Morphogenetic Studies on the Neural Crest of Hynobius Larvae Using Vital Staining and India Ink Labeling Methods

Shigeki Hirano and Toshio Shirai

Department of Anatomy (Prof. T. Shirai), Yamagata University School of Medicine, Yamagata, Japan

Received June 27, 1983

Summary. The neural crest cells of hynobius larvae during the morphogenesis of the neural crest were studied with the following methods: the region of the primordial neural crest was first determined by vital stainings, and then the movement of individual neural crest cells was traced with India ink labeling. The cytoarchitecture and the fine structure of neural crest cells were further observed by light, transmission and scanning electron microscopy.

The primordium of the neural crest is located in the area extending from the lateral part of the neural plate to the medial part of the neural fold. Raven (1931) suggested that the lateral part of the neural fold was a part of the primordial neural crest. The result of our vital staining shows that it is the primordial epidermal ectoderm.

The morphogenesis of the neural crest was found to proceed as follows: at first both neural folds moved to the dorsal mid line of larva, then contacted and fused to each other. The primordial neural crest cells in the neural folds became polygonal, and these cells from both folds mixed and formed a neural crest cell mass. In this process they lost their junctional apparatuses. When larvae developed at the tail bud stage, the cells in the lateral part of the neural crest extended their cytoplasmic processes and began to migrate.

It is well known that neural crest cells are derived from ectodermal cells of neural folds and migrate to areas between the epidermis and the somite or between the somite and the neural tube. The migrant cells in the former area mainly differentiate to melanocytes and the cells in the latter to neurons or glial cells of the peripheral nervous system (Hörstadius, 1950; Weston, 1970; Noden, 1978).

Up to present, most studies on the neural crest have generally been classified into two categories, one being the formation of the neural crest in the early developmental stage. The cytoarchitecture of the neural crest (Raven, 1931; Baker and Graves, 1939), observations on the organization of neural crest cells with scanning electron microscopy (Bancroft and Bellairs, 1976; Lofberg and Ahlfors, 1978; Tosney, 1978, 1982; Anderson and Meier, 1981) and the fine structure of neural crest cells with transmission electron microscopy (Newgreen and Gibbins, 1982) have been reported. The second category is the migration and differentiation of neural crest cells. The migrating pathway of neural crest cells (Johnston, 1966; Noden, 1975; Bronner-Fraser and Cohen, 1980a), the relationships between the migrating neural crest cells and their
surrounding tissues (Le Douarin and Teillet, 1974; Pratt et al., 1975; Weston et al., 1977; Takahashi and Yamadori, 1979; Tosney, 1978, 1982) and the differentiation of neural crest cells (Raven, 1936; Le Douarin and Teillet, 1973) have been studied. There has been some research on the developmental process from the primordium of the neural crest in the neural fold to the migrating neural crest cells (Detwiler, 1937; Schroeder, 1970; Lamers et al., 1981; Rosenquist, 1981). The relationships between the morphogenesis of the neural crest and the migration of neural crest cells have not been fully clarified. For a thorough analysis of the migration and the differentiation of neural crest cells it is necessary to first observe neural crest cells forming a cell mass on the neural tube before their migration. The present investigation deals with the morphogenetic movement of the primordial neural crest marked with vital stainings, and the cytoarchitecture and fine structure of neural crest cells labeled with India ink.

### MATERIAL AND METHODS

Larvae of salamander (*Hynobius lichenatus* Boulenger) were used. The developmental stages in our experiments are shown in Table 1 and Figure 1.

**Table 1.** The stages of development used in our experiments

| Stage  | Description |
|--------|-------------|
| 1      | Neural folds elevate slightly. |
| 2      | Neural folds further elevate and move to the dorsal midline of the larva. |
| 3      | Neural folds come in contact with each other on the dorsal midline of the larva. |
| 4      | Neural folds fuse to each other, and the dorsal part of the larva is covered with epidermis. |
| 5      | Tail bud appears. |

**Vital staining-1. Staining with a piece of agar immersed in neutral red or Nile blue:** After growing to stage 1, the jelly and vitelline membranes of the larvae were removed while they were in a dish filled with Holtfreter's solution. A small piece of agar with neutral red or Nile blue was put on various areas of larvae: on the lateral part of the neural plate, the whole neural fold, the lateral side of the neural fold, and the area from the lateral part of the neural plate to the medial part of the neural fold—at the level of the transitional region between the rhombencephalon and the spinal cord (Fig. 1). After about 15–30 min the piece was taken off. The stained larvae were cultivated at room temperature, fixed with 10% formalin at each stage from stage 1 to stage 5 and cut transversally at the stained level. The marker in each larva was observed.

**Vital staining-2. Staining with injection of Nile blue:** Larvae were prepared with the same method as mentioned above, and 0.01% solution of Nile blue with Holtfreter's solution was injected into the boundary zone between the neural fold and the lateral border of the neural plate with a glass needle at the same level of vital staining-1. Also the stained areas were observed.

**Labeling with India ink:** India ink was longitudinally injected into the neural fold of the larva at the same place of the vital stainings at stage 3 with a glass needle. This India ink was used after filtration. The injected larvae were fixed with 5% glutaraldehyde solution in 0.1 M phosphate buffer, 10% formalin or Bouin's solution at each stage from stage 3 to metamorphosed larvae after 4 months and embedded in Quetol 812 or paraffin. Serial sections were made, and Quetol 812 was removed by Lane and Europa's method (1965). These sections were treated with H₂O₂ to decolorize pigment.
Neural Crest Study with India Ink Labeling

granules and to define the India ink contained in the cytoplasm of neural crest cells, and then stained lightly with hematoxylin.

Preparation for observing the cytoarchitecture of the neural crest: Serial sections of larvae at each stage were stained with toluidine blue.

Preparation for scanning electron microscopy: Larvae at each stage were fixed with 2.5% glutaraldehyde solution in 0.1 M phosphate buffer and postfixed with 1% OsO₄ in the same buffer. In the glutaraldehyde solution, a transverse incision was made into the abdominal part of larvae at the transitional region between the rhombencephalon and the spinal cord; these larvae were cleaved through the incision site with a pair of needles. After the larvae were immersed in isoamyl acetate, dried at critical point and

Fig. 1. Results of the two vital staining methods are summarized. The line in the dorsal view of larvae at each stage indicates the level of the transitional part between the primordia of the rhombencephalon and of the spinal cord. a–g. The dotted areas are the areas stained with vital staining–1. a. The lateral part of the neural plate is stained. This area is found in the wall of the neural tube from stage 1 to stage 5. b. Both the lateral part of the neural plate and the medial part of the neural fold are continuously stained. These areas are in the central part, or in the deep part of the neural crest and the dorsal part of the neural tube at stage 4. c. The medial part of the neural fold is stained. This area is in the central part or the deep part of the neural crest at stage 4. d. The whole neural fold is stained. These areas are in the dorsal parts of the neural crest and of the epidermal ectoderm at stage 4. e. The lateral part of the neural fold is stained. At stage 3, the stained area is in the apical area of the connecting part in the neural fold and its adjoining epidermal ectoderm, and in the upper part of the neural crest and the epidermal ectoderm at stage 4. f and g. The lateral part of the neural fold and its adjoining epidermal ectoderm are stained. These areas are in the epidermal ectoderm at each stage. h. The results of vital staining–2. The area from the lateral part of the neural plate to the medial part of the neural fold and the mesoderm beneath them are stained. At stage 4, the stained area is only in the deep part of the neural crest, or in that part and its wall contiguous to the neural tube. At stage 5, the stained area in the neural crest becomes vague. The extent of the neural crest in each stage is indicated in i. The arrow indicates the extent of the neural fold.
Fig. 2. Neural crest cells labeled with India ink. India ink droplets remain in their cytoplasm without being decolorized by H2O2. 

a. India ink droplets are dispersed into intercellular spaces shortly after injection.

b. Labeled cells are widely distributed in the neural crest after the fusion of both neural folds. The arrowheads point out India ink droplets in the cells. The droplets are clearly seen in the inserted picture.

c. A labeled cell starting to migrate.
coated with gold, the ectoderm of some larvae at stages 4 and 5 was removed with adhesive tape before coating with gold.

**Preparation for transmission electron microscopy:** Larvae of stages 1 and 2 without labeling and larvae labeled with India ink after stage 3 were fixed in the same way as for scanning electron microscopy, embedded in Quetol 812, and ultra-thin sections were made.

**RESULTS**

**Vital stainings**

The ectoderm from the lateral part of the neural plate to the medial part of the neural fold was vitally stained with Nile blue at stage 1, and at stage 4, either the dye manifested itself or disappeared in an area deep in the neural crest (Fig. 1b, c). With this method, however, it could not be determined which part of the ectoderm formed the deep part of the neural crest. The boundary between the neural plate and the neural fold was stained by an injection of Nile blue solution to determine the presumed area for the deep part of the neural crest; the result is shown in Figure 1h. Also, the primordium of the neural crest in the ectoderm of each stage is summarized in Figure 1i based on the results of the two vital stainings. It was located in the lateral part of the neural plate and two-thirds of the medial part of the neural fold in the early neural stage.

When the neural fold elevated in the lateral part of the dorsal ectoderm, the primordium of the neural crest occupied almost its entire fold, but the lateral face of the

![Fig. 3. Fine structure of the primordial neural crest cell in the early stage. I India ink particles, P pigment granule, L lipid droplet, Y yolk granule. × 27,000](image-url)
Fig. 4. The area and cell construction of the neural crest during its morphogenesis. 

The neural crest area and its primordium. 

: The transitional area between the primordia of the neural crest and of the epidermal ectoderm. 

: The transitional area between the primordia of the neural crest and of the neural tube. Each area shown in a-d, was determined from the results of two vital staining methods and in d-e was inferred from the observations of the cell construction. The arrow indicates the extent of the neural fold.
Fig. 5. The cell construction of the neural crest at different stages as observed by scanning electron microscopy. The stages of larvae correspond to those shown in Figure 4.
neural fold was covered with the primordium of the epidermal ectoderm. Throughout the fusion of both neural folds, the two primordial neural crests derived from both folds also fused. These were located between the epidermal ectoderm and neural tube and formed a cell mass. During this process, the stained area remained on one side of the neural fold, but it could not be observed in a cell mass as a neural crest.

**India ink labeling**

The distribution of India ink particles immediately after ink injection into the primordial neural crest region during the fusing process of both neural folds was investigated. The movement of labeled neural crest cells in the neurulation was traced.

In the stage immediately after ink injection into the neural fold region, India ink particles were spread out among primordial neural crest cells in the injected neural fold (Fig. 2a), but they were not found in the intercellular spaces on the other side of the neural fold. Soon after, they appeared as several particles of variable size in the cytoplasm of primordial neural crest cells. The residual particles in the intercellular spaces gradually disappeared by the time of the fusion of both neural folds.

No malformation was found in the injected larvae after metamorphosis, and India ink particles were retained in pigment cells and the neurons and mantle cells of the spinal ganglion.

India ink dropped on a collodion membrane was observed by transmission electron microscopy as an aggregation of particles of 0.05-0.01 μm in diameter. The India ink particles contained in neural crest cells formed an aggregation of particles of the same dimension (Fig. 3).

**Cytoarchitecture and fine structure of neural crest cells**

At the early neural stage, the primordium of the neural crest consisted of pseudostratified columnar epithelial cells (Fig. 4a, 5a), while the primordial neural tube was single columnar, and the epidermal ectoderm single cuboidal.

Glycogen granules, pigment granules, yolk granules, lipid droplets and free ribosomes were observed in primordial neural crest cells, but rough surfaced endoplasmic reticulum was not found (Fig. 3). Slight thickenings of plasma membrane appeared in the area near the top of the neural crest cells and dense materials were distributed in their cytoplasm beneath these thickening areas. These structures seem to be primitive junctional apparatuses (Fig. 6a). Lamellipodia were extended from their apical region and spread out on adjacent neural crest cells (Fig. 6a inset). A structure appearing like a junctional apparatus was also found in plasma membranes of cells consisting of the neural tube and the epidermal ectoderm.

During the movement of both neural folds to midline, many primordial neural crest cells maintained the arrangement of the pseudostratified columnar epithelium, and some neural crest cells changed to polygonal form, and were separated from the epithelial part of the primordial neural crest. At the same time the intercellular spaces among these cells expanded (Fig. 4c). The neural crest cells were labeled with India ink in this stage.

At the time of neural fold fusion on the dorsal midline, almost all neural crest cells changed from columnar epithelial cells to polygonal cells and formed a cell mass as a neural crest beneath the epidermal ectoderm (Fig. 4d, 5d). Some neural crest cells extended their cytoplasmic processes between the surface ectodermal cells. The transitional area from the neural crest to the neural tube was still composed of pseudostratified epithelium. At this time cells labeled with India ink were found on the same side of the neural crest cell mass.
Fig. 6. a. A junctional apparatus between two neural crest cells at the early neural stage. ×23,000. **Inset.** A lamellipodium in the apical region of a primordial neural crest cell at the same stage.  
b. A junctional apparatus is not found in these neural crest cells which contain India ink particles. This stage is consistent with Figures 4d₂ and 5d. ×17,000.  
c. A developed junctional apparatus in a neural tube cell at the same stage. ×50,000.  
d. A desmosome in the epidermal ectoderm at the same stage. ×61,000.
In the middle of stage 4, the neural crest clearly separated from the surface ectoderm, and the labeled cells were scattered in it (Fig. 2b). The ectoderm was removed during this stage, and the neural crest was observed with the scanning electron microscope. The aggregation of polygonal cells formed a ridge of the neural crest cranio-caudally (Fig. 7a). These cells extended some filopodia and lamellipodia to the neighboring cells (Fig. 7a inset). The junctional apparatuses were not found in the contacting faces of these cells (Fig. 6b). The plasma membranes of the neural tube cells were thickened in the apical part near the central canal and many microfilaments assembled under the membrane (Fig. 6c). These structures seem to be junctional apparatuses. Desmosomes appeared in the epidermal cells (Fig. 6d). Also in this stage, the basal lamina was observed on the basal face of epidermal and neural tube cells, but not between epidermal and neural crest cells, neural crest and neural tube cells nor around the remaining area of the neural crest.

In the second half of stage 4, the neural crest was also isolated from the neural tube, and its lateral edge extended to the upper edge of the somite by the beginning of the tail bud stage (Fig. 4e, 5e, 7b). During this period neural crest cells laterally situated in a cell mass projected their cytoplasmic processes between the epidermis and the somite or between the somite and the neural tube. The former processes were slender and extended to the somite (Fig. 8a), and the latter were thick like a club and were exactly on the lateral wall of the neural tube (Fig. 8b). The fine structure of these neural crest cells was almost the same as that of primordial neural crest cells in the neural fold.
DISCUSSION

Vital staining and India ink labeling methods were used to study the morphogenesis of the neural crest. It may be possible to define the area of the neural crest in its morphogenetic process from the fusion of the neural folds to the beginning of neural crest cell migration based on the observation of its cytoarchitecture as studied by Raven (1931) and Schroeder (1970). However, it is difficult to follow the arrangement of individual cells in the primordium of the neural crest from the appearance of the neural folds to their fusion in the dorsal midline without having any stable marker in these cells. During this process, the arrangement of cells consisting of the primordial neural crest gradually changed with the movement of the neural fold. In this study, the area of the primordial neural crest was determined in the neural plate with the vital stainings (Vogt, 1925), and cell composition during the morphogenesis of the neural crest was observed. It is especially necessary to continuously pursue neural crest cells during the period from their mass stage to their separation into individual cells. For this purpose the vital stainings of the primordial neural crest as a mass are inadequate, and marking of individual cells is essential.

Labeling of the cells with India ink was thus used in this study. The difficulty with this method is that only neural crest cells around the injection site are labeled. On the other hand, there are some advantages in this method: the India ink particles are contained unchanged for a long time in the cytoplasm of neural crest cells, and
their grouping and migration can be followed with ease. From the observation of the vital staining and cell construction, stage 3 and the beginning of stage 4 were determined as the most suitable for injection of India ink into the neural fold (Fig. 4). For this study, one side of the neural fold was injected to follow the migration of labeled cells in the formation of the neural crest at stage 3 before both folds fused.

These findings suggest that the primordium of the neural crest is situated in the lateral part of the thickening ectoderm, and the simple cuboidal epithelium adjacent to it represent the primordial epidermal ectoderm (Fig. 4). Raven (1931) called the contact area of both neural folds “Doppelmembran” in which simple cuboidal epithelium formed double sheets, and he concluded that the primordium of the neural crest was derived from this epithelium and the columnar epithelial cells under it, though without confirming this idea with the use of vital staining. From our vital stainings it was clear that the middle part of this epithelium formed the transitional area between the primordia of the neural crest and of the epidermal ectoderm. It seems that the neural crest cells are mainly derived from the lateral part of the neural plate in the early neural stage and the neural fold excluding its lateral part in the middle neural stage, as stated by Fautrez (Horstadius, 1950).

However, the vital staining methods could not in detail follow the movement of individual neural crest cells after stage 5. The labeling of individual cells was available for studying their movement from stage 5 to the beginning of their migration, and it was apparent that the individual cells derived from both neural folds were almost completely mixed in the neural crest mass. This process was confirmed by the disappearance of junctional apparatuses in the plasma membranes of polygonal neural crest cells. Their disappearance might make possible the mixture of these cells in the formation of the neural crest. In fact, their movement has not been shown in previous studies. Without observing the arrangement of individual cells derived from both neural folds in the period up to their migration, Raven (1936), Johnston (1966), Noden (1975) and Rosenquist (1981) reported that the neural crest cells derived from one side of neural folds migrated to spaces of another side between the epidermis and the somite or between the somite and the neural tube. Johnston (Weston, 1970) conjectured the fate of the primordial neural crest in the stage of its epithelial form, but his hypothesis seems incorrect because neural crest cells are mixed while the crest is formed.

Newgreen and Gibbins (1982) concluded that the junctional apparatuses of neural crest cells disappeared 5 hr before they began to migrate. From our observations, the time of their disappearance coincided with that of neural crest cell mixing. From that time to the beginning of their migration, neural crest cells contacted each other by their filopodia or lamellipodia as shown by Bancroft and Bellairs (1976), Löfberg and Ahlfors (1978), Tosney (1978) and Anderson and Meier (1981) (Fig. 7a inset). These findings suggested that the disappearance of the junctional apparatuses had no direct relation with the onset of cell migration from the neural crest, but represented only one of the preparatory changes for migration. The migration of neural crest cells seems to begin at the stage in which the neural crest spreads out laterally and the cells in the lateral part of the crest extend their cytoplasmic processes, one to the space between the epidermis and the somite, and another between the somite and the neural tube. These two processes appeared to be different forms. Bronner-Fraser and Cohen (1980b) suggested that the neural crest consisted of the same potential cells at the onset of their migration, but the difference in the two cytoplasmic processes suggests that their conclusion may deserve further examination.
REFERENCES

Anderson, C. B. and S. Meier: The influence of the metameric pattern in the mesoderm on migration of cranial neural crest cells in the chick embryo. Devel. Biol. 85: 385-402 (1981).

Baker, R. C. and G. O. Graves: The behavior of the neural crest in the forebrain region of amyl- stoma. J. com. Neurol. 71: 389-415 (1939).

Bancroft, M. and R. Bellairs: The neural crest cells of the trunk region of the chick embryo studied by SEM and TEM. Zoon 4: 73-85 (1976).

Bronner-Fraser, M. and A. M. Cohen: Analysis of the neural crest ventral pathway using injected tracer cells. Devel. Biol. 77: 130-141 (1980a).

———: The neural crest: What can it tell us about cell migration and determination? Curr. Topics level. Biol. 15: 1-25 (1980b).

Detwiler, S. R.: Observations upon the migration of neural crest cells, and upon the development of the spinal ganglia and vertebral arches in amylstoma. Amer. J. Anat. 61: 63-94 (1937).

Hörstadius, S.: The neural crest. Oxford Univ. Press, New York-London, 1950.

Johnston, M. C.: A radioautographic study of the migration and fate of cranial neural crest cells in the chick embryo. Anat. Rec. 156: 143-156 (1966).

Lamers, C. H. J., J. W. H. M. Rombout and L. P. M. Timmermans: An experimental study on neural crest migration in Barbus conchonius (Cyprinidae, Teleostei), with special reference to the origin of the enteroendocrine cells. J. Embryol. exp. Morphol. 62: 309-323 (1981).

Lane, B. P. and D. L. Europa: Differential staining of ultrathin sections of Epon-embedded tissues for light microscopy. J. Histochem. Cytochem. 13: 579-582 (1965).

Le Douarin, N. M. and M. A. Teillet: The migration of neural crest cells to the wall of the digestive tract in avian embryo. J. Embryol. exp. Morphol. 30: 31-48 (1973).

———: Experimental analysis of the migration and differentiation of neuroblasts of autonomic nervous system and of neurectodermal mesenchymal derivatives, using a biological marking technique. Devel. Biol. 41: 162-184 (1974).

Löfberg, J. and K. Ahlfors: Extracellular matrix organization and early neural crest cell migration in the axolotl embryo. Zoon 6: 87-101 (1978).

Newgreen, D. and I. Gibbins: Factors controlling the time of onset of the migration of neural crest cells in the fowl embryo. Cell Tiss. Res. 224: 145-160 (1982).

Noden, D. M.: An analysis of the migratory behavior of avian cephalic neural crest cells. Devel. Biol. 42: 106-130 (1975).

———: Interactions directing the migration and cytodifferentiation of avian neural crest cells. In: (ed. by) D. R. Garrod: Specificity of embryological interactions. Chapman and Hall, London, 1978 (p. 4-49).

Pratt, R. M., M. A. Larsen and M. C. Johnston: Migration of cranial neural crest cells in a cell-free hyaluronate-rich matrix. Devel. Biol. 44: 298-305 (1975).

Raven, C. P.: Zur Entwicklung der Ganglienleiste. I. Die Kinematik der Ganglienleistenentwicklung bei den Urodelen. Roux Arch. 125: 210-292 (1931).

———: Zur Entwicklung der Ganglienleiste. V. Über die Differenzierung des Rumpfganglienleistenmaterials. Roux Arch. 134: 122-146 (1936).

Rosenquist, G. C.: Epiblast origin and early migration of neural crest cells in the chick embryo. Devel. Biol. 87: 201-211 (1981).

Schroeder, T. E.: Neurulation in Xenopus laevis. An analysis and model based upon light and electron microscopy. J. Embryol. exp. Morphol. 23: 427-462 (1970).

Takahashi, H. and T. Yamadori: Electron microscope study on the contact of neural crest cells in the early stage of migration in bantam embryo. Arch. histol. jap. 42: 551-562 (1979).

Tosney, K. W.: The early migration of neural crest cells in the trunk region of the avian embryo: An electron microscopic study. Devel. Biol. 62: 317-333 (1978).

———: The segregation and early migration of cranial neural crest cells in the avian embryo. Devel. Biol. 89: 13-24 (1982).

Vogt, W.: Gestaltungsanalyse am Amphibienkeim mit örtlicher Vitalfärbung. Vorwort über Wege
and Ziele. I. Teil. Methodik und Wirkungsweise der örtlichen Vitalfärbung mit Agar als Farbträger. Roux Arch. 106: 542-610 (1925).

Weston, J. A.: The migration and differentiation of neural crest cells. Adv. Morphogenes. 8: 41-114 (1970).

Weston, J. A., J. E. Pinter, M. A. Derby and D. H. Nichols: The morphogenesis of spinal ganglia from neural crest cells. In: (ed. by) Z. W. Hall, R. Kelly and C. F. Fox: Cellular neurobiology. Alan R. Liss, Inc., New York, 1977, (p. 217-226).

平野茂樹
〒990-23 山形市藤原町字向の前
山形大学医学部
第二解剖学教室

Dr. Shigeki Hirano
Department of Anatomy
Yamagata University School of Medicine
Yamagata, 990-23 Japan