo extract] and incubated in a humidified 10% CO₂ atmosphere at 37°C. For some experiments the dishes were coated with collagen [Vitrogen (Collagen Corp.), 100 μ l spread on each dish, polymerized with NH₃ vapors, and dried under a UV light]. Neural tube explants were removed with a glass needle after about 36 hr, leaving rings of mesenchymal cells ("neural tube outgrowths") that were maintained in culture for 3 or 5 more d (totaling 5 or 7 d in culture). The culture medium was replaced with fresh medium on the second day, and for the longer-term cultures, 1 ml of medium was exchanged for fresh medium 3 d later.

Neural crest clusters were isolated from the explanted neural tubes at about 36 hr, as described previously (Loring et al., 1981). Twenty to 30 clusters were cultured in each 35 mm culture dish, either on tissue culture plastic or collagen. The first day of subculture is termed day 2 in the Results, indicating the time at which the neural tubes were explanted. Clusters were cultured for a total of 5 or 7 d (about 3 and 5 d in subculture, respectively).

Tissues used as controls included freshly isolated neural tubes, notochords, and somites, cultured somites (2 and 4 d in culture), and 10 d quail thoracic sympathetic ganglia (5 d in culture). Serum and embryo extract were also examined by HPLC. Of these controls, only the sympathetic ganglia contained detectable amounts of the amines analyzed (see Results).

Preparation of samples and HPLC. Cultures were washed twice in Hanks' balanced salt solution (HBSS) buffered with 10 mM HEPES, then were incubated in 0.5 ml 1 mM EGTA in calcium- and magnesium-free HBSS until the cells began to detach from the dishes. Then 0.5 ml of HBSS was added, and the solution was triturated to dissociate clumps of cells. A sample (50 μ l) was removed to count cells in a hemocytometer. The percentage of pigmented cells was determined by counting cells in each sample with both phase and bright-field optics. The suspension was centrifuged to pellet the cells, the supernatant was removed and immediately replaced with 50 or 100 μ l ice-cold extraction buffer (0.1 N citric acid, 0.1 N Na₂HPO₄, and 0.1 mM EDTA, pH 2, containing 1×10^{-7} M epinine as an internal standard). Samples were frozen on dry ice and kept frozen until analysis. Just before loading on the HPLC column, the samples were thawed, cell debris was pelleted in a microfuge, and the supernatants were centrifuged through a 0.45 μ m filter (Rainin).

A method for determining dihydroxyphenylalanine (DOPA), dopamine (DA), norepinephrine (NE), epinephrine (Epi), and 5-HT in a single HPLC run was devised by combining several published procedures (Hegstrand and Eichelman, 1981; Reinhard and Roth, 1982; Van Valkenberg et al., 1982; Martin et al., 1983). The reversed-phase HPLC system employed an Altex model 100A pump, a sample injector with a 20 μ l sample loop, a 30 cm Whatman Partisil 5CCS/C8 column (protected by a 5 cm guard column), and a BioAnalytical Systems electrochemical detector (model LC-3-120). The mobile phase was 0.1 N citric acid, 0.1 N Na₂HPO₄, 0.1 mM EDTA, 0.2 mM sodium octyl sulfonate, and 5% methanol. The electrochemical detector used a glassy carbon electrode set at 0.72 V. Standard solutions of DOPA, NE, Epi, DA, 5-HT, and epinine containing 2.0 and 20.0 pmol each in 20 μ l were used to determine retention times and the quantitative response of the electrochemical detector. Limits of detection were arbitrarily defined as a clear peak with a height at least 5 times baseline noise. Table 1 shows typical retention times and limits of detection. The identities of peaks from sample extracts were determined by comparing retention times to standards. The identity of each peak in each type of sample was further confirmed by coelution, using a mixture of the sample and the relevant standards. Identified peaks were quantified by determining their areas, using a GTCO digitizer and the measured response to standards. To control for dilution or sample loss, all quantities were

normalized to the internal standard, epinine (2 pmol/20 μ l), which was included in each sample. Typically, recoveries were greater than 75%. To compare quantities of amines found in different cultures, values were normalized to cell number and expressed as pmol/10⁵ cells.

DL- β -3,4 dihydroxyphenylalanine (DOPA), 3-hydroxytyramine HCl (dopamine), (–)-arterenol bitartrate (NE), epinephrine bitartrate, deoxyepinephrine HCl (epinine), and 5-hydroxytryptamine creatinine sulfate (5-HT) were obtained from Sigma. Sodium octyl sulfonate was obtained from Aldrich Chemical Corp.

Cell culture for immunohistochemistry. Neural tubes were isolated as described above, except that shorter pieces of the trunk were used (the last 5 somites and the segmental plate). Neural tubes were cultured on plastic or on collagen. Twenty or 36 hr after culturing the neural tubes, explants were removed (see above), leaving neural tube outgrowths that were cultured for an additional 1–5 d. In some cases, explants were not removed, and were included in immunohistochemical analysis. Clusters that appeared on the explants were removed at 20 or 36 hr and subcultured on plastic or collagen for an additional 1–5 d. Usually, 2–5 clusters were cultured in 3 ml of medium in each 35 mm dish, but 6 of the clusters isolated at 20 hr were cultured singly on collagen in 0.3 ml wells (Lab-Tek Chamber/Slides, Miles). The small wells minimized loss of cells and seemed to promote differentiation, at least of pigment cells (see Results).

Cryostat sections. Thoracic spinal cords and associated ganglia were dissected from quail embryos at 9 d of incubation, and were fixed overnight at 4°C in 4% paraformaldehyde in PBS. Tissues were washed extensively in PBS, then incubated overnight in 12% sucrose at 4°C. After several hours at 4°C in 30% sucrose, tissues were embedded in cryoprotectant (OCT compound; Tissue-Tek) and frozen in liquid nitrogen. Serial 20 μ m sections were made with a Bright cryostat and mounted on gelatin-coated slides. Slides were stored at –20°C for 1 d prior to immunohistochemistry.

Immunohistochemistry. Cell cultures were washed twice in HBSS buffered with HEPES, then fixed for several hours in 4% paraformaldehyde in PBS. Cryostat sections were thawed, rinsed in PBS, and immersed in 0.4% paraformaldehyde for 10 sec to fix the sections to the slides. After 2 rinses in PBS, cultures and sections were incubated in 2 primary antisera, HNK-1 antibody (hybridoma ascites, diluted 1:1000) and anti-5-HT (ImmunoNuclear; diluted 1:500). Antibodies were applied either sequentially (HNK-1 in PBS/0.5% BSA, followed by anti-5-HT in PBS/BSA with 0.4% Triton X-100) or simultaneously (in 0.4% Triton), with the same results. Incubations in primary antisera were at ambient temperature for 2–4 hr. After rinsing in PBS and 0.5% BSA in PBS, cultures were incubated for 1 hr at ambient temperature in a 1:50 dilution of 2 secondary antisera in 0.4% Triton X-100 in PBS: fluorescein-conjugated goat anti-rabbit immunoglobulins (FITC-GAR) for anti-5-HT, and rhodamine-conjugated goat anti-mouse IgG + IgA + IgM (RITC-GAM) for HNK-1 (both from Cappel). After antibody incubation, cultures and sections were rinsed in PBS and mounted in a solution of 70% glycerol containing 0.1 M NaHCO₃ and 2% *n*-propylgallate. Fluorescence was observed and photographed with an epifluorescence Leitz Orthoplan microscope.

Controls included incubation of samples with secondary antisera only, and preincubation of the anti-5-HT antibody with a 5-HT-BSA conjugate (ImmunoNuclear). No antibody staining was observed in controls.

Results

Cultures analyzed by HPLC

We used reversed-phase HPLC with electrochemical detection to compare the presence of catecholamines and 5-HT from 2 sources of cultured neural crest cells: outgrowths from neural tube explants and subcultured neural crest clusters. These 2 types of cultures were described by Loring et al. (1981). Briefly, cells emigrated onto the substratum from explanted neural tubes, and by 1–2 d, when the neural tube explant was usually removed, there was a halo of migrating cells; these cultures are termed "outgrowths." In the same cultures, after about 1 d, spherical clusters of 200–300 cells could be seen on the explanted neural tubes; these spheres are termed "neural crest clusters." Neural crest clusters, when isolated and subcultured, formed circular multilayered colonies of small, stellate cells. Pigmented cells

Table 1. Typical retention times and limits of detection

Amine	Retention time (min)	Limit of detection ^a (pmol)
DOPA	3.7	0.11
NE	4.3	0.14
Epi	5.9	0.16
DA	10.3	0.20
Epinine	13.5	0.25
5-HT	33.0	0.24

^a Limit of detection was arbitrarily set at 5 times the baseline noise.

appeared in most of both kinds of cultures after 4 or 5 d. Table 2 shows the number of cells and percentage of pigment cells in each culture that was analyzed by HPLC. Though the percentage of pigmented cells was variable among replicate cultures, both outgrowth and cluster cultures generally had more pigment cells at 7 than at 5 d.

Catecholamine and 5-HT content

We detected and quantified the endogenous levels of DOPA, a precursor of both other catecholamines and of melanin pigment, and the neurotransmitters DA, NE, Epi, and 5-HT. Cultures of 10-d thoracic sympathetic ganglia were used as a positive control to demonstrate that this method could reliably detect catecholamines and 5-HT from an embryonic tissue source. DA, NE, Epi, and 5-HT were detected in sympathetic ganglia and their identities confirmed by coelution with standards (not shown). DOPA was not detected, nor did these ganglia contain pigment cells. We also analyzed extracts of culture medium, notochords, and somites. Neither catecholamines nor 5-HT were detected in these extracts, demonstrating that culture medium and non-neural embryonic tissues did not contain detectable levels of these compounds, although they did contain unidentified compounds that were also observed in neural crest cultures (not shown).

Figure 1 shows chromatograms of standards and an extract of 7 d neural tube outgrowths, and illustrates the separation and detection of DOPA, NE, DA, and 5-HT. Epi was not detected in any of the neural crest cultures.

Catecholamine content of outgrowths and clusters

No catecholamines were detected in freshly isolated neural tubes nor in neural tube outgrowths after only 2 d in culture. Similarly, no catecholamines were detected in clusters when they were analyzed just after isolation at 2 d. Figure 2 shows the results for cultures grown on plastic substrata. Catecholamines were detected in both outgrowths and clusters at 5 d *in vitro* (3 d after isolation of the clusters). In outgrowths, DOPA, DA, and particularly NE increased further by 7 d in culture. In cluster cultures, however, the amounts of catecholamines were similar at 5 and 7 d. Qualitatively similar results were found for cultures grown on collagen (Fig. 3).

Since we knew the number of pigmented cells in each sample, we compared the content of DOPA (a precursor of melanin pigment) with the percentage of pigmented cells, and found no positive correlation between the number of pigment cells and the DOPA content of the culture. Neural crest clusters contained about twice as much DOPA as neural tube outgrowths on day 5, but about 5 times as many pigment cells (Fig. 2, Table 2). At

Table 2. Number of cells and percentage of pigment cells in neural tube outgrowths and neural crest cluster cultures

Type of culture ^a	Cells/culture × 10 ⁵	Pigment cells (%) ^b
Day 5		
Outgrowth, plastic	4.5	1.1
	4.5	0.7
	1.5	2.8
	1.9	0.9
	1.9	0.9
Outgrowth, collagen	3.3	2.0
	2.2	1.5
Clusters, plastic	1.5	4.5
	1.3	7.7
Clusters, collagen	1.2	6.7
	1.2	11.0
Day 7		
Outgrowth, plastic	2.3	9.2
	1.1	10.0
	0.7	2.0
	2.2	21.0
	1.5	2.0
	0.5	0.0
Outgrowth, collagen	0.8	7.1
	2.7	5.5
	1.1	24.5
	1.1	14.0
	1.6	0.0
Clusters, plastic	3.5	23.8
	3.4	18.8
	0.4	28.8
Clusters, collagen	2.0	20.3
	2.1	25.1
	0.5	27.8
	0.6	52.2
	2.0	30.1

^a The total number of cells in each culture included in the HPLC analysis is listed. See Materials and Methods for the method used to count cells.

^b The percentage of pigmented cells in each culture.

day 7, the DOPA content of outgrowths was actually somewhat higher than that of clusters, but outgrowths had only about a third as many pigment cells.

5-HT was detected in outgrowths but not in neural crest clusters

Although DOPA, DA, and NE were detected in both neural tube outgrowths and in cluster cultures, 5-HT was found only in outgrowths, and only at the 7 d time point. Figure 3 shows the content of catecholamines and 5-HT in outgrowth and cluster cultures grown on both plastic and collagen for 7 d. 5-HT was detected in ten of eleven 7 d outgrowth cultures, but in none of the 8 cluster cultures. Because 5-HT was the only compound found in one but not the other of the 2 sources of neural crest cells, immunohistochemistry was used to confirm this result. As shown below, immunohistochemistry was a more sensitive method than HPLC for detection of 5-HT (see Discussion).

Figure 1. Representative HPLC chromatograms. The left edge of the graphs represents the time at which samples were injected. *A*, Response of standards: 20 pmol in 20 μ l of DOPA, NE, Epi, DA, epinine, and 5-HT. *B*, Response of 20 μ l of extract from 7 d neural tube outgrowths. Responses at 2 sensitivities are included to show both large and small peaks clearly. Arrowheads mark the elution times of the standards shown in *A*. DOPA, NE, DA, epinine (2 pmol internal standard), and 5-HT are detectable, but not Epi. Coelution with standards (not shown) confirmed the identities of the peaks.

Anti-5-HT and HNK-1 antibody staining

The types of cultures analyzed and the immunohistochemistry results are illustrated schematically in Figure 9. The results of antibody staining for each type of culture are presented below.

Neural crest clusters contain HNK-1-positive cells but no 5-HT-positive cells. Neural crest clusters were subcultured from neural tubes at either 20 or 36 hr, and stained with HNK-1 and anti-5-HT antibodies after an additional 3–5 d in culture (a total culture time of 5–6 d). As reported previously (Vincent and Thiery, 1984), HNK-1 antibody stained the majority of cells in the first few days of subculture (not shown), but at 6 d, after pigmentation had begun, only a few cells in each colony were HNK-1-positive (Fig. 4*B*). Table 3 shows the number of cluster colonies we examined and the number with pigmented cells and 5-HT. None of the 49 cluster colonies examined contained any 5-HT-positive cells (see Fig. 4*A*). The time at which the clusters were subcultured (20 or 36 hr) made no difference in anti-5-HT staining, but we did observe a difference in the differentiation of pigmented cells in clusters that were removed for subculture

Table 3. Neural crest clusters contain pigmented cells but not 5-HT-positive cells

Time of subculture (hr)	Culture time (d) ^a	Fraction of colonies ^b with	
		5-HT+ cells	Pigment cells
36	5	0/18	18/18
36	6	0/25	25/25
20 ^c	6	0/10	6/10

^a Total time in culture, beginning when the neural tubes were explanted.

^b The second number in each fraction is the total number of colonies, each derived from a single cluster.

^c The six 20 hr clusters that contained pigment cells were cultured in 0.3 ml of medium; the 4 that had no pigment cells were cultured in 3 ml of medium. See text for details.

at the 2 different times. Six of the 10 colonies of 20 hr clusters were cultured in a small volume of culture medium (0.3 ml), and all of these contained pigmented cells. The other 4 were cultured in a larger volume (3.0 ml), and none of these contained pigmented cells after 6 d in culture (see Table 3).

Neural tube outgrowths contain 5-HT-positive, HNK-1-negative neurons. In contrast to the cluster cultures, the neural tube outgrowth cultures contained 5-HT-positive cells as early as 4 d *in vitro*. All of the 43 outgrowth cultures contained 5-HT-positive cells (Table 4). The antibody-positive cells had a distinct morphology: they were usually bipolar, with long, thin varicose processes (Fig. 5, *A*, *B*; see also Fig. 7*A* for higher magnification). HNK-1 antibody also stained cells in outgrowth cultures. At 6 d, many of the HNK-1-positive cells appeared to be neurons (Fig. 5*D*; see also Fig. 7*B* for higher magnification). We saw no cells that were stained with both HNK-1 and anti-5-HT antibodies (see below).

Distribution of 5-HT-positive cells in outgrowth cultures. The 5-HT-positive cells in outgrowths appeared to be close to the original position of the explants, so we cultured some outgrowths without removing the neural tubes to determine whether any 5-HT-positive cells developed in the explants themselves. We saw a few 5-HT-positive cells within the explants as early as 3 d in culture, and at 4 d we observed a large number of stained cells within the explanted neural tubes. Figure 5 shows the distribution of such cells in an explanted neural tube and associated outgrowth; the cells are arranged in a “river” along the ventral edge of the explant. In some 5 d cultures, we counted the number of 5-HT cells associated with the neural tube explant and with the outgrowth (Table 5). Three hundred to 500 5-HT-positive cells appeared in each culture; of these 20–60% were in the

Table 4. Outgrowths from neural tubes contain 5-HT-positive cells and pigment cells

Time of removal of explant (hr)	Substratum	Culture time (d)	Fraction of cultures with	
			5-HT+ cells	Pigment cells
36	Plastic	4	9/9	6/9
36	Plastic	6	15/15	15/15
36	Collagen	4	5/5	3/5
36	Collagen	6	4/4	4/4
20	Collagen	6	10/10	10/10

Figure 2. Catecholamine content of cells cultured on plastic substrata. The amount of each of the catecholamines in cultures of neural tube outgrowths and neural crest clusters at 3 time points was determined as described in Materials and Methods, and normalized to the number of cells in each culture. The bars are the means $\pm$ SEM of 2–6 replicate cultures analyzed separately by HPLC. The number of cells in each of these cultures is listed in Table 2.

explant itself. Of the cells that did move away from the explant, none moved very far; all 5-HT-positive cells occupied the inner half of the halo of migrating cells.

5-HT-positive, HNK-1-negative cells appear in cultures of ventral, but not dorsal, neural tube fragments. To determine directly a possible source of the 5-HT-positive cells in outgrowth cultures, we cut isolated neural tubes lengthwise into 3 pieces. The dorsalmost and ventralmost portions were a quarter or less of the total height of the neural tube (Fig. 6). The middle portion was discarded and the dorsal (clearly containing neural crest) and ventral (clearly excluding neural crest) fragments were cultured for 3–6 d and then double-labeled with HNK-1 and anti-5-HT antibodies. There were HNK-1-positive cells in both types of cultures (Figs. 7B; 8B), but 5-HT-positive cells appeared only in ventral neural tube cultures (Table 6). The distinct morphology of these cells (Fig. 7A) was similar to that of 5-HT cells in outgrowth cultures (Fig. 5B). As in outgrowth cultures, there was apparent exclusion of staining with the 2 antibodies. Because the cultures were multilayered, and HNK-1-positive and 5-HT-positive neurons were often fasciculated, we cannot be absolutely certain that no cell stained with both antibodies. However, in less dense areas of the cultures, it was clear that none of the 5-HT-positive cells was HNK-1-positive (Fig. 7, A, B). No pigmented cells appeared in ventral neural tube cultures (Table 6).

In contrast to the ventral neural tube cultures, no 5-HT-positive cells were observed in the dorsal neural tube cultures. Also,

unlike the ventral neural tubes, pigment cells appeared in all of the dorsal neural tube cultures (Fig. 8C, Table 6).

For a schematic illustration of the types of the cultures analyzed, see Figure 9.

5-HT neurons in the spinal cord of 9-d quail embryos

To determine whether quail embryos, like chick embryos (Wallace, 1985), possess serotonergic neurons within the spinal cord, we double-labeled cryostat sections of 9-d quail thoracic spinal cord with anti-5-HT and HNK-1 antibodies. We observed 5-HT neurons in the ventral spinal cord, ventral to the central canal (Fig. 10A). They were usually bipolar and often one process was closely associated with the central canal (Fig. 10B). Like the 5-HT-positive cells in outgrowths and ventral neural tube cultures, the cells in the spinal cord were HNK-1-negative (Fig. 10C). Other cells in the spinal cord, the nerves, and the dorsal root ganglia did stain with HNK-1, as also reported by Vincent and Thiery (1984). No 5-HT-positive cells were found in the dorsal root ganglia or nerves, but the sympathetic ganglia did contain 5-HT antibody staining (not shown).

Discussion

The purpose of this work was to study the differentiation of the multipotent population of neural crest cells, which gives rise to most peripheral neurons of vertebrates. We restricted our *in vitro* analysis to the thoracic neural crest, whose normal derivatives are believed to be relatively few: sympathetic and dorsal

Figure 3. Catecholamine and 5-HT content of cells cultured on plastic and collagen substrata. Neural tube outgrowths and neural crest clusters were cultured on either plastic or collagen substrata for 7 d before HPLC analysis. The amine content was normalized to the number of cells in each culture (listed in Table 2). The bars are the means $\pm$ SEM of 3–6 replicate cultures analyzed separately by HPLC. The culture substratum produced no significant difference in content of any of the amines (Dunnett *t* test).

root ganglia, adrenal medulla, and pigment cells (LeDouarin, 1982). Since neural crest cells are a transient, ill-defined population of embryonic cells (see below), we used several well-established techniques to obtain the cells, and compared the

subsequent differentiation of the populations that resulted. Our results (1) confirm earlier reports of differentiation *in vitro* of catecholamine-containing cells and pigment cells from undifferentiated precursors, (2) suggest that 5-HT-containing cells that develop in some types of thoracic “neural crest” cultures are not neural crest cells, (3) cast doubt on previously accepted operational definitions of neural crest cells *in vitro*, and (4) show that cells do migrate from the ventral (non-neural crest) part of the neural tube *in vitro*, as well as suggest that a similar phenomenon may occur *in vivo*.

Neural tube outgrowths and neural crest clusters both contain catecholamines; only outgrowths contain 5-HT

Two commonly used sources of undifferentiated neural crest cells in culture are (1) cells that emigrate onto culture substrata from explanted neural tubes, termed “neural tube outgrowths,” and (2) “neural crest clusters” that arise in culture as aggregates of several hundred cells that loosely adhere to explanted neural tubes. In order to determine the similarities and differences in neurotransmitter accumulation between outgrowths and neural crest clusters, we analyzed both by 2 methods: HPLC, to detect and quantify endogenous levels of catecholamines and

Table 5. Distribution of 5-HT-positive cells in neural tube cultures

Culture	Number of 5-HT+ cells (% of total)		Extent of outgrowth (mm) ^a	Farthest 5-HT+ cells (mm) (% of distance) ^b
	On neural tube	In outgrowth		
1	161 (52)	146 (48)	1.9	0.6 (32)
2	84 (21)	308 (79)	1.6	0.6 (38)
3	106 (40)	161 (60)	2.1	0.8 (38)
4	278 (58)	199 (42)	1.6	0.8 (50)
5	206 (40)	310 (60)	2.4	0.8 (33)

^a The outgrowth was roughly circular; the extent of outgrowth is the length of the radius from the explanted neural tube to the cells that had migrated the farthest from it in an area that contained 5-HT-positive cells.

^b The distance migrated by cells that were 5-HT-positive was compared to the greatest distance migrated by cells in the same radius.

Table 6. 5-HT-positive cells appear in explanted neural tubes and neural tube fragments

Type of culture	Culture time (d)	Fraction of cultures with	
		5-HT+ cells	Pigment cells
Neural tube explant and outgrowth	3	4/4	0/4
	4	11/11	5/11
	6	9/9	9/9
Ventral fragments of neural tubes ^a	3	1/10	0/10
	4	10/10	0/10
	5	9/9	0/9
	6	10/10	0/10
Dorsal fragments of neural tubes ^a	3	0/9	0/9
	4	0/8	0/8
	6	0/11	11/11

^a Ventral fragments are the ventral quarter of isolated neural tubes. Dorsal fragments are the dorsal quarter of the same neural tubes. See Figure 6.

5-HT, and immunocytochemistry, a more sensitive but non-quantitative method to assay for 5-HT.

Content of catecholamines and 5-HT measured by HPLC

We measured by HPLC the normal content of catecholamines and 5-HT in cultures containing neural crest cells. It is important to note that this method reveals the total content of the compounds in cells, not the accumulated synthesis from radiolabeled precursors, as was reported earlier for neural tube outgrowths (Maxwell et al., 1982) and for neural fold cultures (Smith and Fauquet, 1984). This is the first time that neural crest clusters have been analyzed by HPLC. At the earliest time points analyzed, freshly isolated neural tubes and their progeny, outgrowths and clusters at 2 d, had no detectable catecholamines or 5-HT. On the fifth and seventh day of culture, both outgrowths and clusters contained the catecholamines DOPA, DA, and NE. This result is in qualitative agreement with the observations made by Maxwell et al. (1982) and Smith and Fauquet (1984). In agreement with Maxwell (see Fig. 4 of Maxwell et al., 1982), but not with Smith and Fauquet, we detected no epinephrine. This difference in results is not surprising, because, unlike the work reported here and that of Maxwell et al., Smith and Fauquet cultured the cells in the presence of added glucocorticoid. It has been shown that glucocorticoids increase the expression of the adrenergic phenotype in neural crest derivatives (for review, see Bohn, 1983).

Because we counted the number of pigmented cells in each culture before analyzing it by HPLC, we were able to compare the degree of pigmentation with the amount of DOPA in the cultures (see Fig. 3 and Table 2). DOPA is the precursor of melanin pigment, as well as of DA and NE (Bagnara et al., 1979). We found no correlation between DOPA content and the degree of pigmentation of the cultures. In spite of the fact that clusters almost invariably contained more pigmented cells than the outgrowths, the DOPA content at 7 d was actually slightly lower in the cluster cultures. This suggests that fully differentiated pigmented cells are not the ones accumulating DOPA in these cultures. A speculative interpretation is that pigment cell precursors (and perhaps precursors to catecholaminergic cells) are the cells that are accumulating DOPA at an early stage of differentiation.

Figure 4. A neural crest cluster isolated from a neural tube explant after 2 d and cultured for an additional 4 d before double-labeling with anti-5-HT and HNK-1 antibody. *A*, Anti-5-HT staining. No cells are stained with this antibody. The bright spots are debris, not cells. *B*, HNK-1 staining of the same microscope field, showing 5 antibody-positive cells. *C*, The same microscope field as in *A* and *B*, viewed with bright-field optics. Pigment cells are clearly visible. The positions of the HNK-1 positive cells are indicated with arrowheads. Few if any pigment granules can be seen in the HNK-1-positive cells. Bar, 100 μ m.

Also of interest is the difference in NE content between outgrowths and clusters. Outgrowths had greater than 20-fold more NE than clusters. It has been suggested (Norr, 1973; Teillet et al., 1978; Loring et al., 1982; Smith and Fauquet, 1984) that

Figure 5. A neural tube explant and associated outgrowth cultured for 6 d before double-labeling with anti-5-HT and HNK-1 antibodies. *A*, Low-power micrograph showing most of the neural tube explant (NT) and some of the outgrowth (O) stained with anti-5-HT antibody. Part of the notochord was left associated with the neural tube to allow clear identification of the dorsal–ventral axis after culture. The neural tube lies on its side and is twisted; the ventral part is at the top on the left side of the micrograph, and the twist has resulted in the ventral part being at the bottom on the right side of the micrograph. Notice that the 5-HT-positive cells within the neural tube explant are mainly in the ventral portion. There are also many 5-HT-positive cells among those that migrated into the outgrowth. *B*, Higher-power micrograph of the left edge of the explant shown in *A*, stained with antibody against 5-HT. Numerous 5-HT-positive cells and processes can be seen within both the explant and the outgrowth. Labels as in *A*. *C*, The same microscope field as *B*, viewed with bright-field optics, and showing the distribution of pigment cells. Pigment cells appear to colocalize with the HNK-1-positive cells (*D*) in the outgrowth, but, unlike the HNK-1-positive cells, almost no pigment cells are visible within the explanted neural tube. Bars, 100 μ m (*A*, *D*). *D*, The same microscope field as *B* and *C*, showing the distribution of HNK-1-positive cells. HNK-1-positive cells can be seen in both the outgrowth and the neural tube explant. The 5-HT-positive and HNK-1-positive cells clearly do not codistribute in the outgrowth (compare with *B*). However, because these cultures are multilayered, we cannot be absolutely sure that none of the individual cells are stained with both antibodies (see also Fig. 7).

Figure 6. Freshly isolated neural tube that will be dissected into dorsal and ventral neural tube fragments. The dorsal (*D*) and ventral (*V*) portions have been partially dissected and will be removed and cultured separately. The *middle portion* will be discarded. The notochord (*N*) was left partially attached to the neural tube (*NT*) during the dissection to identify the ventral margin of the neural tube, but will be completely removed before culture. Bar, 100 μ m.

cellular environment affects the differentiation of catecholaminergic cells. If we assume that the NE-containing cells in the outgrowths and the clusters are derived from the same precursor,

that is, undifferentiated (and perhaps undetermined) neural crest cells, then it is possible that the outgrowths provide a more suitable environment for catecholaminergic expression. Alternatively, the clusters may contain a smaller proportion of catecholaminergic precursor cells. There is at present no means to distinguish between these 2 possibilities.

We believe that the most important result of our HPLC analysis is not the similarities in catecholamines, but the striking difference in 5-HT content between neural tube outgrowths and neural crest clusters. We detected 5-HT in outgrowths at 7 d but not at 5 d. The amount, about 5 pmol/ 10^5 cells, was small, but comparable to the amounts of DOPA and DA detected. The cluster cultures contained no detectable 5-HT, although the culture conditions were clearly conducive to 5-HT accumulation by outgrowth cells. We tested the possibility that the outgrowth cultures simply retained the 5-HT neurons because the substratum had become more adhesive owing to production by the neural tube of adhesive macromolecules such as collagen (Trelsted et al., 1973). No 5-HT was detected in clusters cultured on collagen, suggesting that the neurons were not selectively lost.

The observation that 5-HT was present in neural tube outgrowths but not in neural crest cluster cultures is of particular interest. This 5-HT content, unlike catecholamines and melanin pigment, marks a phenotype shared by embryonic spinal cord neurons (Wallace et al., 1986) and putative neural crest derivatives in the PNS (LeDouarin, 1982), and is thus not a definitive marker for the neural crest. The qualitative difference between

Figure 7. Ventral neural tube culture at 6 d, double-labeled with anti-5-HT and HNK-1 antibodies. *A*, Anti-5-HT staining. Note the bipolar neurons with long, thin varicose processes. The *vertical arrowheads* point to 5-HT-positive cells and processes that are clearly HNK-1-negative (see *B*). The *horizontal arrowheads* point to the positions of cells that can be seen in *B* to be HNK-1-positive, but do not stain with anti-5-HT. *B*, The same microscope field, showing HNK-1 antibody staining. Though numerous cells with neuronal morphology can be seen, the antibody staining clearly does not codistribute with the anti-5-HT staining shown in *A*. *Arrowheads* mark the cells described in *A*. There were no pigment cells in this culture, so a bright-field micrograph is not shown. Bar, 100 μ m.

Figure 8. Dorsal neural tube culture at 6 d, double-labeled with anti-5-HT and HNK-1 antibodies. *A*, Anti-5-HT staining is absent. *B*, HNK-1 staining of the same microscope field. The white cells are antibody-positive; black pigment cells are also visible. *C*, The same microscope field as *A* and *B*, viewed with bright-field optics. Pigment cells are clearly visible. None of the darkly pigmented cells are stained with HNK-1 antibody (compare *B* and *C*). Bar, 100 μ m.

CULTURES DERIVED FROM NEURAL TUBES

Figure 9. Summary diagram of most of the types of cultures examined by immunohistochemistry, showing the methods of obtaining the cultures and the major results. Cultures (from left, bottom row): Dorsal neural tube fragments (including the neural crest), which produced HNK-1-positive cells and pigment cells, but not 5-HT-positive cells; ventral neural tube fragments (excluding the neural crest), which contained HNK-1-positive cells and 5-HT-positive cells, but not pigment cells; neural tube outgrowths, which produced HNK-1-positive cells, 5-HT-positive cells, and pigment cells; and neural crest cluster cultures, that, like the dorsal neural tube cultures, contained HNK-1-positive cells and pigment cells, but not 5-HT-positive cells. Not shown is the fifth type of culture analyzed, outgrowths from which the neural tube explant was not removed. This type of culture contained all 3 cell types, like the neural tube outgrowths (see Results). The *stippled cells* represent pigment cells, the *black neurons* are 5-HT-positive cells, the clear neurons are HNK-1-positive, and the clear non-neuronal cells have none of these markers.

outgrowth and cluster cultures reported here raises the possibility that the 5-HT reported to be present in neural tube outgrowths (Maxwell et al., 1982; Sieber-Blum et al., 1983), and possibly other neurotransmitters, such as ACh (Kahn et al., 1980; Maxwell et al., 1982) and peptides (Maxwell et al., 1984) might be derived from spinal cord cells that migrate *in vitro* and populate the outgrowths. Because of the implications of this possibility for studies of neural crest differentiation in general, we sought to confirm this result and to determine the source of 5-HT containing cells in outgrowths by using a more sensitive method of detection of 5-HT, immunocytochemical analysis.

Detection of 5-HT by immunocytochemistry

Using an antibody to 5-HT, we were able to detect 5-HT in outgrowths as early as 4 d *in vitro*. Our HPLC analysis did not demonstrate 5-HT in outgrowths before the seventh day of culture. This observation clearly shows that 5-HT was present but was not detected in cultures that we analyzed by HPLC; immunohistochemistry was a more sensitive detection method for HT. Although this result calls into question the HPLC data that suggested that 5-HT was absent in cluster cultures, our immunohistochemical analysis of cluster cultures was consistent

with the HPLC results; no 5-HT-containing cells were detected in any of our cultures of clusters. Since the culture conditions we used allowed differentiation of outgrowth cells into 5-HT neurons, it seems unlikely that they were inadequate for serotonergic differentiation of cluster cells. We did, however, explore the possibility that we had isolated neural crest clusters too late to allow the cells to differentiate into their full range of possible derivatives. Recent evidence suggests that the ultimate differentiation of cluster cells may be influenced by the time spent in close association with each other. Vogel and Weston (1986) compared clusters that were isolated and subcultured as soon as they were distinguishable on explanted neural tubes (about 18–20 hr after the neural tubes were explanted) with clusters isolated at later times. Using an antibody marker for neurons, they report that more neurons and fewer pigmented cells differentiate from clusters subcultured at earlier times, compared to those subcultured later, and suggest that cell-cell interaction may promote melanogenesis and deter neuronal differentiation. To test the possibility that the time of isolation of the clusters we cultured (36 hr) was affecting their ability to differentiate, we isolated clusters after only 20 hr *in vitro*. To discourage any influence of cells on each other, we cultured some single 20 hr

Figure 10. A cryostat section through the thoracic spinal cord of a 9-d quail embryo. *A*, Anti-5-HT staining. Three 5-HT-positive bipolar neurons (arrowheads) can be seen in the spinal cord, ventral to the central canal (*c*). The double arrowheads point to small 5-HT-positive cells within blood vessels, which are thrombocytes or mast cells. The asterisk indicates an area of the cord that contains many 5-HT-positive fibers, but no cell bodies. A similar distribution of serotonergic cells and fibers has been reported in the chick embryo spinal cord (Ho and LaValley, 1984; Wallace, 1986). *B*, A higher-power micrograph of the ventral portion of the spinal cord section shown in *A*. The arrowhead points to one of the cells that is shown to be HNK-1-negative in *C*. *C*, HNK-1 antibody staining. The arrowhead points to the position of the 5-HT-positive cell indicated in *B*. There is no HNK-1 staining in this portion of the spinal cord, although staining of the central canal (*c*) and other cells in the cord is apparent. Bars, 100 μ m (*A*, *C*).

clusters in a large volume of culture medium, to prevent conditioning of the medium by the cells. We found that these clusters did not become pigmented in 6 d, in agreement with Vogel and Weston's results, but in contrast, clusters cultured in a small volume of culture medium contained many pigmented cells after 6 d. This result supports the idea proposed by Vogel and Weston, that cell-cell interaction in neural crest clusters promotes differentiation into pigment cells. However, in neither case did any of the cultures contain 5-HT-positive cells, supporting the idea that cluster cells do not include serotonergic differentiation in their developmental repertory.

What is the source of 5-HT cells in neural tube outgrowths?

One interpretation of the results discussed so far is that outgrowths contain not only neural crest cells, but also spinal cord precursors that are able to migrate from the neural tube *in vitro* and produce 5-HT in the outgrowth. We took 2 approaches to confirm or disprove this possibility. First, we cultured neural tubes, and instead of removing the explant after a day or two, we left the explant with the outgrowth and counted both the number of 5-HT-positive cells that migrated into the outgrowth and those that remained with the explant itself. In all cases, a large proportion of the 5-HT-positive cells were found in the explant itself, and those that did migrate did not move very far with respect to other outgrowth cells. There are 2 possible explanations for this observation: either the 5-HT-positive cells are neural crest cells that do not migrate well in culture, or alternatively, the antibody-positive cells are precursors of spinal cord neurons that do migrate to a certain extent *in vitro*. To distinguish between these 2 possibilities, we took a direct approach: we examined the 2 possible sources of these cells, neural crest cells and spinal cord precursors, separately. To do this, we cut isolated neural tubes into dorsal fragments (clearly containing neural crest cells) and ventral fragments (clearly devoid of neural crest cells) and cultured them separately. The results were consistent and remarkable: *all* of the ventral fragments produced 5-HT-positive cells; *none* of the dorsal fragments (containing neural crest cells) contained any 5-HT-positive cells. This result strongly supports the idea that serotonergic cells in outgrowths are not derived from the neural crest.

If they are not neural crest cells, then what is the normal fate of the 5-HT cells found in neural tube cultures? We found neurons in the spinal cord that are probably the *in vivo* equivalent of the 5-HT cells found in outgrowths. It has been reported that 5-HT neurons normally exist at all axial levels of the spinal cord, as well as in the brain stem in chick embryos, and such cells have been detected with antibodies as early as day 7 of incubation (Wallace et al., 1986). Furthermore, these cells are localized in the ventral part of the spinal cord, and in the brain stem, 5-HT cells have been reported to migrate ventrally and laterally within the confines of the CNS (Wallace, 1985). We report here the existence of 5-HT neurons in the spinal cord in the quail. Like the chick neurons reported previously, the 5-HT neurons in the quail occupy the ventral portion of the spinal cord. Their morphology (long varicose processes) is similar to that of all the 5-HT neurons we observed in culture, in outgrowths, within neural tube explants, and in ventral neural tube fragments. One of the characteristics of the 5-HT neurons we studied in culture is that they do not stain with the "neural crest antibody," HNK-1. The neurons we detected in the spinal cord share this characteristic, providing further evidence that they

are the same cells *in vivo*. We have not yet examined peripheral 5-HT neurons (for example, enteric neurons) that are believed to be neural crest-derived (Gershon, 1981; LeDouarin, 1982). If they do express the HNK-1 marker, it would indicate a molecular difference and thus might provide a means of distinguishing CNS from PNS 5-HT neurons.

How can cells be identified as neural crest in vitro?

The work reported here questions the ability of researchers to identify neural crest cells *in vitro*. Below are listed the commonly used operational definitions of neural crest cells, with our comments on their usefulness in light of the results reported here:

1. *Neural crest cells are cells that emigrate from explanted neural tubes in vitro (neural tube outgrowths).* Though migratory ability is certainly a characteristic of neural crest cells *in vivo*, our results strongly suggest that ventral neural tube cells migrate *in vitro* from explanted neural tubes.

2. *Neural crest cells are cells that are labeled with the "neural crest" antibodies NC-1 and HNK-1.* Although it has been shown that neural crest cells *in vivo* are labeled with these antibodies (Tucker et al., 1984), other cells are labeled as well (Vincent and Thiery, 1984; Loring and Erickson, 1987), and ventral neural tube (non-neural) crest cells are labeled *in vitro* (Loring and Erickson, 1987, and this report). Thus, a cell in culture cannot be identified as a neural crest cell on the basis of NC-1 or HNK-1 staining alone.

3. *Neural crest cells are cells derived from dorsal neural tubes or neural folds.* Dorsal neural tube cultures contain pigment cells (a definitive neural crest phenotype), as well as nonpigmented cells. We could not specifically examine cultures of dorsal neural tubes or neural folds for the presence of non-crest cells. However, because there is presently no way to identify potential neural crest cells within the neural tube or neural folds, and hence to isolate them in pure form, it is extremely likely that such cultures contain non-neural crest cells.

4. *Neural crest clusters are neural crest cells.* Since it has been reported that all cells in neural crest clusters will differentiate into pigment cells under certain culture conditions (Glimelius and Weston, 1981; Loring et al., 1981), there is currently little doubt that clusters contain exclusively neural crest cells. Cluster cells differentiate into catecholaminergic cells as well (Loring et al., 1982), and we confirm and extend that observation in the present study, demonstrating that clusters are not purely premelanocytes. In spite of the fact that they are an artifact of tissue culture, and that they certainly do not contain all the neural crest cells derived from a neural tube (outgrowths from the same neural tubes contain pigmented cells as well), neural crest clusters remain the most certain means of obtaining identifiable neural crest cells from neural tubes *in vitro*.

Do non-neural crest cells emigrate from neural tubes in vivo?

Besides the implications for *in vitro* studies of neural crest cell differentiation, this work also raises questions about migration of non-neural crest cells. First, why should non-neural crest cells migrate *in vitro*? *In vivo*, neural crest cells seem to be restricted in their migrations by basal laminae, which they may be unable to penetrate (Erickson, 1987; for review, see Erickson, 1986). Normally, during neural crest migration *in vivo*, a basal lamina surrounds all but the most dorsal part of the neural tube (Martins-Green and Erickson, 1986, 1987). However, the enzymes used to isolate neural tubes for culture degrade the basal lamina

around the neural tube (unpublished observations). Perhaps ventral spinal cord cells are, like the neural crest, prevented from escaping the CNS *in vivo* because of the surrounding basal lamina, and its disappearance *in vitro* allows their emigration. Second, the results reported here raise the possibility that cells other than the neural crest may normally migrate from the neural tube during embryonic development. Although an abundance of evidence from transplantation and ablation experiments supports the idea that all peripheral ganglia of the trunk are derived from the neural crest (that is, dorsal neural tube), there is no evidence showing that *all* of the cells in trunk PNS are neural crest-derived. In fact, the origin of one cell type, the supportive cells of the ventral spinal nerves, remains in doubt. Based on his transplantation experiments, Weston (1963) suggests that ventral root supportive cells may be derived from the ventral, not the dorsal, neural tube (suggested also by Keynes, 1987; Loring and Erickson, 1987; see also LeDouarin, 1982). These cells might emigrate from the ventral neural tube along the ventral roots, emerging through the breaks in the basal lamina created by the growing axons. It seems possible that among the cells that emigrate from the ventral neural tube are the 5-HT cells that we report here to be migratory *in vitro*.

Are there serotonergic cells in the thoracic PNS?

If 5-HT neuron precursors are among the ventral neural tube (non-neural crest) cells that emigrate from the neural tube *in vivo*, then what might their ultimate location be after migration? Although the 5-HT neurons in the enteric nervous system (Gershon, 1981) are believed to be derived from the neural crest, the vagal and lumbosacral neural crest, not the thoracic neural crest, give rise to enteric ganglia and plexuses (LeDouarin, 1982). The superior cervical ganglion (SCG) of the rat contains a small number of neurons that synthesize 5-HT, and others that use 5-HT as a false transmitter (Happola et al., 1986; Sah and Matsumoto, 1987). In the rat embryo, the SCG may be derived from both cervical and thoracic neural crest (Rubin, 1985), but in avian embryos, evidence suggests a strictly cervical origin of the SCG (Yip, 1986). There is some evidence that derivatives of the thoracic and upper lumbar neural crest normally contain serotonergic cells. Mondragon and Wallace (1985) report a transient expression of 5-HT immunoreactivity in thoracic sympathetic ganglia of the chick embryo, and also report that the adrenal medulla contains 5HT cells. In the rat, adrenal chromaffin cells clearly contain 5-HT *in vivo* (Holzwarth et al., 1984). It is not yet clear whether the chromaffin cells can synthesize as well as take up and store 5-HT (Verhofstad and Jonsson, 1983; Holzwarth and Brownfield, 1985). We have found no published report of 5-HT in the sympathetic ganglia or adrenal chromaffin cells of quail embryos. In preliminary experiments, we have detected 5-HT in embryonic quail thoracic sympathetic ganglia and adrenal medulla *in vitro* and *in vivo* (unpublished observations).

Whether or not the serotonergic neuron precursors actually migrate from the ventral neural tube *in vivo*, there is a growing suspicion that some cells do normally migrate from this area (Weston, 1963; Keynes, 1987; Loring and Erickson, 1987). This work raises the intriguing possibility that the "neural trough," as well as the neural crest, may be a significant contributor of precursors to the PNS of vertebrates.

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