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What insights into the phenomena of cell fate determination and cell migration has the study of the urodele neural crest provided?

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ABSTRACT In this review we ask whether studies on the development of the urodele neural crest (NC) have provided special insights into the fate and migration of these cells when compared to other amphibian embryos or those of higher vertebrates. We recognize that during the first half of this century and even before, urodele embryos were the favorite objects of experimental embryology for studying the development of mesenchymal derivatives and their participation, together with mesodermal mesenchyme, in the development of the neuro- and viscerocranium. Furthermore, the NC was discovered to be the source of cranial sensory and spinal ganglia, and the influence of the somites on the localization of the latter was clearly pointed out. In addition, pioneering studies were devoted to the NC-derived pigment cells. Investigations in this field concentrated on their migration in the embryo and *in vitro*, and on the mechanisms underlying larval pigment pattern formation. It is mainly in these three areas that the urodele embryo has served as a tool for gaining major results and defining the concepts of classical embryology. Even today, when the interest has shifted towards the molecular biology in *Xenopus*, chicks and mice, the urodele embryo with its large cells, convenient for injections, is a potential model for future lineage studies and knockout experiments. And furthermore, as important concepts of vertebrate development are defined in the urodele, future studies in these embryos may link the disciplines of development and evolution.

KEY WORDS: *Urodele amphibians, neural crest, migration, differentiation*

Introduction

The neural crest (NC) represents a transient embryonic structure specific to vertebrates. It consists of a cord of ectomesenchymal cells lying on top of the neural tube. From this site, NC cells migrate into various body regions and give rise to neurons and glial cells of the peripheral nervous system, connective and supportive tissue, pigment cells and a variety of other structures (Weston, 1970; Le Douarin, 1982; Hall and Hörstadius, 1988).

In this review we ask what insights into early embryonic processes, such as cell migration and cell differentiation, have emerged from studies of the NC in urodele amphibians. The urodele species used for experiments are mainly those of the genus *Taricha* (Californian newt), *Triturus* (the European newt) and *Ambystoma* (the Mexican axolotl). The development of these species has been staged in normal tables (*Taricha torosa*: Twitty and Bodenstein, 1962; *Triturus alpestris*: Epperlein and Junginger, 1982; *Ambystoma mexicanum*: Bordzilovskaya *et al.*, 1989).

It is certainly naive to assume that the NC of a particular genus of vertebrates could supply us with major knowledge about the developmental processes of NC cells. Research on embryos within the different classes of vertebrates, whether mammals, birds or amphibians, is so much intermingled and the biological processes are so similar that certain insights and results are often obtained only by comparative observations. To a lesser extent we will be discussing observations of that type. A major part, however, includes experiments which were designed for and carried out only in urodeles, either because this order has served traditionally as an experimental system or because it provides unique advantages over other systems.

Among the experiments first carried out in urodeles were those on the migration and differentiation of the cranial NC and on the formation of cranial ganglia in *Ambystoma punctatum* (Stone, 1926; *punctatum* is *maculatum* today), and those on the three-dimensional reconstruction of the head skeleton (Stone, 1926; Raven, 1931). The first fate map for NC cells was designed in a urodele neurula

Abbreviations used in this paper: NC, neural crest; CSPG, chondroitin sulfate proteoglycan; GFAP, glial fibrillar acidic protein; GAG, glycosaminoglycans; LSM, laser scanning microscope; BM, basement membrane.

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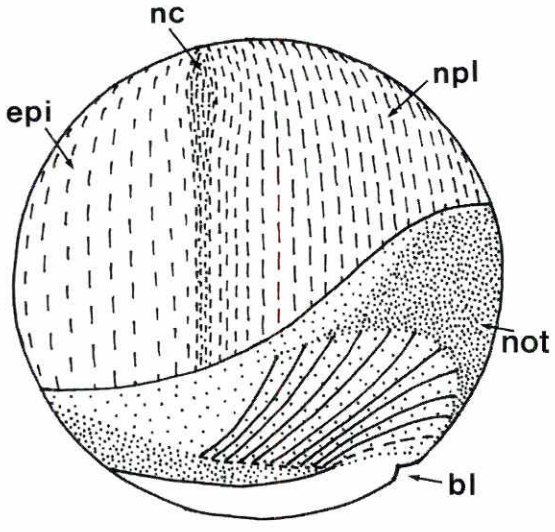


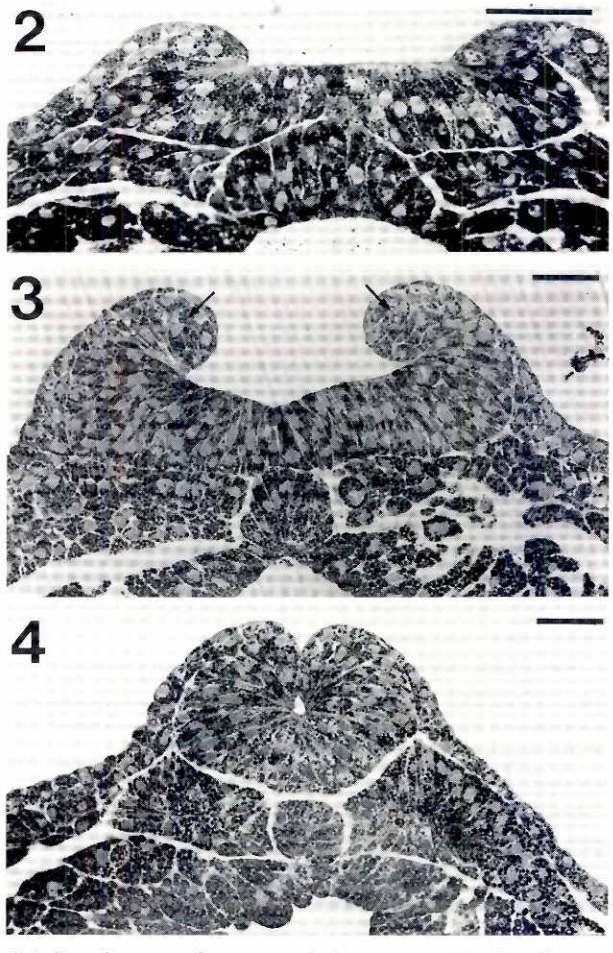
Fig. 1. Localization of the prospective neural crest (nc) in a urodele gastrula. *Using vital dyes, the NC has been traced back to this stage. It is situated between the prospective epidermis (epi) and the prospective neural plate (npl). not, notochord; bl, blastopore. Redrawn after Vogt (1929).*

(Hörstadius and Sellman, 1946), and among the first amphibian NC cells in culture were those of *Ambystoma* (Twitty and Bodenstein, 1939; Twitty and Niu, 1948). Apart from this direct involvement of urodele embryos in certain aspects of NC cell research, embryological research generally during the first half of this century was directed towards amphibians. Leading embryologists worked with amphibians: Harrison in the United States in the field of experimental neurogenesis and tissue culture; Spemann in Germany on early organ determination (Hamburger, 1988).

Among those urodele NC derivatives which have attracted special interest are the pigment cells. They have been studied within a long tradition (Twitty, 1936, 1966; Epperlein and Löfberg, 1990) and offer many advantages over pigment cells of chickens and mice. Whereas pigment cells in the latter embryos consist of only the melanocytes, which are often outwardly invisible or visible only with difficulty, the pigment cells in amphibian embryos comprise three different cell types, melanophores, xanthophores and iridophores, which are clearly visible from outside (see Frost-Mason and Mason in this issue). In urodeles, pigment cells are mainly used for studying cell differentiation, cell-matrix interactions and pigment pattern development. Their arrangement in various pigment patterns serves as a model system for studying mechanisms of NC cell differentiation and distribution. These mechanisms could be valid also for other types of pattern, such as the visceral arches formed by NC-derived chondrocytes or the chain of dorsal root ganglia formed by NC-derived neurons and glial cells. These latter patterns develop within the embryo, thus excluding direct observation, but we assume they are subject to similar morphogenetic principles as pigment cells during pigment pattern formation.

Cell fate determination

One of the most important questions for the genetic and developmental analysis of the NC relates to its lineage and differentiation into various derivatives. *Is the NC already set apart in one defined blastomere, or is it mixed from the material of several blastomeres and present only at later stages, during gastrulation or neurulation?* The only experiments tracing urodele NC cells back earlier than the neurula stage were done by Vogt (1929). Applying vital dyes (externally) to the early gastrula, he defined the prospective NC as a narrow stripe of ectodermal cells between the prospective epidermis and the prospective neural plate (Fig. 1). In the urodele embryo, NC cells were thought to be induced by the lateral margins of the chordamesoderm (Raven and Kloos, 1945), i.e., at the margin of neuralizing and epidermalizing influences (Rollhäuser-ter-Horst, 1977, 1979; Moury and Jacobson, 1989). NC cells in *Ambystoma mexicanum* were shown to develop from both epidermal and neural plate material (Moury and Jacobson, 1990). Before the neural folds become elevated, their inner margins are delineated by a greater number of pigment granules (of maternal origin). Already at this stage the prospective neural fold contains NC material. Still later, the NC cells are located in the apical part of the elevated folds (Figs. 2-4). The identification of NC cells within the folds based solely on morphological criteria is



Figs. 2-4. Development from neural plate to neural tube. *Transverse sections through neurula stages (prospective midtrunk region) of Ambystoma mexicanum. Early (stage 14; **2**), middle (stage 16; **3**) and late neurula (stage 18; **4**). Prospective NC material is contained in the apical neural folds (arrows) but cannot be identified solely on morphological criteria. Bars, 100 μm*

difficult, as these cells are in close proximity to the prospective neural plate and epidermis. In all the "crest" cultures or "crest" transplants of early embryologists, an amount of epidermal and neural plate tissue was probably present, in addition to NC cells. The accompanying tissues in these fold explants, however, may have had a profound influence on the determination and migration of NC cells when studying "autonomous" differentiation of NC cells *in vitro* or at a heterotopic site in the embryo. For example, a piece of epidermis differentiating in an "NC" culture may have attracted pigment cells and supported their differentiation.

Resolving the issue of when the NC first emerges will require suitable external or internal markers for undifferentiated NC cells or for NC derivatives. In a classic experiment in urodele embryos Raven (1933) transplanted prospective NC material (as delineated by Vogt) from one early gastrula into the prospective epidermal area of another and found no indication of differentiation. The transplant merely formed epidermis. If, however, a piece of head neural fold was transplanted into the prospective trunk epidermal area of another neurula, it developed into neuronal, cartilage and pigment cells (Raven, 1933). In these experiments no particular tracer but the potency of naturally differentiating NC cells was used as criterion. It is possible that the delineation of the prospective NC in the early gastrula may have been inexact (because of the use of *diffusible* vital dyes in Vogt's experiments), and the NC cells may have been present much earlier than in the gastrula. Intracellular injections into single blastomeres with a lineage tracer (horseradish peroxidase), as first applied in *Xenopus* by Moody (1987a,b; Moody and Kline, 1990), would therefore be useful in urodele embryos. Moody had shown that NC cells derive from certain blastomeres in 16- or 32-cell stage embryos. Unfortunately, attempts to localize NC cells or NC-derived neurons in the neurula or at subsequent stages with modern immunohistochemical markers (e.g., antibodies against HNK-l, cadherins, neurofilaments), mostly fail in urodeles (in contrast to the chick) because of the absence of immunological cross-reactivity of the antibodies. In later stages, however, when the NC cells are already determined or covertly differentiated, several histo- and immunohistochemical markers are effective for staining the urodele NC. They are tyrosinase (shown with the dopa-test in melanophores), pterins (shown with the pterin-fluorescence in xanthophores), chondroitin-sulfate proteoglycan (CSPG; chondrocytes), tyrosine hydroxylase (sympathetic ganglia), tubulin (spinal ganglia) and glial fibrillar acidic protein (GFAP; glial cells). Applications of these markers will be mentioned below in the sections on differentiated NC cells.

How is the NC lineage established in the urodele?

Lineage analysis in urodeles or amphibians in general is not as advanced as in the chick. Many fewer experiments have been performed, particularly in culture, and the differentiating conditions of sensory, sympathetic or enteric neurons or the glial and neuronal cell relationship are largely unexplored. Following our own observations in *Triturus* and *Ambystoma* and a lineage model for the chick (Weston, 1991), we assume that the prospective connective and supportive tissues are first segregated from the cranial NC. Neurogenic precursors (sensory and autonomic ganglia) and non-neurogenic elements (glial and pigment cells) are then generated from the trunk NC. Pigment cells are also derived from the head NC, but to a lesser degree. The mesenchyme from the trunk NC develops into the connective tissue cells of the dorsal fin (Stone, 1926; Raven, 1931; Hörstadius and Sellman, 1946; Bodenstein, 1952). Trunk crest cells cannot migrate out or give rise to cartilage when transplanted into a head neural fold position and head neural fold cannot migrate or form cartilage in the trunk (Hörstadius and Sellman, 1946). This finding was recently refuted (Selleck and Epperlein, 1996).

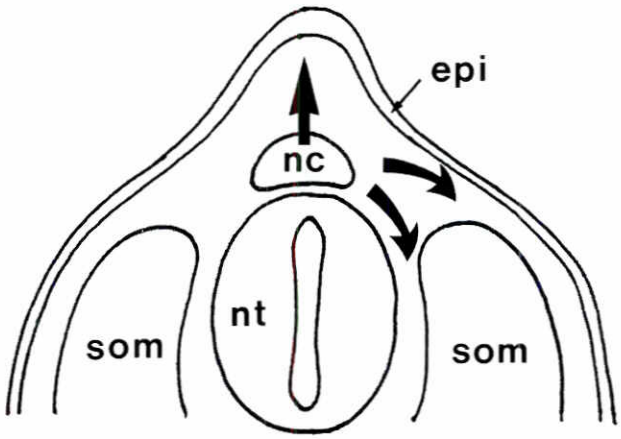


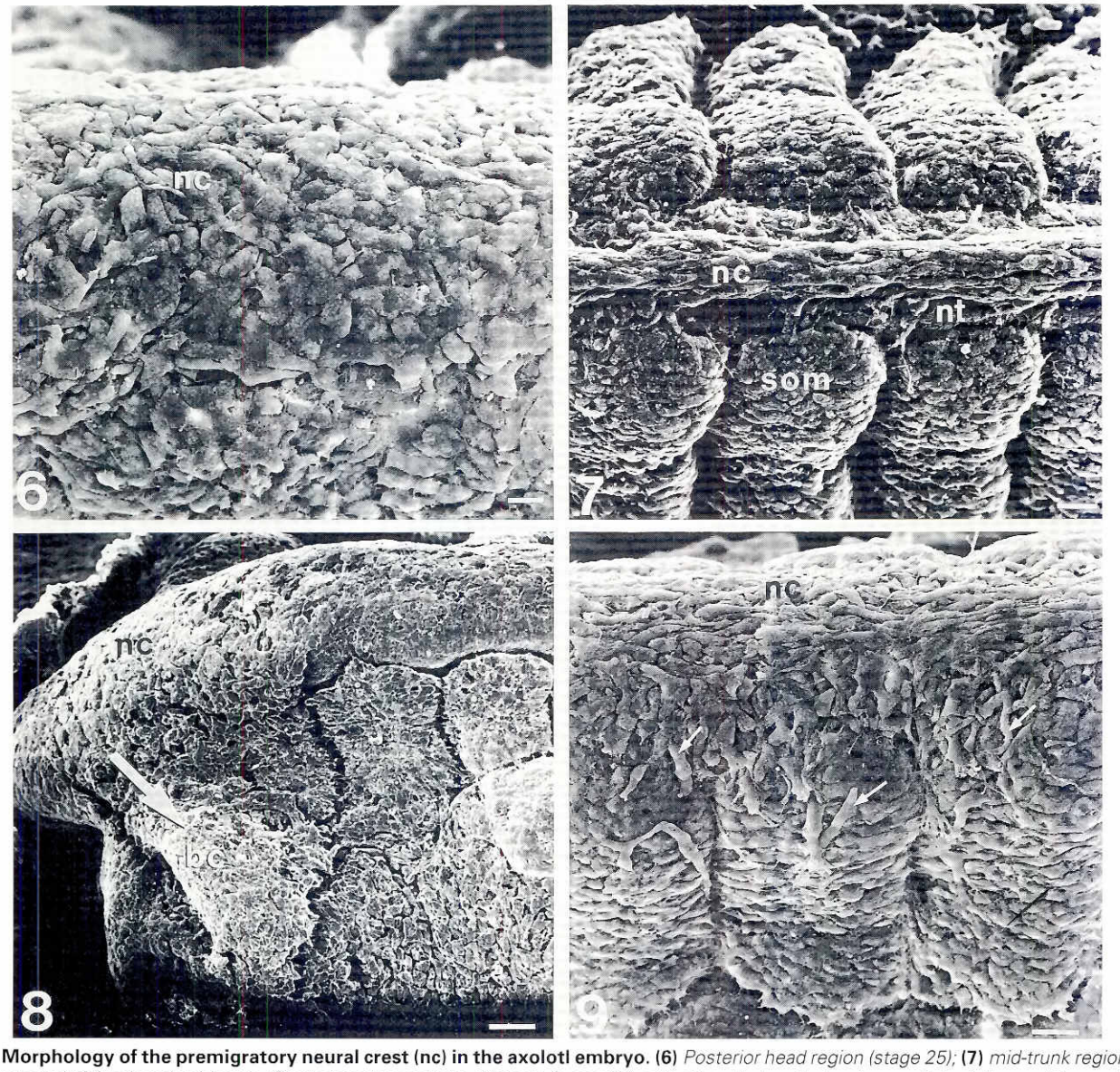
Fig. 5. Migratory routes of NC cells in the trunk of a urodele embryo (premigratory crest stage; schematic representation of a transverse section). *The routes are directed dorsolaterally (between somites, som, and epidermis, epi), ventromedially (between the neural tube, nt, and somites, som) and dorsally (into the developing dorsal fin). Redrawn from Epperlein and Löfberg, 1990.*

How is determination/differentiation of the NC correlated with migration?

As a rule, undifferentiated NC cells migrate on defined routes (Fig. 5) until they settle and differentiate in a specific body region. This is true, for example, for the ganglia of the peripheral nervous system. Pigment cells in the axolotl embryo, however, may be determined and may differentiate in their premigratory position on the neural tube. This is particularly obvious in the white axolotl mutant, where the pigment cells do not migrate out under the epidermis. Thus, certain NC cells may be pluripotent and differentiate on a specific migratory route or at a final site of location. Alternatively, subsets of NC cells may be determined or may already differentiate in the premigratory position of the NC and migrate as differentiated cells, like the pigment cells.

Cell migration patterns

When the neural folds fuse into the neural tube, their apical parts join and give rise to the NC, which is then expelled to a site on the dorsal surface of the neural tube. The structure of the premigratory crest varies depending on its position in the head or trunk of the embryo or the species investigated. On the head neural tube of both *Triturus* and axolotl embryos, the NC cells form a flat epithelial-like sheet (Fig. 6) prior to or at early migratory stages. In the trunk of *Triturus*, the NC is also a flat epithelium-like layer, but in the trunk of the axolotl it forms a multilayered cellular string (Fig. 7). In the epithelial arrangement, NC cells have an irregular shape, whereas in the cellular string they are elongated spindle-shaped cells with their anterior-posterior axes parallel to the neural tube. In contrast to *Xenopus* or the chick, where some NC cells may start

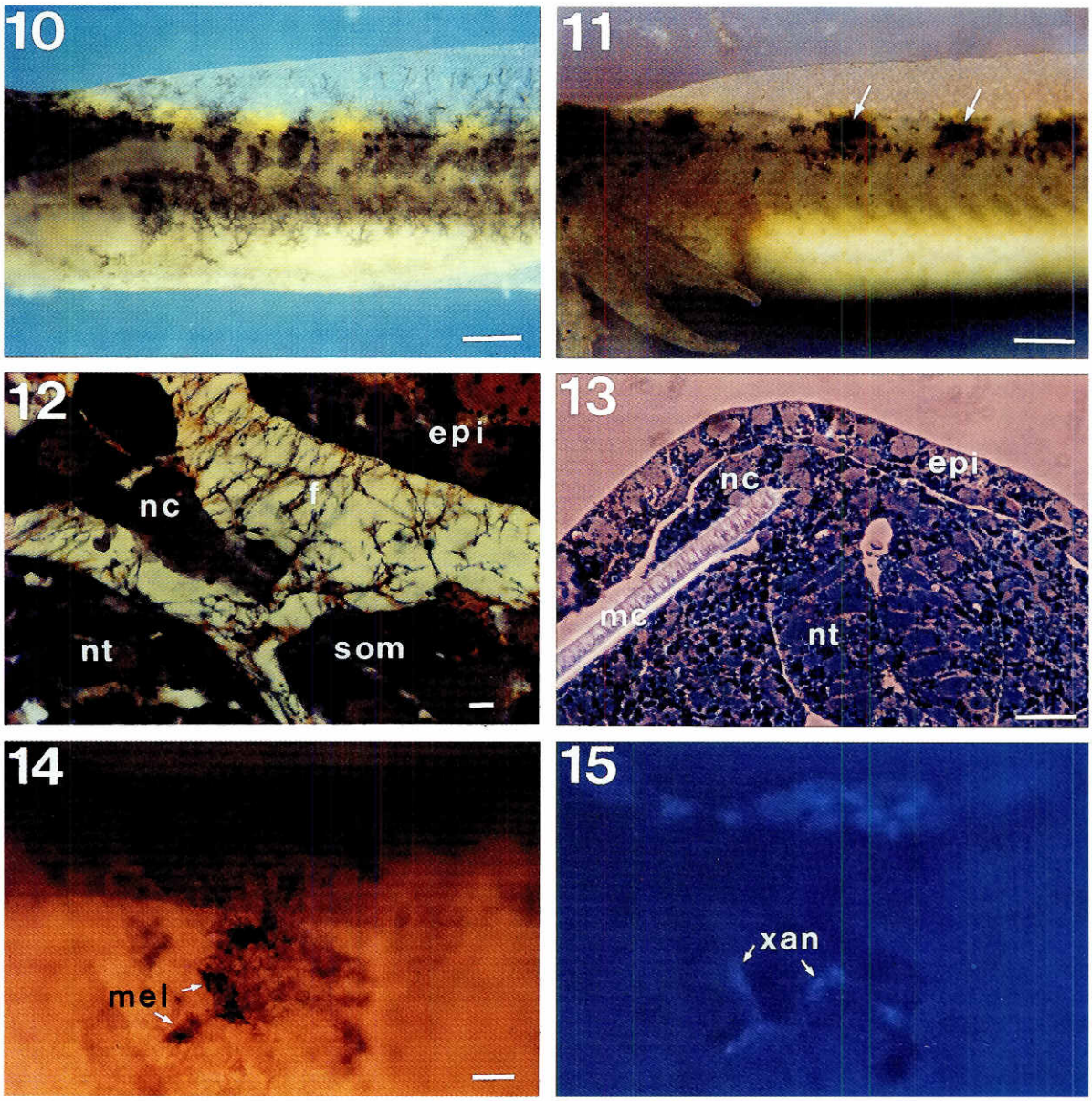


Figs. 6-7. Morphology of the premigratory neural crest (nc) in the axolotl embryo. (6) *Posterior head region (stage 25);* **(7)** *mid-trunk region (stage 30), after removal of the dorsal epidermis. On the head neural tube, NC cells form a flat epithelium, but on the trunk neural tube (nt), a multilayered cellular string. som, somite. Bars: (6) 25 μm; (7) 50 μm.*

Figs. 8-9. Lateral migration of NC cells in the axolotl embryo. *In the head region (stage 25;* **8***), the NC forms massive cellular tongues (e.g. the branchial crest, bc); in the trunk (stage 32;* **9***), migration occurs as single cells (arrows). Bars: (8) 100 μm; (9) 50 μm.*

migration before the folds are fused, NC cells in the amphibian species mentioned here only start to migrate after a definite crest has formed. Lateral migration of these cells commences in a cranio-caudal wave. In the head, they form massive cellular tongues (mandibular, hyoid and branchial crest; Fig. 8), whereas in the trunk they migrate as single cells (Fig. 9). In the trunk, NC cells orient their long axes dorsoventrally when starting to migrate laterally over the dorsolateral portion of the neural tube. During advanced migration in the axolotl (stage 35), several elevated NC aggregates occur at irregular distances along the trunk neural tube (see Fig. 28). Such aggregates have only been found in axolotl embryos and in those of other ambystomatids (Epperlein and Löfberg, 1984, 1990; Olsson and Löfberg, 1992; Olsson, 1994; Epperlein *et al.*, 1995). The aggregates form a prepattern for the larval pigment pattern and will be dealt with later. One could speculate that the differential migration of NC cells from the neural tube (cell streams in the head versus single cells in the trunk) may be under the control of *hox* gene expression (Prince and Lumsden, 1994).

When reaching the apex of the somites, the NC cells either continue to migrate laterally between epidermis and somites, or they turn medially, between neural tube and somites (Fig. 5). Those cells using the lateral route are already determined or differentiated; they are pigment cell precursors or pigment cells. Those migrating ventromedially are undifferentiated and become chromaffin cells or neuronal and glial cells of the peripheral nervous system. A third migratory route runs into the dorsal fin, where trunk NC cells form loose connective tissue.



Figs. 10-15. Normal and experimentally stimulated migration of NC cells and NC- derived pigment cells in axolotl embryos. (10) *Dark (wild-type) larva (stage 41; head to the left outside) showing the barred pigment pattern with alternating transverse bands of black melanophores and yellow xanthophores. Xanthophores are still confined to the region on and above the dorsal neural tube, whereas melanophores become distributed in the lateral trunk and dorsal fin as well.* **(11)** *White mutant larva (stage 41) in which the pigment cells cannot migrate laterally and dorsally because of a defective subepidermal ECM. They remain confined to their original position on the dorsal neural tube, where they form mixed chromatophore groups (arrows).* **(12)** *Normal NC cell migration showing a NC cell (nc) at the apex of the somites (som) in a transverse section through the trunk of an axolotl embryo (stage 34). ECM fibrils (f) consisting mainly of collagen and glycosaminoglycans are enhanced with ruthenium red staining. Nt, neural tube; epi, epidermis.* **(13)** *Stimulation of NC cell migration in a white embryo (stage 30). A stream of NC cells (nc) is observed on a microcarrier (mc, consisting of nuclepore filter material) which was preconditioned with subepidermal ECM from a dark donor. For conditioning, the microcarrier was implanted in a dark embryo at stage 25 and removed at stage 30. It was then inserted in the white host at stage 25. nt, neural tube; epi, epidermis.* **(14, 15)** *Stimulation of NC migration in a white embryo (same part of anterior trunk region in both figures; stage 35). A microcarrier containing dorsolateral subepidermal ECM from a dark donor (implanted at stage 25, explanted at stage 30) had been inserted under the epidermis of the white host embryo (stage 25) close to the NC. Prospective melanophores (mel) were made visible with the dopa reaction* **(14)** *and xanthophores (xan) with pterin fluorescence* **(15).** *The outlines of the microcarrier can be seen in* **14**. *Bars: (10,11) 0.5 mm; (12) 10 μm; (13) 100 μm; (14,15) 100 μm.*

What is the evidence for NC cell migration and what, in particular, have urodele embryos contributed? Apart from the pigment cells, which are externally visible, all other NC derivatives can be observed only with difficulty, as they have no external marker and disappear either on the dorsal or ventromedial pathways. It is mainly for this reason that special markers have to be found for these cells. The markers are applied directly (intracellular dye injections) or used for labeling the tissue in intra- or interspecific

grafting experiments. The markers include, for example, cellular or nuclear size, vital dyes, radioactive precursors, chromatin differences, retroviral infection or fluorescent tracers (for review see Epperlein and Löfberg, 1990, 1993; Epperlein *et al.*, 1995). In urodeles, markers more reliable than nuclear size or vital dyes (used in the 1930s) have been applied by Chibon (1967) and Vogel and Model (1977) who used radioactive precursors. With this method the routes of NC migration in the head and trunk of the embryos and the major NC derivatives could be ascertained. More recently, a relatively stable fluorescent cell membrane marker (PKH 26; Horan and Slezak, 1989), has been used to stain NC cells in the axolotl (Epperlein *et al.*, 1995). With this marker, the main routes of NC cell migration could be confirmed. As the dye is too diffusely distributed around the cells, problems concerning the distribution and progeny of single cells can be solved only by single cell injections. Previously, this technique had, in amphibians, been successfully applied only to *Xenopus* embryos using fluorescent dextran (Collazo *et al.*, 1993). This method should be tested in the axolotl, where the NC cells are much larger and the embryonic development much slower (more experiments possible at a certain stage). A variety of problems may be solved, such as the different modalities of crest migration in the head and trunk, for example.

The mechanisms which operate when NC cells lose their contact with the neural tube and develop new specific affinities for the different migratory routes are largely unknown. *Do the NC cells initiate their own migration or are they stimulated by the embryonic environment, or are both mechanisms involved?* Classical and more recent explanatory concepts include negative chemotaxis (Twitty, 1944), contact inhibition (Abercrombie and Heaysman, 1954; Abercrombie, 1961), contact guidance (Weiss, 1945, 1961), haptotaxis (Carter, 1965) or spatial restrictions (Newgreen, 1989). Although useful in part, none of these concepts can provide a sufficiently unifying explanation for the adhesive changes and the directional migration of NC cells occurring at the turning point from premigratory to migratory stages.

According to more recent experimental results in dark and white axolotl embryos (Figs. 10, 11), the dorsolateral migration of NC cells is stimulated by the extracellular matrix (ECM) of the dorsolateral epidermis (Fig. 12). If microcarriers are loaded *in vivo* with subepidermal ECM of dark embryos and transplanted to younger dark or white embryos (Löfberg *et al.*, 1985, 1989a), dorsolateral NC cell migration is stimulated (Fig. 13) during a limited period of development. This result was particularly surprising in the white mutant, where NC cells do not normally leave their position on the dorsal neural tube and migrate laterally. Using special histochemical methods for revealing pigment cells (dopa test for melanophores and pterin fluorescence for xanthophores), NC cells (or propigment cells) migrating laterally could be demonstrated prior to their visible differentiation on a microcarrier containing subepidermal ECM from a dark donor (Figs. 14, 15).

In another set of experiments, differences between the composition of the ECM of the lateral and ventromedial NC route in dark embryos were shown to be responsible for the different fate of NC cells (Perris *et al.*, 1988). In the experiments, pure populations of premigratory NC cells were confronted either with subepidermal ECM *in vitro* and developed into pigment cells, or they were grown on ventromedial matrix and gave rise to neuronal cells (Fig. 16).

The structure and composition of the subepidermal ECM in dark and white embryos have been investigated with various methods. These include morphology (TEM, SEM with conventional and cryofixation), immunohistochemistry (various antibodies against ECM components: glycosaminoglycans (GAG), proteoglycans and glycoproteins) and biochemistry (one- and two-dimensional gel electrophoresis; see Epperlein and Löfberg, 1990, 1993; Perris *et al.*, 1990). A variety of differences between the ECMs of dark and white embryos could be revealed. However, the identification of a particular component as being mainly responsible for the differences in the migratory behavior of NC cells has not been achieved. It may be questioned whether major differences exist at all between the two subepidermal ECMs.

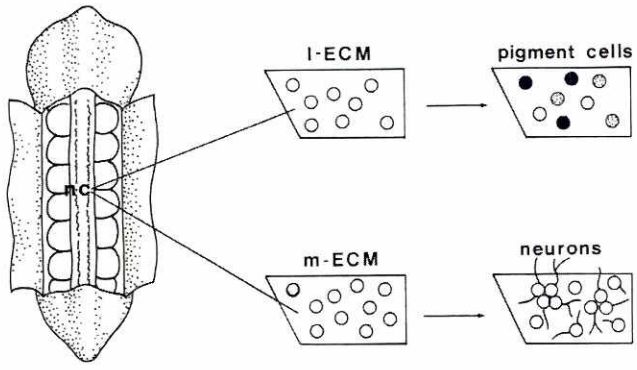
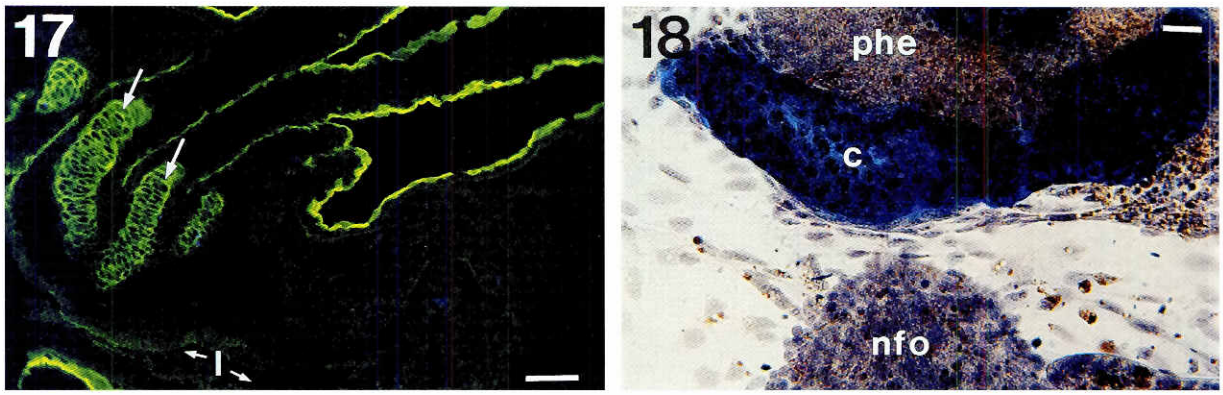


Fig. 16. The effect of different ECMs on the fate of axolotl NC cells. *Premigratory NC cells from an axolotl embryo (stage 25; left) were seeded on microcarriers preincubated in a dark embryo (stage 25) with dorsolateral (subepidermal) ECM (l-ECM) or with ventromedial ECM (between neural tube and somites; m-ECM). On l-ECM, some NC cells differentiated into pigment cells (melanophores, filled circles; xanthophores, dotted circles); on m-ECM, some NC cells differentiated into neurons (cells with processes). Undifferentiated cells, open circles. Redrawn after Perris et al. (1988).*

Experimental evidence suggested that the white ECM may be only transiently defective (Löfberg *et al.*, 1989b), since older white ECM can also stimulate NC cell migration in younger white hosts. Therefore, the ideal ECM component responsible for the stimulation of NC cell migration in the axolotl embryo should be demonstrated first in the wild-type and, a few stages later, in the white mutant. To complete this approach, however, the loss of cell surface receptors in NC cells for this component must be demonstrated, as the NC cells in the white mutant are capable of migration only during a limited period.

Mesenchymal derivatives from the neural crest

The head NC in amphibians (and also in higher vertebrates) contributes to large parts of the connective tissue and skeletal elements in the neuro- and viscerocranium. This was first postulated for urodeles by Platt (1893) and was later confirmed (Landacre, 1921; Stone, 1922, 1926; Hörstadius and Sellman, 1946; de Beer, 1947). Because of this potency, the NC was first denoted as "mesectoderm." Today the term "ectomesenchyme" is used more often (see Le Douarin, 1982). In fact, it took nearly 30 years to recognize the NC as a source of mesenchymal cells as first suggested by Platt (1893). Usually the mesenchyme is derived from mesoderm and develops, for instance, into fibroblasts, myoblasts or chondroblasts. Since the crest gives rise, apart from mesenchyme, to a variety of non-mesenchymal derivatives such as neurons and pigment cells, a terminological difficulty arises if the



Figs. 17-18. Development of visceral cartilage in urodele embryos. (17) *Frontal cryostat section through the gill region of a 15 mm axolotl larva stained immunohistochemically with anti 6-CSPG (positive reaction of the cartilage matrix, arrow). l, lumen of foregut.* **(18)** *In vitro, visceral cartilage (c; stained with Alcian Blue) develops from head NC cells leaving a head neural fold explant (nfo) and establishing contact with a fragment of pharyngeal endoderm (phe). Bars: (17) 100 μm; (18) 30 μm.*

term "ectomesenchyme" is extended to include the entire NC epithelium, which is often done. *It would be better, therefore, if the NC were to be regarded as a blastema.*

A prognostication of the eight arbitrary sectors of the head neural folds in the urodele embryo was achieved by the fate map of Hörstadius and Sellman (1946), which was based on a great number of transplantation, extirpation and vital dye labeling experiments. Later this fate map was improved by the more detailed studies of Chibon (1967) using radioactive labels in *Pleurodeles* (another urodele). From these studies it became clear that the neurocranium is derived from mesenchyme of both mesodermal and NC origin, whereas the mesenchymal derivatives of the viscerocranium consist solely of NC material.

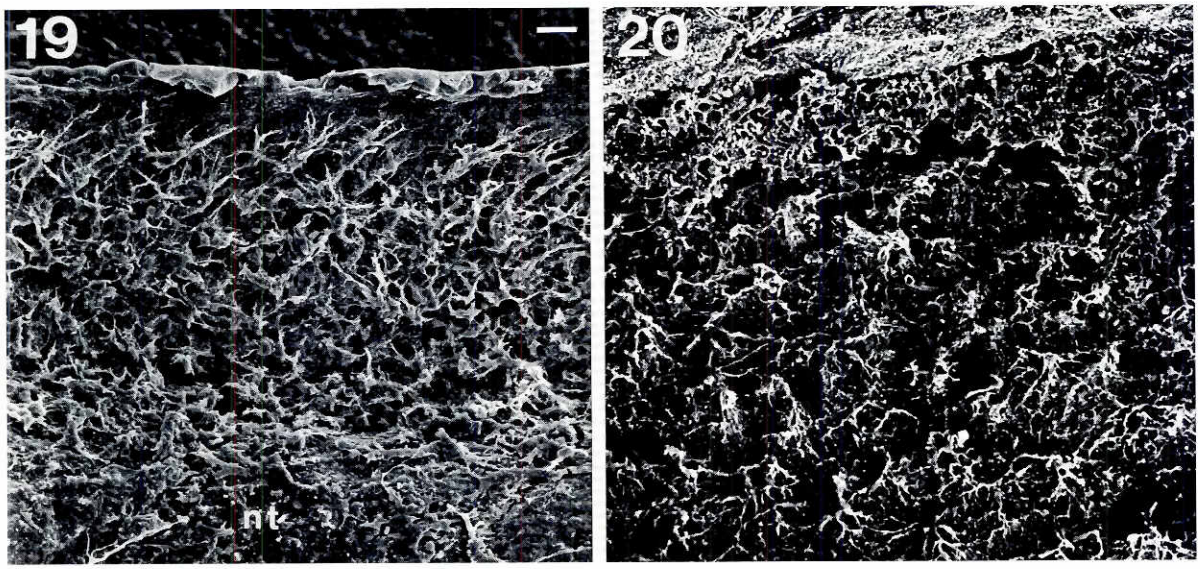
As shown for *Ambystoma*, NC cells also give rise to odontoblasts (Stone, 1926; Raven 1931). In xenoplastic transplantations between axolotl and *Triturus* embryos, Raven (1935) found axolotl NC cells in tooth papillae of *Triturus* hosts. Sellman (1946) has demonstrated that three factors are necessary for the development of the urodele tooth: the foregut endoderm, oral ectoderm and NC mesenchyme. Since anurans do not develop teeth, urodele embryos could be particularly suitable for studying the interactions of these three factors at a molecular level.

The investigation of visceral cartilage development *in vivo* is difficult, as the dynamics of the process cannot be followed and as suitable immunohistochemical markers for chondrocytes were either unavailable for a long time or did not react with urodele tissues. In Figure 17, the differentiation of NC cells into visceral arches is shown by using an antibody against CSPG, a component of the ECM surrounding chondrocytes. That the presence of pharyngeal endoderm is necessary to induce the formation of cartilage from NC cells has been demonstrated in organ culture (Okada, 1955; Wilde, 1955) and tissue culture (Holtfreter, 1968; Drews *et al.*, 1972; Epperlein, 1974; Epperlein and Lehmann, 1975). In tissue culture, particularly, some of the conditions of cartilage formation could be explained. If a head neural fold explant is cultured at a short distance from an explant of pharyngeal endoderm, NC cells may migrate from the neural fold explant towards the endoderm and make contact with it. However, directed NC cell migration towards the endoderm (based on chemotactic affinity or a gradient) was not observed, nor did chondrocytes develop from NC cells having no contact with the endoderm. Differentiation of cartilage was accompanied by changes in the adhesion of NC cells. Some of those that had previously been randomly distributed in the culture system lost their motility and condensed when contacting the inducing endodermal tissue (Fig. 18).

In the urodele trunk, NC cells also give rise to mesenchymal derivatives which form the connective tissue of the dorsal fin (Raven, 1931; DuShane, 1934, 1935). Fin formation seems to depend on an inductive interaction between the NC and the dorsal epidermis (Bodenstein, 1952). NC cells apparently first induce the epidermis to start fin development. In a second step, the epidermis may act as a reciprocal inductor and assign to the NC cells their future differentiation into the mesenchymal core of the dorsal fin. The dorsal fin of axolotl larvae might serve today as a suitable system for investigating the interaction of pigment cells and mesenchymal cells with ECM components in the SEM and laser scanning microscope (LSM). In Figures 19 and 20, the connective tissue in the dorsal fin of dark (Fig. 19) and white mutant axolotl larvae (Fig. 20) is compared in the SEM after removing one side of the epidermis. In white larvae only fibrocytes are found in the dorsal fin, whereas in dark larvae there is a mixture of both fibrocytes and pigment cells.

Neuronal and glial derivatives of the neural crest

The NC gives rise to neuronal and glial derivatives in the head and trunk of urodele embryos. As relatively little can be said about glial cells in amphibians, and as most studies were not carried out in urodeles, these cells are only briefly mentioned here. The glia derived from the crest comprises Schwann cells (ensheathing nerves of the peripheral nervous system) and satellite cells of the sensory and autonomous ganglia (Le Douarin, 1982). LM- and TEM-investigations on Schwann cells dealt mainly with the process of myelinization in tadpole fins in *Rana* and *Xenopus* (Harrison, 1924; Speidel, 1932; Billings-Gagliardi *et al.*, 1974) or *Ambystoma* (Yntema, 1943). Hardly anything is known about the position (see below) and structure of dorsal root ganglia, for example, or about the proportion of satellite and neuronal cells. In Figures 21 and 22 we compare glial and neuronal cells in spinal ganglia using specific antibodies against GFAP (glial cells) and tubulin (neurons). More



Figs. 19-20. SEM micrographs of the dorsal fin in the mid-trunk region of a dark and white axolotl larva (stage 40) after removal of one side of the epidermis. (19) *In dark larvae, NC-derived pigment cells (melanophores, xanthophores) are mixed with NC-derived fibrocytes. The three types of cell are indistinguishable in the SEM.* **(20)** *In white larvae, only fibrocytes occur in the dorsal fin. nt, neural tube. Bars, 25 μm.*

intensive studies on glial cells, however, are available in higher vertebrates than in amphibians (see Pannese, 1974, for review).

The origin of cranial sensory ganglia in both anuran and urodele embryos has been discussed for more than 100 years. Controversies about the origin were due to inexact labeling methods. The ganglia were assumed to be derived from NC cells, from placodal material (inner layer of the epidermis) or from both sources (see Epperlein and Löfberg, 1993, for review). With the invention of the more exact biological labeling system of *Xenopus laevis/X. borealis* (Thiébaud, 1983), the origin and composition of the sensory ganglia in the head of *Xenopus* embryos could be studied more precisely than before (Sadaghiani and Thiébaud, 1987). In addition, with an antibody against an epidermal protein (Cornish *et al.*, 1992), labeling of the epidermal portion of the ganglia in *Xenopus* could be achieved immunohistochemically, without any grafting. According to these results in *Xenopus*, which seem to be relatively accurate, the sensory cell bodies of cranial nerves I and VIII are derived from epidermal placodes, whereas the ganglia of nerves V, VII, IX and X are of dual (NC/placodal) origin.

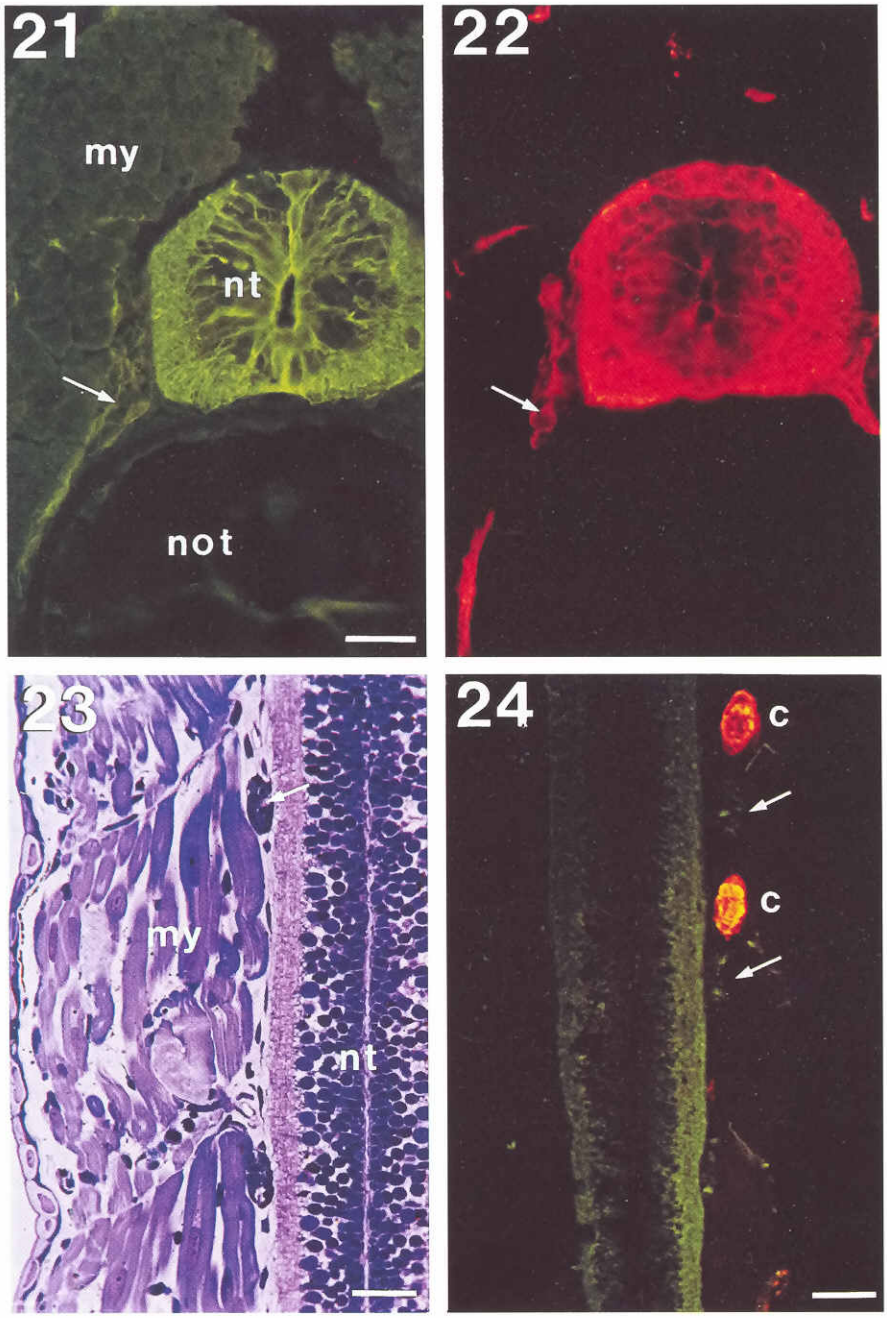
For the urodele embryo, no biological labeling method exists, as in *Xenopus*, with which the origin and composition of cranial sensory ganglia could be studied, and antibodies against the epidermis, although available (Epperlein *et al.*, unpublished), have not yet been used. Injections into NC cells have also not yet been applied. Nevertheless, the problem could be solved much more easily than in *Xenopus*, where a lateral crest exists in addition to a medial one (Schroeder, 1970; Hausen and Riebesell, 1991), which could be contained in 'epidermal' grafts designed to distinguish between the crest and/or epidermal origin of cranial ganglia in grafting experiments.

In the trunk of urodele embryos, a chain of sensory or spinal ganglia develops (Detwiler, 1934, 1937). These ganglia are derived from the NC string and become separated into single structures when NC cells are migrating ventromedially towards the somites. For several years it had been debated whether in chick embryos NC cells migrate rostrally through the somites with their migratory route defined or caused by tenascin (Rickmann *et al.*, 1985; Tucker and McKay, 1991). That is not apparently the case. In urodele embryos it was not known whether the NC cells coalescing into spinal ganglia migrate adjacent to the rostral, medial or caudal portion of the somite. As was reported by Detwiler (1934), the removal of somites in *Ambystoma* leads to fewer, but larger and irregularly segmented ganglia; the addition of somites leads to development of supernumerary ones. The position of the ganglia in *Ambystoma* in relation to the somites is currently under investigation with immunohistochemical methods and 3D-reconstruction (Berge and Epperlein, in preparation). In axolotl larvae (stage 42), the ganglia are localized adjacent to the rostral part of the myotome (Fig. 23), whereas in younger stages they seem to lie more central. In older larvae (Fig. 24), cartilage deriving from the sclerotome and later incorporated into the vertebrae can be demonstrated in addition to the ganglia, though opposite the caudal part of the myotome.

A primary sensory system precedes the spinal ganglia and degenerates when the spinal ganglia are functioning (stages 40-45). It consists of Rohon-Béard neurons, giant neurons being localized in the dorsal part of the neural tube. They can be demonstrated with anti-tubulin immunostaining and DiI labeling (Berge, Epperlein and Selleck, unpublished). Like the spinal ganglia these neurons also develop from the NC (DuShane, 1938; Chibon, 1966; Vogel and Model, 1977).

The sympathetic system of the axolotl is not present until there is a larval length of 24 mm. This has been demonstrated by Vogel and Model (1977) using orthotopic transplantation of tritiated-thymidine labeled neural folds. The sympathetic system is preceded by chromaffin cells already appearing in 14 mm larvae. Both sympathetic neurons and chromaffin cells are derived from the NC and diffusely distributed along the aortic region. According to Vogel and Model, the two cell types can be distinguished with certainty only with TEM. More recently, monoclonal antibodies against tyrosine hydroxylase were used to identify these cells (Fig. 25).

The NC contribution to the enteric nervous system in urodeles



Figs. 21-22. Immunostaining of spinal ganglia (arrows) and spinal nerves on adjacent transverse cryosections through the mid-trunk region of an axolotl larva *(about 15 mm long) with anti-glial-fibrillar acidic protein (GFAP; for glial cells,* **21***) and anti-tubulin (for neurons,* **22***). my, myotomes; not, notochord; nt, neural tube. Bars, 50, μm.*

Figs. 23-24. Localization of spinal ganglia in the mid-trunk region of axolotl larvae. (23) *In a frontal section (JB4-embedding, Azur B-eosin staining) through a 15 mm larva (level of the neural tube, nt), the ganglia (arrow) are found opposite the rostral part of the myotomes (my).* **(24)** *In a frontal cryosection through a 17 mm larva cartilage nodules (c; not yet present at 15 mm length) are revealed with anti-6-CSPG opposite the caudal portion of each myotome. The spinal ganglia (arrows) are revealed with anti-tubulin immunostaining. Bars, 50 μm.*

is not known. However, conditions may be similar to those in *Xenopus*, where the NC origin of enteric neurons has been demonstrated with the *Xenopus laevis/borealis* marking system by colocalizing *borealis* and HNK-I positive cells (Epperlein *et al.*, 1990) and by using single-cell injected embryos (Collazo *et al.*, 1993). In Figure 26 neuronal nets are demonstrated in the intestine of an axolotl larva by using an anti-tubulin antibody.

Pigment cells derived from the neural crest

As mentioned above, three types of pigment cells occur in amphibian embryos; melanophores, xanthophores and iridophores (see Frost-Mason and Mason in this issue). Their origin from the NC was established by DuShane (1934). The lateral migration of melanophores under the skin, where these cells are most easily recognized from outside, has been used by various investigators for studying migration, interaction and localization of NC derivatives. Twitty (1936, 1945, 1966) was the first to study divergent ways of pigment cell patterning by using two species of the Californian newt, *Taricha torosa* (larvae with a prominent dorsal and a weak lateral longitudinal melanophores stripe) and *Taricha rivularis* (larvae with a uniform distribution of melanophores). *Twitty tried to find out whether the pigment patterns are controlled by the pigment cells themselves (intrinsic control) or by the embryonic environment (extrinsic control).* In his first experiments, Twitty exchanged trunk neural fold material between *rivularis* and *torosa* neurulae and always found donor-type patterns. This result could only be explained by a cell-intrinsic mechanism of pattern formation. Melanophores in both *rivularis* and *torosa* tailbuds go through a period of diffuse arrangement, but only *torosa* melanophores develop further and react to environmental cues. Thus, *rivularis* embryos were, in fact, unsuitable for testing the environmental influence on pigment pattern formation in Twitty's experiments. By exchanging neural folds between embryos of different urodele species, the melanophores of which could react to external cues, Rosin (1943) showed that mostly host-type patterns developed. This result clearly emphasized the extrinsic control of pigment pattern development. Dalton (1950, 1953), by comparing pigment pattern formation in dark and white axolotl embryos, could demonstrate that pigment cell migration is controlled by the epidermal environment. Dalton's studies were later extended and further refined by investigators working with axolotls and other urodele species (Keller *et al.*, 1982; Tucker and Erickson, 1986a,b; Epperlein and Claviez, 1982; Epperlein and Löfberg, 1984, 1990, 1993; Epperlein *et al.*, 1995). The interest in these later studies has shifted towards cell surface differences between melanophores and xanthophores, both of which interact during pigment pattern

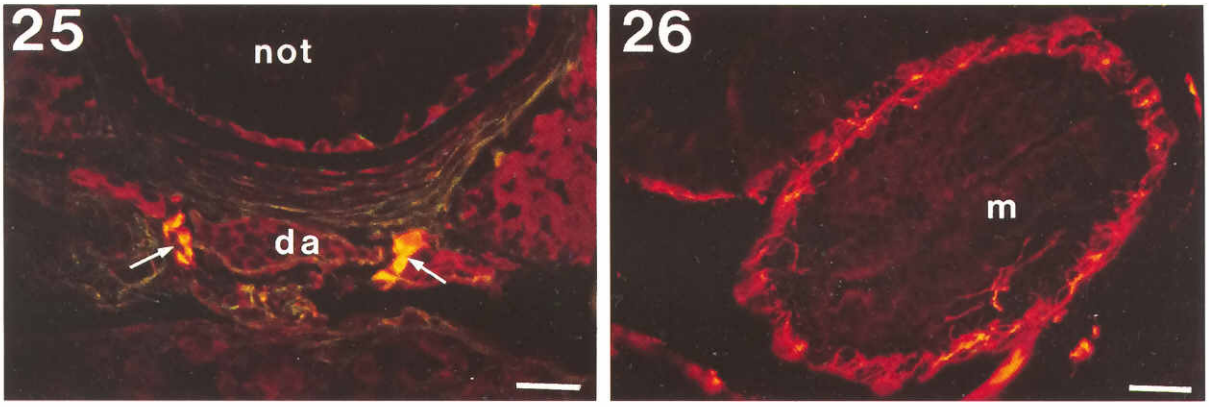


Fig. 25. Transverse cryosection through the trunk of an axolotl larva (24 mm) showing tyrosine hydroxylase (TH) positive cells (sympathetic neurons or chromaffin cells; arrows) at the dorsal aorta (da). *The cells are stained with an antibody against TH (gift of Dr. Ziller, Nogent) and visualized with a Cy-3 secondary antibody. The section is counterstained with anti-fibronectin (FITC conjugated second antibody). not, notochord. Bar, 50 μm.*

Fig. 26. Transverse cryosection through the trunk of an axolotl larva (24 mm) showing anti-tubulin positive neuronal nets predominantly in the outer (myenteric) part of the intestine. *m, mucosa. Bar, 50 μm.*

formation, and to molecular cues in the extracellular environment (ECM, cytokines), to which the pigment cells might react.

In their studies on pigment pattern formation in *Taricha torosa*, Tucker and Erickson (1986a,b) suggested that the melanophores forming the dorsal stripe possess long searching processes (as opposed to xanthophores) with which to recognize and move towards adhesive subepidermal substrates (fibronectin) opposite the dorsal somite edges. The melanophores in the lateral stripe seem to be trapped by the pronephric duct, although even after its ablation further ventral migration of melanophores was still impeded. The mechanism or components inhibiting ventral migration need, therefore, to be defined. Why xanthophores but not melanophores may invade the dorsal fin could be explained by assuming (from evidence *in vitro*) that only these cells can invade a GAGrich substrate (present in the fin).

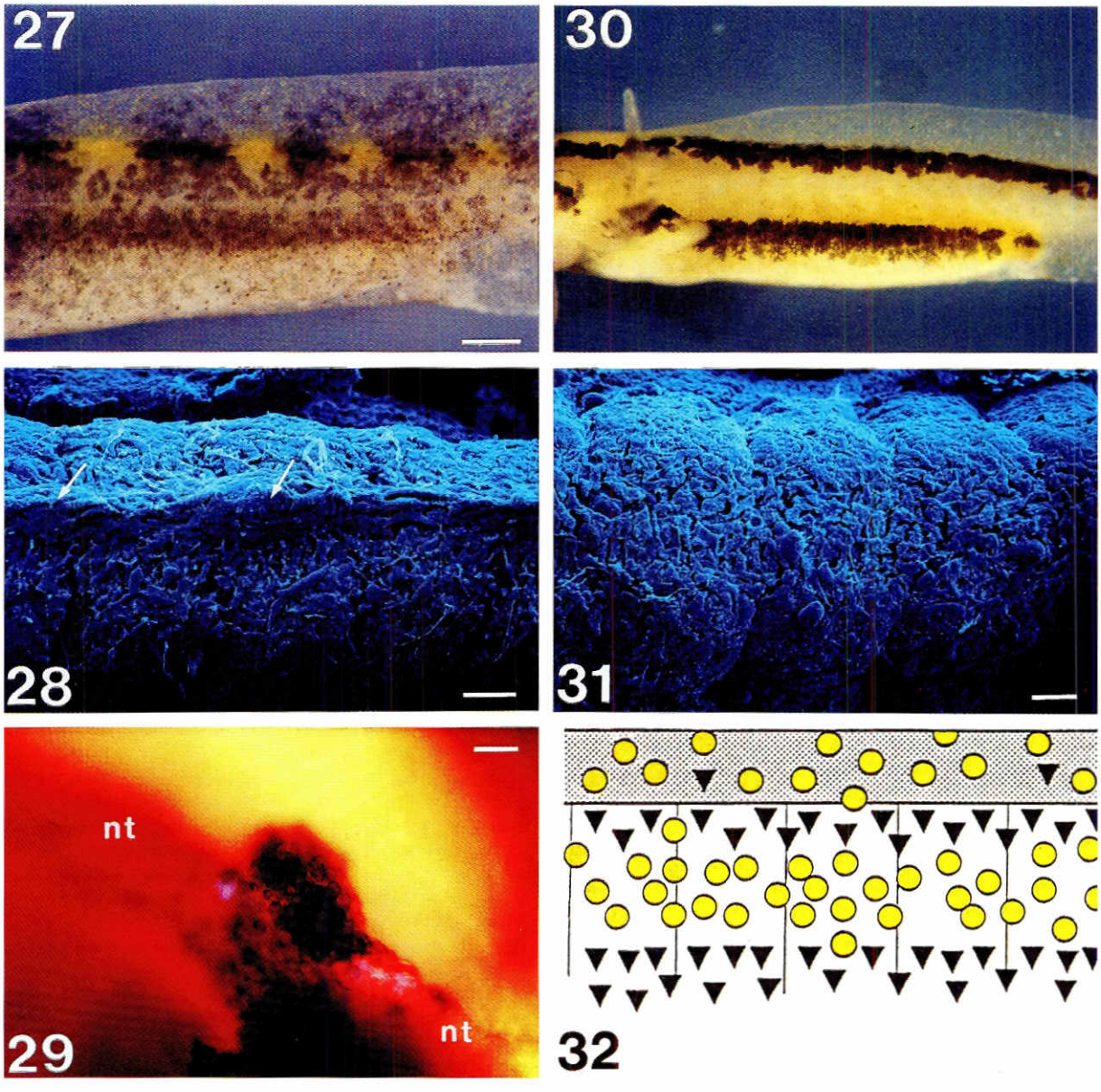
The arrangement of melanophores (and xanthophores) in alternating vertical bars, as in *Ambystoma mexicanum* (Fig. 27), or in horizontal stripes, as in *Triturus alpestris* (Fig. 30) was studied more recently by Epperlein and Löfberg (1990). *The principal question was how the NC, being a basic structure in embryos of both species, can give rise to different pigment patterns. Is the NC itself different, or have the differences to be sought in the mode of NC segregation and distribution of the pigment cells? Or does the embryonic environment contain different cues?*

In dark (wild-type) embryos of *A. mexicanum*, the melanophores are the first to migrate out and become scattered over the entire dorsolateral trunk (stage 35). At this stage, several mixed chromatophore aggregates are recognizable along the premigratory NC (see Figs. 28, 29). They constitute a prepattern for the barred or transverse band pigment pattern of the axolotl larva. The formation of the mixed aggregates seems to be caused by xanthophores adhering more tightly to each other and to the neural tube than do melanophores (Fig. 29). During further development, xanthophores spread more and more radially, while melanophores recede towards adjacent regions populated by those melanophores which had dispersed earlier from the NC. As a consequence of this separation or sorting out of melanophores and xanthophores, alternating transverse bands of melanophores and xanthophores are formed.

In *Triturus*, melanophores and xanthophores are mixed in the premigratory NC, which shows a homogeneous distribution (Fig. 31). From stage 28 onwards (early tailbud), the pigment cells start to migrate laterally and become distributed over the flank. Then melanophores aggregate into compact bands or stripes at the dorsal and lateral somite margins (Figs. 30, 32; stage 34). Xanthophores withdraw from the sites of melanophore stripes and recede into the interband area or the dorsal fin. Stripe formation of melanophores seems to be induced by specific cues arising in the subepidermal ECM along the trunk at stage 31/32 (Epperlein and Löfberg, 1990). According to the results of transplantation experiments, SEM and immunohistochemical findings, pigment pattern formation in *Triturus* can be explained as follows. Melanophore distribution changes from uniform to banded, because the appearance of tenascin (TN) and CSPG in the interband region (probably of mesodermal origin) prevents their adhesion to a substrate still rich in fibronectin (FN). Melanophores thus recede to the more adhesive dorsal and lateral areas with unchanged distribution of FN (these are the cues for stripe formation). In the dorsal fin, TN and CSPG prevent the invasion of melanophores. Ventral to the lateral stripe, melanophores are absent because the substrate is unsuitable for migration.

Conversely, where melanophores occur, xanthophores are excluded. Xanthophores, however, may persist in the interband area and dorsal fin, i.e., in regions containing TN and CSPG. *A significant implication arising from this "exclusion" hypothesis is that melanophores and xanthophores possess different cell surface receptors for different components of the ECM.*

If both patterns are compared, the decisive difference is that, in *T. alpestris*, randomly scattered chromatophores follow different environmental cues when sorting out into the longitudinal zones, whereas in the axolotl alternating transverse melanophore and xanthophore bands do not arise in response to environmental influences but because of cell-cell interactions. As transplantation of NC cells between *Triturus* and *Ambystoma* has shown, *Triturus* melanophores can form broad longitudinal stripes in the axolotl. Thus the stripe forming cues do not seem to have been lost in the axolotl, although axolotl melanophores are unable to respond to these cues in their own embryo.



Figs. 27-29. Larval pigment pattern and its development in the Mexican axolotl, *Ambystoma mexicanum* (wild-type). (27) *In dark axolotl larvae, the definite barred pigment pattern becomes established at stage 41 (see also Fig. 10). It consists of alternating transverse bands of melanophores and xanthophores along the dorsal trunk.* **(28)** *At stages 33-35, after removal of the dorsal epidermis (SEM micrograph), several pigment cell aggregates (arrows) stand out from the NC string. They form a prepattern of the barred pigment pattern.* **(29)** *Enlargement of one aggregate (mixed chromatophore group) on the dorsal trunk neural tube (nt) after removal of the dorsal epidermis. LM micrograph showing blue pterin fluorescence of xanthophores in a dopa-stained embryo. Melanophores are situated more peripherally, xanthophores are located more basally and centrally (not visible).*

Figs. 30-32. Larval pigment pattern and its development in the newt, *Triturus alpestris*. (30) *In the trunk (stage 34), the definite larval pigment pattern consists of melanophores forming a distinct dorsal and lateral longitudinal stripe with yellow xanthophores distributed between the stripes and in the dorsal fin (hardly visible).* **(31)** *SEM micrograph of a dorsal mid-trunk region at stage 28 with the premigratory NC cells forming a flat sheet.* **(32)** *Melanophores (black triangles) and xanthophores (yellow circles) which were previously mixed and scattered over the lateral trunk, have sorted out in response to their specific cues. Bars: (27,30) 0.5 mm; (28,29,31) 50 μm.*

Conclusion

On reflecting generally about NC cell migration, differentiation and cell-matrix interactions, one might speculate that possibly subsets of NC cells develop different cell surface receptors for different environmental cues. These cues might be present or activated as cytokines in different compartments of the embryo, e.g., in the ECM and basement membranes (BM) of the epidermis, somites, neural tube or dorsal fin. For a better understanding of cell migration and differentiation, these cell-cell and cell-matrix interactions should therefore be studied (1) by isolating and comparing the surface receptors of migrating NC cell populations (melanophores, xanthophores or neurons); and (2) by investigating the local milieu (compartment) of adjacent tissues and their BM and associated

ECM. For instance, the extent to which the outer BM of the somites and the opposing subepidermal BM contribute components to the common interstitial ECM (=ECM of the lateral route of migration) between both BMs is unknown. Knowledge of the fine regulation of ECM components (or cytokines in general) would be important for understanding the mechanisms that control the migration of NC cells.

Acknowledgments

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