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Guest Editor, Marianne Bronner

***The “beginnings” of the neural crest***

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ANR, anterior neural ridge; CNS, central nervous system; DRG, dorsal root ganglion; E, embryonic day; EMT, epithelial-to-mesenchymal transition; ENS, enteric nervous system; Fgf, fibroblast growth factor; FSNC, facial skeletogenic neural crest; Mab, monoclonal antibody; NC, neural crest; NCC, neural crest cells; PNS, peripheral nervous system; r, rhombomere; Shh, Sonic hedgehog.

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## ***Abstract***

### Foreword

The neural crest has been the main object of my investigations during my career in science, up to now. It is a fascinating topic for an embryologist because of its two unique characteristics: its large degree of multipotency and the fact that its development involves a phase during which its component cells migrate all over the embryo and settle in elected sites where they differentiate into a large variety of cell types.

Thus, neural crest development raises several specific questions that are at the same time, of general interest: what are the mechanisms controlling the migratory behavior of the cells that detach from the neural plate borders? What are the migration routes taken by the neural crest cells and the environmental factors that make these cells stop in elected sites where they differentiate into a definite series of cell types?

When I started to be interested in the neural crest, in the late 1960s, this embryonic structure was the subject of investigations of only a small number of developmental biologists. Fifty years later, it has become the center of interest of many laboratories over the world.

The 150<sup>th</sup> anniversary of its discovery is a relevant opportunity to consider the progress that has been accomplished in our knowledge on the development of this ubiquitous structure, the roles it plays in the physiology of the organism through its numerous and widespread derivatives and its relationships with its environment, as well as the evolutionary advantages it has conferred to the vertebrate phylum.

I wish to thank Pr Marianne Bronner, Chief Editor of *Developmental Biology* and Special Issue Guest Editor, for dedicating a special issue of this journal to this particular structure of the vertebrate embryo.

In the following pages, Elisabeth Dupin and I will report some of the highlights of our own acquaintance with the neural crest of the avian embryo, after retracing the main trends of the discoveries of the historical pioneers.

Nicole M. Le Douarin

## **Introduction**

The neural crest (NC), or at least its precursor, the neural fold, which delimits the “neural plate” at the origin of the central nervous system (CNS), was designated by the embryologist P. Raven<sup>1</sup> one of the “primary organs” of the vertebrate embryo. It emerges early during development and is very conspicuous on the dorsal side of the amphibian embryo, which was the common experimental model of the pioneers of experimental embryology. The NC is however a transient structure that disappears as its component cells start to migrate away from their source and invade the developing body. Within a short time, all the NC cells (NCC) have left the neural epithelium and the NC has ceased to exist as a unitary structure. This is why it had escaped the attention of the embryologists of most of the nineteenth century.

In his monograph, a landmark in the history of our knowledge on the NC, entitled “*THE NEURAL CREST; its properties and derivatives in the light of experimental research*”, published in 1950, Sven Hörstadius states that: “*Although it has been the subject of intense study for many decades, ... outside the circle of investigators in this field, it (the NC) is hardly known at all. This lack of knowledge is only to be expected, since the neural crest is, as a rule, only very sparsely treated by the authors of text-books.*” This was accounted for by the fact that, at the time of neural tube closure, the cells forming the lateral borders of the CNS migrate away from their source and, in most cases, become undetectable.

In 1868, one hundred and fifty years ago, the German embryologist and anatomist Wilhem His was the first to distinguish and describe the NC in the chick embryo. He noticed a band of particular material lying on top of the neural tube, underneath the superficial ectoderm (*Hornblatt*), which he called “*Zwischenstrang*”. The ectoderm was thus divided into three different territories: the neural plate, the two strips -that later join- of the *Zwischenstrang*, and the *Hornblatt*. He also found that the NCC migrating away from their source accumulated laterally to the neural tube, at the site where the spinal ganglia develop. For this reason, the name of “ganglionic crest” was coined to designate this transitory structure (His, 1868).

His’ view of the nature of the NC was not immediately adopted by the embryologists of the time. Kastschenko (1888), Balfour (1881), among others, considered that it was an outgrowth of the neural tube itself that was giving rise to the dorsal roots of the spinal ganglia. In 1950 however, Hörstadius wrote: “*It seems clearly established to day that the neural crest forms a special rudiment already present in the open neural plate stage*”, a view that has been fully confirmed in modern time, by the pattern of gene expression in the dorsal ectoderm at early developmental stages (Nieto et al., 1994; Sauka-Spengler and Bronner-Fraser, 2008; De Croze et al., 2011; Milet et al., 2013; Simoes-Costa and Bronner, 2015; Roellig et al., 2017).

Another remarkable event in the NC history was the demonstration of its capacity to produce mesenchymal cells. It is interesting to notice that the participation of this ectodermal structure to the skeleton was one of its first roles to be evidenced... It began with the claim, by Kastschenko (1888), that some of the head mesenchyme of the Selacian embryos originate from the NC. Some time later, Goronowitsch (1892, 1893) recognized, from his own observations in teleosts and birds, that the cephalic NC was providing the head with mesenchymal cells, but he denied the same origin for the spinal ganglia. These discrepancies generated intense debates and it was Miss Julia Platt, one of

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<sup>1</sup> Cited in Horstadius, 1950.

the very rare women involved in research at that time, who found that, not only ganglia and nerves, but also mesenchymal cells participating in bones and cartilages of the visceral arches, were of ectodermal origin (Platt, 1893, 1897). She considered that most of this participation arose from the lateral head ectoderm and proposed the term of “*mesectoderm*” for the mesenchyme originating from the ectoderm and of “*mesentoderm*” for the mesoderm-derived mesenchyme. Investigations were further pursued on several vertebrate species. Some authors supported the ectodermal origin of bones via the NC whereas others denied the possibility that mesenchyme could be derived from ectoderm (see Landacre, 1921; Stone, 1922; Holmdahl, 1928). A violent controversy ensued whose roots were to be found in the power of the germ layer theory put forward in 1828 by Karl von Baer, according to which homologous structures in different animals are formed of material arising from the same germ layers. This affirmation was rapidly considered a “*law*” that clearly found an exception in the NC, a so far neglected structure.

Many investigations were conducted especially in amphibians and fishes in the first decades of the twentieth century (see Landacre, 1921, Stone, 1922 for the observations on frog and *Amblystoma jeffersonianum*). The discovery of the contribution of the NC to the peripheral nervous system (PNS) ensued. This was made possible by devising techniques to trace the migration of the cells arising from this discrete and transitory structure.

### ***Devising strategies to follow the migration of the neural crest cells and discover their fate***

Apart from mesenchymal cells in the head, the NC was later on, shown to be essential for the development of several other cell types and structures such as the sympathetic and parasympathetic nervous systems, the sheath cells of Schwann, lining the peripheral nerves, and the pigment cells. This research required the possibility to follow the NCC as they migrate within the tissues of the developing embryo. The migration starts as soon as the NC is formed during neural tube closure. It can take place sometime before the neural folds are completely joined, depending on the species, and, within a species, on the different levels of the neural axis. As they start migrating, the NCC, undergoing epithelial-to-mesenchymal transition (EMT), lose their epithelial arrangement and move away within the tissues of the embryo. Several techniques were designed to disclose the fate of these migratory cells.

#### ***Ablation of the neural tube or the neural crest***

In situ destruction or extirpation of segments of the neural tube or of the NC only, followed by the observation of the deficits consequent to the operation, has been used in some species by pioneer researchers. This technique has provided some fundamental knowledge; however, the results obtained are approximate since extirpation of embryonic areas can trigger regulatory mechanisms able to restore deletions.

Considering the case of the ontogeny of the spinal ganglia will show how difficult it was to decipher the developmental history of the derivatives of the NC. The assumption by His that the spinal ganglia were formed by the accumulation of cells navigating from the NC, deduced from the observation of the crest cells gliding on the dorsal neural tube surface, did not convince all the embryologists of his time. Kastschenko (1888) and some others thought that the NC material was only giving rise to the dorsal roots of the spinal ganglia, the latter ganglia being supposed to derive from either the mesoderm

(the myotomes) or the neural tube itself. The problem raised was thus to know whether the NC was an outgrowth of the spinal cord, or a separate rudiment? In other words, from which part of the ectoderm was the NC derived? In the 1930s, observations carried out on various vertebrate species led to the conclusion that, in agreement with His' description in the chick, the NC "runs like a peripheral band around the whole neural plate"<sup>2</sup>. It was only several years later, that Harrison produced the indisputable proof that His was right in identifying the NC as the source of the cells forming the dorsal root ganglia (DRG). He removed the dorsal half of the spinal cord in the frog embryo, just after neural tube closure; this resulted in the absence of DRG at the level of the operation. Removal of the ventral part of the neural tube did not prevent DRG development, but was followed by the absence of the ventral root of the spinal nerves at the level of the extirpation, while the sensory nerves were not affected (Harrison, 1938).

### ***Application of vital dyes***

During the first decades of the 20<sup>th</sup> century it was discovered that, in addition to the spinal ganglia, the NC is also involved in the development of the sympathetic nervous system, the Schwann sheath cells that protect peripheral nerves, the teeth, pigment cells and, in fishes, the dorsal fin. This was related to the significant progress that arose from the use of in situ labeling of definite regions of the neural anlage with vital dyes. As an example of this approach, Detwiler (1937) stained the NC in *Amblystoma* by pressing small blocks of agar impregnated with vital stains (black and red) placed respectively on the neural plate and the neural folds. The aim of the experiment was to try and discriminate the contribution of these two structures to the head skeleton and the peripheral ganglia. The results however were far from clear and hardly reliable, since the diffusion of the dyes was difficult to control and the contamination of the NCC by mesoderm-derived cells during the migration process could not be excluded.

Examples of the efficiency of this technique exist however; such is the case for the thorough study performed, later on, by Hörstadius and Sellman (1941, 1946) on the contribution of the NC to the visceral arch cartilages in *Amblystoma*. This series of experiments gave rise to fundamental results that the authors confirmed by extirpation experiments.

All these techniques were essentially concerned with the fate of the NCC and their role in the construction of the embryo. They did not address the process and pattern of NCC migration in their early phase and at the single cell level. Moreover, around the 1950s and even much later, many questions on the fate of NCC remained controversial. Such was the case of the origin of Schwann cells lining the peripheral nerves and of sympathetic neuroblasts, which were claimed by some authors to arise from both NC and neural tube, or from the neural tube alone. For example, in 1947, Yntema and Hammond found a NC origin "most probable" for the sympathetic ganglia while in the same year, Rita Levi-Montalcini reported that extirpation of the neural tube and NC on the length of ten to twelve segments in the chick embryo resulted in normal development of the sympathetic system while the spinal ganglia were absent in the operated region (Levi-Montalcini, 1947). Yntema and Hammond's experiment consisted in removing the NC bilaterally from the thoracic and lumbar regions; they found that not only the sympathetic neurons but also the chromaffin cells of the suprarenal gland and the aortic chromaffin bodies, failed to develop (Yntema and Hammond, 1947). These

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<sup>2</sup> Quoted from Hörstadius, 1950.

discrepancies are likely to be due to the regeneration of remaining NCC in Levi-Montalcini's experiments, where the removed segment of neural tube was much shorter than in those performed by Yntema and Hammond.

The problem of the origin of the Schwann cells lining the peripheral nerves has also given rise to controversies. It was, for long, considered that they were derived from mesodermal mesenchyme. Harrison (1904) however put forward the notion that they were likely to be produced, at least partly, from the NC. This opinion was based on experiments where he removed the dorsal part of the neural tube (including the NC) in frog embryos and found that the ventral roots of the nerves at the level of the operation were devoid of their protective layer of Schwann cells. These cells were, at a time, considered as giving rise to the peripheral axons, but this view was abandoned, thanks to the famous Harrison's demonstration, through the first in vitro tissue culture experiments in history, that the outgrowth theory of nerve fiber formation was correct (Harrison, 1907, 1910). Detwiler and Kehoe (1939), who used vital dyes to stain the NC in *Amblystoma*, further confirmed Harrison's view. These authors found that both the DRG and the Schwann cells lining the nerves at the same level were carrying the dye. Other experiments however, like those of Raven (1936, 1937), based on the construction of chimeric embryos, did not confirm this view of a NC origin for Schwann cells. Raven thought that they were derived from cells lying on the surface of the lateral walls of the neural tube. Harrison had also noticed the migration of Schwann cell precursors from the ventral part of the spinal cord, but this was taking place at a later stage and involved only a small number of cells.

In summary, it is noticeable that the theory according to which Schwann cells might be derived from mesodermal mesenchyme persisted up to the 20<sup>th</sup> century.

The question of the development of the intrinsic gut innervation has also been the subject of controversies. Some authors, such as van Campenhout (1930) in frog and Yntema and Hammond (1954, 1955) in the chick, have proposed that enteric plexuses derived from the NC. Others have considered that a source of the gut plexuses could be the neural tube (Jones, 1942) or the mesoderm (Keunig, 1944).

This short glance on the pioneer studies on the NCC, extending over nearly a century (from 1868 to 1950) - from their discovery up to Sven Hörstadius' review-, shows that the active research, aimed at establishing the pathways NCC follow during their migration all over the body and their contribution to organs and tissues, had beautifully progressed, while leaving numerous unsolved questions. Moreover, there was still the possibility that, at that time, the whole panorama of the NC-derived cell types had not been fully discovered. There was a critical need to produce an appropriate means to follow the NCC during the full period of their migration.

Much later in the history of NC research, in the late 1980s, labeling cells with vital dyes has found new developments through a method pioneered by Marianne Bronner and Scott Fraser. It consists in microinjection of a fluorescent dye into single cells, thus allowing to visualizing NCC and their progeny (Bronner-Fraser and Fraser, 1988, 1989; Fraser and Bronner-Fraser, 1991; Collazo et al., 1993; Raible and Eisen, 1994; Schilling and Kimmel, 1994). In combination with novel techniques of imaging, this method has provided the opportunity to follow the dynamics and fate acquisition of NCC in vivo.

***Constructing embryonic chimeras to follow the migration of the neural crest cells***

**The early times:** From 1931 on, Raven introduced the construction of chimeric embryos into the NC field, a method that had previously been used by embryologists, namely by Hans Spemann in his famous experiments on *primary induction* in amphibian embryos. Definite fragments of the premigratory NC were exchanged between *Amblystoma* and *Triturus*. The recognition of cells belonging to host or donor was based on the difference in the size of their nucleus. The nuclei had to be measured and their “nuclear values”- length and breadth multiplied- determined (Raven, 1931). This method made it possible to estimate the origin of groups of cells but the species origin of single cells in the chimeric tissues was doubtful. Raven’s experiments consisted in exchanging the neural fold on one side, before closure of the neural tube: he found a mixture of host and donor cells in the DRG, which he interpreted as the contribution of NCC from the site opposite to the graft. Later on, bilateral extirpation of the NC along a sufficient length performed by Dushane (1938) in *Amblystoma punctatum*, resulted in complete absence of DRG. Thus the origin of the spinal ganglia from the NC was definitively established.

The problem of the origin of the tooth papillae deserved to be mentioned here.

Vertebrate teeth result from the juxtaposition of two primordia, one of epithelial origin forming the enamel organ (from ectoderm or endoderm, according to the position of the tooth in the mouth), and tooth papilla, which produces dentine and pulp. As early as 1897, Platt working on *Necturus*, proposed that both odontoblasts and pulp were derived from ectomesenchyme, hence, of NC origin. This view was confirmed later by Adams (1924) and De Beer (1947) through the construction of chimeric embryos: first, extirpation of the NC resulted in strong reduction of teeth on the operated side (Stone, 1926; Raven, 1931); furthermore, xenoplastic transplantation of *Amblystoma* NC into *Triturus* embryos was followed by colonization of the tooth papillae of the host by donor cells (Raven, 1935). These findings were confirmed by others using different experimental approaches (Hörstadius and Sellman, 1946; Chibon, 1966, 1970).

**Thirty years later:** A significant progress was made through the use of autoradiographic techniques following injection of tritiated (<sup>3</sup>H)-thymidine to label dividing neuroepithelial cells (Sauer and Walker, 1959; Sidman et al., 1959). The labeling of a tissue in a donor embryo allowed defined territories to be transferred to an unlabeled recipient by grafting. This technique was initiated by Jim Weston to follow the migration of NCC (Weston, 1963) and later on, used by Johnston (1966) and Noden (1975) in chick, and by Chibon (1964, 1967) in *Pleurodeles*. It has, however, several drawbacks: it is not stable, since the labeling gets diluted through proliferation of the grafted embryonic cells; it is not strictly cell-specific because, in case of death of the grafted cells, their radioactive DNA is released in the intercellular medium and can be captured by host dividing cells. Since the radioactivity is concentrated in the most radiosensitive cellular structure, nuclear DNA, the question of toxicity has also to be considered. According to Weston, conditions could be found that did not seem to hinder the normal morphogenetic processes.

Several results can be credited to this technique, such as the confirmation of the NC origin of spinal ganglia, sympathetic neuroblasts and pigment cells (Weston, 1963). Regarding the Schwann cells, their derivation from a mesodermal source was definitively excluded and their origin from the NC recognized for most of them, but some doubt subsisted: “Some Schwann sheath cells...were shown to emerge from the neural



tube along with the ventral nerve fibers, and the possibility is raised that sheath cells are neuroglia solely of neural tube origin”<sup>3</sup>.

Following Hörstadius and Sellman (1941, 1946), a thorough series of studies in *Pleurodeles* using <sup>3</sup>H-thymidine labeled NCC implanted into a non-labeled host, was pursued by Chibon (1966, 1967), leading to further document the origin of the head skeleton and showing a major role of the NC in this process. According to this author, the neurocranium has a mesodermal origin, except -partly- for the trabeculae cranii and the auditory capsules, which are mostly derived from the anterior head NC; the visceral skeleton, formed of six visceral arches and two basibranchials, in contrast, appears to be derived essentially from the NC-derived mesectoderm.

### ***The quail-chick marker system***

An input was given to the study of the NC and its role in the development of amniote vertebrates, when a novel cell marking system was devised in 1969 (Le Douarin, 1969). It is based on the particularity of cell nuclei of a bird commonly raised for human food supply, the Japanese quail (*Coturnix coturnix japonica*). I noticed the presence of a large nucleolus in all embryonic and adult cell types of this species of bird and further found that the unusual size of this organelle resulted from association of a mass of heterochromatin with the nucleolus proper (essentially made up of RNA). This particularity is of rare instance in the animal kingdom (Le Douarin, 1971a) and does not exist in the chick (Le Douarin, 1969, 1971b). When quail cells are transplanted into a chick embryo or associated with chick tissues in *in vitro* cultures, cells of the two species retain their nuclear characteristics and can be identified in chimeras after Feulgen-Rossenbeck staining (Le Douarin, 1973). This staining procedure is specific for DNA and reveals the large mass of heterochromatin associated to the nucleolus. Quail and chick cells, side by side in chimeric tissues can therefore be easily identified, since the natural nuclear labeling of quail cells is conspicuous enough to enable identification of a single quail cell located in chick tissues, provided that the section includes the nucleolus. A significant improvement in the identification of the cells of the two species occurred with production of monoclonal antibodies able to recognize species-specific antigens expressed by one, but not the other, species. The antibody against a perinuclear antigen, QCNP (for Quail non Chick Peri-Nuclear), prepared by B. Carlson and J. Carlson, turned out to be particularly useful to analyze the chimeric tissues from mid-1990s onward.

### ***Application of the quail-chick marker to NC ontogeny***

The ability to distinguish quail from chick cells on tissue sections prompted me and my coworkers to investigate systematically the fate of the NCC in the avian embryo, by constructing chimeras in which a fragment of the neural tube (or neural fold at the head level) of chick was removed prior the onset of NCC emigration at this level of the neural axis, and then replaced by its exact counterpart from a quail embryo at the same developmental stage. The quail neural tube had to be cleaned of contaminating mesodermal cells. This was achieved by using trypsin to separate the tube from the surrounding tissues. To estimate the accuracy of the results deduced from these experiments, the same type of grafts had to be performed in both directions, i.e. from quail to chick and from chick to quail. Although, at hatching, a chick weighs about three times more than a quail, the developmental rates of the two species are very close

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<sup>3</sup> quoted from Weston, 1963 p. 306

during the first half of the incubation period, thus ensuring that the general pattern of development is established normally in the chimeras.

When I became interested in the NC, in 1968, only little information was available about its contribution to embryogenesis in amniote vertebrates. Moreover, knowledge about the NC in lower vertebrates was only partial. It seemed therefore that the identification of the derivatives of the NC in birds was a chapter of embryology that had to be further documented, and the quail-chick marker system seemed to be perfectly suited to do so.

Quail-chick chimeras were constructed as represented in **Figure 1**. The stability of the cell labeling provided by the tissue association of these two species of birds, allowed the migration and fate of the NCC to be followed during the whole incubation period and even post hatching. The chimeras were able to hatch and displayed a normal growth and behavior before being, unexpectedly, subjected to immunological rejection (Kinutani et al., 1986).

The systematic exploration of the fate and migration pathways of NCC along the entire neural axis led us to establish which cell types originate from the NC and from which level of the neural anlage they migrate. We could also find NC derivatives that were so far unknown, such as carotid body and calcitonin-producing cells of the ultimobranchial body (Le Douarin and Le Lièvre, 1970, 1971, Le Douarin et al., 1972, 1974; Pearse et al., 1973, Polak et al., 1974).

Our studies on the development of the PNS led us to recognize the precise levels of the neural axis, its various component cells originated from. It turned out that definite regions of the spinal cord and medulla oblongata were dedicated to yielding the sympathetic, parasympathetic or enteric ganglia, as represented in **Figure 2**. For example, the so-called “vagal” level of the NC, extending from somites 1 to 7 and roughly corresponding to the level of emergence of the vagus nerve, provides the entire gut with NCC that later on, differentiate into the two enteric plexuses (Le Douarin and Teillet, 1971). This showed that the pre- and post-ganglionic neurons of the enteric nervous system (ENS) originate from the same level of the neural tube. In other systems, it was recently shown that NCC located along peripheral nerves gave rise to pigment cells (Adameyko et al., 2009, 2012). In addition, parasympathetic neurons originated from NCC that had migrated along peripheral nerves whose cell bodies reside in the CNS (Espinosa-Medina et al., 2014, 2017; Dyachuk et al., 2014). These findings suggested that the precursors of the enteric ganglia would navigate within the gut along the axons of vagal neurons. Actually, this was recently shown to occur in NCC migrating from somites 1 and 2, which travel along the vagus nerve and give rise to about half of foregut enteric neurons in the chick and mouse (Espinosa-Medina et al. (2017).

We found that, in birds, an additional contribution to the ENS arises from the lumbosacral NC located caudally to the level of somite 28. The vagal and lumbosacral levels of the CNS thus contain the preganglionic neurons of the entire ENS, which, in the gut environment, make contacts with the cholinergic and peptidergic neurons of the enteric plexuses.

A third region of the NC, located between somites 18 and 24, was found to provide the suprarenal gland with adrenal cells producing adrenaline and noradrenaline, together with the sympathetic ganglia corresponding to this axial level, without any contribution to the ENS (Teillet and Le Douarin, 1974). It was designated “adrenomedullary” level of the NC. More posterior levels of the NC generate the accessory aggregates of catecholaminergic cells that develop along the dorsal aorta.

These results were published in the *Journal of Embryology and experimental Morphology* in 1973 (Le Douarin and Teillet, 1973). During the following summer that I spent at Woods Hole, I had the great pleasure to meet the reviewer of this paper, Pr Yntema, who had worked on the development of gut innervation several years before (Yntema and Hammond, 1954, 1955). He said he enjoyed reading the paper, which extended the results he had obtained (using ablation of segments of the neural tube as an experimental approach), by showing that the NC is the exclusive origin of the enteric plexuses.

### ***Developmental plasticity of PNS neural precursors***

The fate map of NC derivatives revealed that NCC arising from the vagal and the lumbosacral levels contribute to the ENS, whereas the entire trunk NC participates in the formation of sympathetic adrenergic derivatives. This raised the question as to whether the neuronal precursors fated to differentiate into adrenergic or cholinergic (enteric) neurons were committed prior to their migration. The alternative being that, according to the level of the neural tube from which they emerge, NCC followed defined migration pathways leading to a particular destination. In this case, the type of differentiation they adopt would depend on signals that they would receive during migration or at their destination (or both). The heterotopic transplantation between quail and chick embryos of the adrenomedullary level to the vagal level of the NC, and vice versa, resulted in differentiation of NCC according to their novel position along the neural axis and not to their fate in normal development: thus, neuroblasts originating from the adrenomedullary level of the NC transplanted to the vagal level, invaded the gut and differentiated into neurons that did not contain detectable traces of catecholamines, but showed physiological characteristics of normal cholinergic parasympathetic gut neurons. Conversely, the vagal NC transplanted more caudally between somites 18 and 24, colonized the adrenal gland and differentiated into adrenomedullary cells.

These data demonstrated the initial plasticity of the autonomic neuroblasts and the influence of environmental cues on their terminal differentiation (Le Douarin and Teillet, 1974; Le Douarin et al., 1975).

### ***The neural crest, source of mesenchymal cells and its contribution to the vertebrate head***

In modern times, the first investigations of NC contribution to the head mesenchyme of avian embryos was achieved by performing isotopic grafts of the neural folds from chickens labeled with <sup>3</sup>H-thymidine into unlabeled embryos (Johnston, 1966; Noden, 1975). These experiments confirmed the participation of the NC to head morphogenesis, but did not allow full identification of the NC contribution to head and neck structures as dilution of the radioisotope marker did not provide long-term labeling of the grafted cells.

Christiane Le Lièvre and I, and also, Johnston (1974) and Noden (1978), undertook experiments aimed at deciphering the role of the cephalic NCC in morphogenesis of the head from the early 1970s onward (Le Lièvre and Le Douarin, 1974, 1975; Le Lièvre 1974, 1978). These investigations were followed later on with Gérard Couly (Couly et al., 1992, 1993, 1998, 2002; Ruhin et al., 2003). Our aim was to see to what extent the NC contributes to the head skeleton and what was the respective role of ectoderm (via the NC) and mesoderm, in forming cephalic skeleton and connective tissues. For this purpose (in contrast to previous experiments involving the

graft of small fragments of the neural tube labeled with 3H-thymidine), entire encephalic vesicles were exchanged between stage-matched quail and chick embryos. This allowed us to see whether the bones were entirely or only partly derived from the NC (Le Lièvre and Le Douarin, 1974, 1975; Le Lièvre 1974, 1978).

The quail-chick chimera experiments have evidenced the dynamics of the wave of NCC, which progressively invades the space between the neural primordium and the superficial ectoderm; these experiments also showed that the role of the ectomesenchyme was quantitatively far more significant than previously thought and that the tissues of NC origin in the head were very diversified. The respective contribution of ectomesenchyme and mesoderm to the various cephalic structures could be established, thus revealing that the NC is a major player in the construction of the vertebrate head, including the skeleton and soft tissues (**Figure 3**).

Apart from the striated muscles of the branchial arches and the vascular endothelium of all the blood vessels, which were derived from the host mesoderm (Couly et al., 1995), the great surprise was that, not only most of the skeletal tissues, but also the connective tissues of the facial area, including dermis and connective cells in muscles, were of NC origin. The cephalic NC also yields adipocytes in the face and neck (Le Lièvre and Le Douarin, 1975; Billon et al., 2007). In addition, brain pericytes and meninges of the telencephalon are NC-derived (except for the blood vessel endothelium); in all the other parts of the CNS, meninges are of mesodermal origin (Le Lièvre, 1974; Le Lièvre and Le Douarin, 1975; Couly et al., 1992, 1993, 1995; Etchevers et al., 1999, 2001).

The striated myocytes forming the iridal muscles as well as the ciliary bodies and the corneal endothelium and stroma, are additional derivatives of the cephalic NC (Creuzet et al., 2005).

### ***The neural crest and the evolution of vertebrates***

These embryological results together with other considerations led Gans and Northcutt (1983) to put forward their concept of the vertebrate “New Head”, according to which emergence of the NC was essential for the evolutionary transition from protochordates to vertebrates. The extant form of cephalochordates, the amphioxus, is a basal chordate devoid of a NC. This is also the case for the urochordates, although NC-like cells have been reported in ascidians (Jeffery et al., 2004, 2008; Abitua et al., 2012; Stolfi et al., 2015). Amphioxus has a poorly developed encephalic vesicle and is devoid of the sense organs that characterize the vertebrate head. According to Gans and Northcutt, the development of a head in the chordate phylum is linked to the appearance of the NC and coincides with a change in life style. Amphioxus is filter-feeder while vertebrates became predators. This was made possible thanks to acquisition of sense organs (vision, smell, audition), which developed from ectodermal placodes that, like the NC, are vertebrate innovations. The vertebrate brain became more and more complex and efficient through the development of associative neural structures, especially in the forebrain, midbrain and cerebellum.

The work that we have done in the late 1980s and early 1990s, which consisted in constructing the fate map of the anterior neural plate, brought about interesting data along this line (**Figure 4**).

By constructing the fate map of the anterior part of the neural plate and thus following the development of the early neural primordium, we have shown that, like in its original configuration in jawless vertebrates (hagfish and lampreys), the anterior most part of the early neural primordium corresponds to the diencephalon (thalamus,

hypothalamus and pituitary gland). The diencephalon corresponds to the anterior end of the notocord while the telencephalon is mostly derived from the lateral and rostral areas of the neural plate (Couly and Le Douarin, 1985, 1987).

Olivier Pourquié in his post-doctoral work demonstrated that the differentiation of the paraxial mesoderm (cephalic and somitic) into cartilage and bone depends upon a signal arising from the notocord (Pourquié et al., 1993), later identified as the secreted molecule sonic hedgehog (Shh). The notocord thus accounts for the formation of the vertebral column and occipital region of the skull. The evolution of the vertebrate phylum is characterized by development of the cerebral hemispheres, peaking in humans. We focused our attention on the early developmental steps of the cerebral hemispheres, and showed that they arise from the lateral areas of the anterior neural plate. After fusion of the neural folds and formation of the encephalic vesicles, these lateral areas undergo intensive growth, so that they develop rostrally beyond the tip of the notocord and the adenohypophysis, in order to form the telencephalon. The adenohypophysis anlage becomes “buried” inside the stomodeal cavity while maintaining its close relationships with the floor of the diencephalon (which yields the hypothalamus) (**Figure 4**).

In the absence of notocord and mesoderm at the telencephalic level, no skeleton of mesodermal origin develops to cover the “new brain” that appeared and enlarged in vertebrates during the course of evolution. The cells of NC origin covered this “new brain”, by producing forebrain meninges and part of the skull (optic and nasal skeleton, frontal and parietal bones). Thus, co-evolution of the anterior brain and of the NC was critical for the development of higher cognitive functions in the most recent forms of vertebrates. Notably, this considerable development of the forebrain has been accompanied by the emergence of sense organs, i.e., the eyes that originate from the diencephalon and the smell organs, whose precursor cells are localized in the anterior neural fold.

The fate map of the neural plate that we published in 1985 and 1987 (Couly and Le Douarin, 1985, 1987) (**Figure 4**) has served as reference for many investigations on the forebrain in mammals, birds as well as xenopus, by other groups.

Acquisition of higher brain functions in vertebrates was matched by a change in life style as compared to the filter-feeder ancestors, the cephalochordates. Vertebrates became able to seek their food and later, even became predators. Their facial skeleton, which is entirely derived from the NC (for references, see Le Douarin, 1982), involves the first organ of predation, the jaw, which is well developed in some teleost fishes. *Evolution of the vertebrate head is therefore characterized by an increased participation of ectoderm via the neural primordium.* The latter not only generates the brain, but also the NC, which is critical to construct the face and yields a large part of the heart and head vasculature (endothelium excluded).

In conclusion, the embryological analysis of NC ontogeny has shed new light on the evolution of the vertebrate phylum. This was recently confirmed by a series of studies that we performed on the role played by the NC on the development of the vertebrate brain.

### ***The neural crest: a signaling center regulating the development of the pre-otic brain***

The origin of this work stems from the observation by several authors (see Le Douarin and Kalcheim, 1999 for a review) that *Hox* genes, which play a critical role in patterning the body in all Bilateria, have, in vertebrates, their anterior limit of

expression between the two rostral most rhombomeres (r1 and r2). This means that most cephalic structures develop in a *Hox*-free domain. As far as the NC is concerned, the cells that migrate to the facial primordium and construct the facial skeleton are *Hox*-negative, whereas those forming most of the hyoid cartilage and the so-called “cardiac NC”, contributing to the conotruncus of the heart (Kirby et al., 1983), express *Hox* genes of the first four paralogous groups. Each rhombomere (or pair of rhombomeres) is characterized by a combinatorial *Hox* gene expression (also designated as “*Hox* code”) (Hunt and Krumlauf, 1991). Mutational analyses carried out in the mouse had shown that *Hox* genes were critical for patterning these NC derivatives as well as for the development of the vertebral column from the paraxial mesoderm, and of the brain stem from the rhombencephalon (Le Douarin and Kalcheim, 1999; Narita and Rijli, 2009 and references therein).

The *Hox*-negative, anterior domain of the cephalic NC, which is responsible for the building of the face and of part of the skull (Couly et al., 1993, 1998, 2002), designated as FSNC (for facial skeletogenic NC), is clearly patterned by another genetic system, which involves vertebrate homologues of drosophila *Orthodenticle* and *Emtyspiracle* genes: *Otx1*, *Otx2* and *Emx1*, *Emx2*, respectively.

The question that we raised was the following: is it possible to get normal development of head and face skeleton if the anterior *Hox*-negative NC is replaced by premigratory NC belonging to the *Hox*-positive domain (e.g., r4-r8)? In previous work, we had shown that the rostral most neural fold, down to the level where the epiphysis forms (i.e., at the mid-diencephalic level), does not undergo EMT and therefore, does not produce NCC (Couly and Le Douarin, 1985, 1987). We first investigated whether, after excision of the FSNC (from posterior half of diencephalon to r1-r2 included), the remaining rostral and/or caudal portion of the neural folds that were left in situ, would regenerate NCC able to construct the head skeleton. The result was that the facial rudiment remained empty of NCC and did not grow following ablation of the FSNC prior to the onset of NCC emigration; hence, a complete absence of facial structures ensued (Couly et al., 2002). In addition, the brain was the site of major defects resulting in anencephaly: the telencephalon was severely reduced as well as thalamus and optic tectum, and the pre-otic brain remained open (Creuzet et al., 2002).

Moreover, when a fragment of the *Hox*-positive neural fold was grafted rostrally to replace the anterior cephalic NC, morphogenesis of the head was equally disrupted. In contrast, within the *Hox*-negative domain (i.e. belonging to the FSNC), the NC exhibited a high regeneration capacity. For example, a quarter of the endogenous *Hox*-negative NC, whatever its level of origin (e.g., diencephalic, mesencephalic or anterior rhombencephalic), was able to reconstitute the entire facial and lower jaw skeleton and to restore brain morphogenesis (Creuzet et al., 2002). Therefore, the information encoding any particular element of the facial skeleton does not belong to the NC proper, but rather is imposed by extrinsic cues. The foregut endoderm was shown to play a role in this morphogenetic process (Couly et al., 2002) and we could demonstrate that one of the signals involved is *Shh* (Brito et al., 2006, 2008). Moreover, gain of function experiments in which *Hox* genes were selectively electroporated into the *Hox*-negative domain of the NC, have shown that expression of these genes impairs formation of facial cartilages and bones (Creuzet et al., 2002).

The role of the rostral cephalic NC (FSNC) in brain development was further investigated at the molecular level. One of the immediate effects of FSNC excision was to dramatically reduce the production of Fibroblast growth factor-8 (*Fgf8*) in the anterior neural ridge (ANR) and, to a lesser extent, in the isthmus, two regions of the brain anlage

that have been recognized as “*brain organizers*” through the production of this signaling molecule. In the absence of the cephalic NC, the branchial arch ectoderm was also deprived of *Fgf8* mRNA. The action of *Fgf8* on both facial and brain development is therefore critical and the dramatic phenotype resulting from FSNC removal can be rescued by exogenous *Fgf8* provided to the operated embryos through *Fgf8*-soaked beads placed in close contact to the ANR (Creuzet et al., 2004, 2006).

From the time they start to migrate onward, cephalic NCC regulate expression of *Fgf8* by the ANR, via the production of anti-Bmp4 secreted molecules, like Noggin and Gremlin. Bmp4 being a strong antagonist of *Fgf8* production (Ohkubo et al., 2002), excision of the cephalic NC results in failure of the development of telencephalon, thalamus and optic tectum, i.e. brain regions derived from the lateral territories of the rostral neural plate. *Fgf8* is crucial for this process and NCC play a regulatory role in the production of this signaling molecule during the early stages of neurogenesis (Creuzet, 2009; Le Douarin et al., 2012 for a review).

Therefore, the role of the cephalic NC in head development is not restricted to providing the cells that build the skeleton and connective tissues of the face. The NC also acts as an *organizing center* able to regulate the activity of both the ANR and the isthmus in brain patterning.

### ***Cell culture of neural crest cells: toward the demonstration of multipotent neural stem cells in the PNS***

Spectacular advances in the discovery of the various NC derivatives as described above, have made crucial the questions of the multipotency of the NCC and of the mechanisms leading NCC to adopt such diversified fates after migration.

The understanding of NCC lineage diversification and, particularly an attempt to identify the fate of single NCC, faces several hurdles: the early NCC in the dorsal neuroepithelium are scarce and overtly similar; in addition, they rapidly start migrating away from the neural folds; moreover, although some aggregate not far from their origin to form the ganglia of the PNS, most of the NCC undergo a wide dispersion in embryonic tissues, populating various distant sites, such as the entire skin and the whole length of the gut.

The large diversity of cell types arising from NC progenitors and the impressive journey of NCC migrating all over the body, are reminiscent of the properties displayed by the *hemopoietic stem cells* of the bone marrow, which produce all the blood cell lineages along life. They were the first tissue stem cells identified in higher vertebrates, mainly thanks to in vitro colony assays and in vivo tracing experiments after cell injections (Till and McCulloch, 1961; Bradley and Metcalf, 1966; reviewed by Metcalf, 2007).

Inspired by these findings as well as by in vitro clonogenic methods developed for human epidermal progenitors (Barrandon and Green, 1985), we began in the late 1980s to investigate avian NCC developmental potentials using in vitro clonal cultures (Baroffio et al., 1988, 1991; Dupin et al., 1990). Single NCC were isolated from the quail embryo during their migration from the mesencephalon and plated individually under microscopic control (Baroffio et al., 1988, 1991; Dupin et al., 1990). After NCC growth in standardized conditions appropriate for the generation of the main NC-derived phenotypes, the resulting clones were analyzed for the presence of various differentiated cell types, thereby providing a retrospective identification of the potentials of the NCC founders. The main result was a striking heterogeneity of the

clones arising from NCC, both regarding size and cellular composition. Several types of multipotent mesencephalic NCC were evidenced, which yielded diverse combinations of glial cells, autonomic neurons, melanocytes and cartilage cells (Baroffio et al., 1988, 1991; Dupin et al., 1990). Noticeably, chondrogenic mesenchymal cells and neural and/or melanocytic cells originated from common progenitors, not from lineage-restricted precursors.

These results revealed the presence of multipotent NCC at early migratory stages, thus providing a direct support for a stem cell lineage model of NCC diversification (Le Douarin et al., 2004; Dupin et al., 2010). In the same time, and using a similar approach of *in vitro* single cell cultures, Sally Temple demonstrated the existence of neural stem cells in the mammalian embryonic brain, able to produce both CNS neurons and glial cells (Temple, 1989).

Together with improvements of culture conditions and assays for recombinant growth factors, the discovery of antibodies suitable for the detection of various phenotype-specific antigens, have led researchers to offer a more comprehensive picture of the developmental repertoire of NCC in avian and mammalian species. This picture underlines a large diversity of multipotent and oligopotent NC progenitors, some of which display self-renewal, a stem cell cardinal feature (for references, see Dupin and Sommer, 2012; Dupin and Coelho-Aguiar, 2013; Dupin, et al., 2018).

In the head, further *in vitro* studies led us to show that the vast majority of cephalic avian NCC are highly multipotent and capable of differentiating into peripheral neurons and glial cells, melanocytes and mesenchymal cells belonging to the myofibroblast/smooth muscle, chondrocyte and bone cell lineages (Calloni et al., 2007, 2009). These data lend further support for a common cellular origin of cranial PNS and craniofacial mesenchymal tissues of vertebrates.

In the trunk, multipotency or, at least, bipotency of NCC had been suggested since pioneer *in vitro* studies of single avian NCC (Cohen and Konisgberg, 1975; Sieber-Blum and Cohen, 1980). Cohen and Konisgberg (1975) had devised a simple and efficient technique to isolate and culture NCC, based on the observation that they have the ability to migrate *ex vivo*, i.e., from a neural tube explanted *in vitro* (provided it is isolated prior to NCC delamination); after 12-48 hours, the resulting outgrowth of NCC adherent to the culture dish, could be detached and subcultured, while easily eliminating the neural tube that remained epithelial. This technique is still the cornerstone for many *in vitro* culture studies of avian and mammalian NCC.

In the avian model, NCC at trunk level comprised multipotent progenitors that produced glial cells, neurons, melanocytes and smooth muscle cells, while others were only bipotent, yielding glia and melanocytes or glia and myofibroblasts (Lahav et al., 1998; Trentin et al., 2004). Furthermore, most clonogenic cells of the quail trunk NC were able to differentiate into both neural cell types and osteoblasts (Coelho-Aguiar et al., 2013), thus revealing that avian trunk NCC have dormant mesenchymal developmental potentials. In mammals, Stemple and Anderson (1992) were the first to identify trunk NC stem cells that clonally generated glia, autonomic neurons and smooth muscle cells and were able to self-renew in culture. This type of NC stem cell also gave rise to sensory neurons (Lee et al., 2004; Kleber et al., 2005).

*In vitro* culture of single NCC offered the possibility to analyze the precise role of signaling factors on the maintenance and differentiation of distinct types of NC progenitors (reviewed by Dupin and Sommer, 2012; Dupin et al., 2018). Regulation of NCC self-renewal by particular combinatorial signaling cues or single growth factors was evidenced in multipotent NC stem cells of the rodent trunk NC (Stemple and



Anderson, 1992; Kleber et al., 2005) and in premigratory cranial NCC, isolated in the chick and derived from human ES cells (Keruoos et al., 2015). In birds, bipotent progenitors for melanocytes and glial cells (Trentin et al., 2004) and for glia and smooth muscle cells (Bittencourt et al., 2013) also exhibited self-renewal in vitro, therefore showing that a variety of NC oligopotent progenitors are endowed with stem cell properties.

These data raise the question as to whether similar progenitors and stem cells are present and function in vivo. A first direct evidence for in vivo multipotency and heterogenous potentialities of the early NCC was provided by lineage fate mapping in the chick embryo, in which single premigratory NCC were labeled by intracellular microinjection of a fluorescence dye or using diluted concentrations of a retroviral tracer (Bronner-Fraser and Fraser, 1988, 1989; Frank and Sanes, 1991). More recently, the use of mouse genetic lines driving multiple fluorescent reporters in the early NC allowed to disclose the multipotency of premigratory and migratory trunk NCC in the mammalian embryo (Baggiolini et al., 2015).

Remarkably, the presence of multipotent progenitors extends far more the initial stages of NC migration from the neural primordium. NCC with multiple developmental potentials and self-renewal ability in culture could be first isolated from NC derivatives such as the rat sciatic nerve and embryonic gut, by using fluorescence-activated cell sorting (Morrison et al., 1999; Bixby et al., 2002; Kruger et al., 2002). New advances in genetic methods that provide a permanent marking of the early NCC in the mouse, allowed to tracing their fate until adulthood (Danielian et al., 1998; Chai et al., 2000; Jiang et al., 2000, 2002). These methods led to demonstrate that some NCC with stem cell features persist, not only in NC derivatives (e.g., PNS and ENS ganglia, cornea, carotid body, dental pulp) but also virtually in all tissues of the body (reviewed by Dupin and Coelho-Aguiar, 2013; Motohashi and Kunisada, 2015). Such widespread distribution of NC multipotent cells can be attributed to their migration along the PNS nerves and nerve terminals, which form a network innervating all organs and tissues.

### ***Concluding remarks***

Early vertebrates underwent a major explosion and became able to occupy most of the ecological domains open to large animals on land, in ocean and in the air.

The emergence of the first vertebrates, jawless fishes, from their ancestors the protochordates, was accompanied by the appearance of a special set of cells, the NCC. These cells are found in the extent jawless lampreys and hagfish, and very likely, were also present in the oldest fish-like vertebrates, the ostracoderms. The NCC have been shown responsible for the development of a large array of cell types and structures in the vertebrate embryo. The prominent contribution of the NC, revealed by embryological studies, to the vertebrate head, has been particularly significant in this respect. It led, in 1983 Gans and Northcutt to put forward the notion that this structure played a paramount role in the remarkable evolutionary success of the vertebrate phylum.

The discovery of this peculiar embryonic structure by Wilhem His, 150 years ago, is therefore an anniversary that is worth celebrating.

The number of researchers involved in NC research has known a spectacular increase from the last 1960s onward. The diversity of the scientific approaches that are now directed to decipher the various characteristics of this structure is very remarkable.

Among the fields that have been opened during the last decades is the disclosure of the identification of the gene regulatory network, which controls the specification of the NC territory within the early developing embryo.

The routes followed by the migrating NCC have been difficult to identify. The use of various ways to trace their migration has revealed that, in the trunk, some of the NCC migrate along a dorso-ventral route, between neural tube and somites; while, in the head, NCC move massively underneath the ectoderm. However, the means through which they travel long distances to reach the ventral side of the body and its appendages has remained mysterious. Recently, an important contribution to our knowledge of the migratory behavior of the NCC was to show that they transiently adopt the “Schwann cell precursor” phenotype and travel along the peripheral nerves and nerve terminals, which progressively become ubiquitously present in the body.

The widespread distribution of the NCC has been the reason to designate them as a *fourth germ layer*, a term particularly appropriate in view of the variety of tissues and cell types they generate; noticeable is the fact that the NC is recognized as the source of the first osseous tissue (dentine) that appeared in vertebrate evolution.

When they reach their destination, NCC proliferate abundantly. The tissues and derivatives they produce are so diversified and widely distributed that they can be said to accomplish a second round of development in the post-phylogenetic period, which results in a considerable increase in the complexity of the vertebrate body.

Several benefits were conferred to vertebrates by the “acquisition” of a NC. One is the coordination of their physiological functions via the PNS. Another benefit resides in their better adaptation capacities to the variations of the environment. For examples, the vasomotricity of their peripheral blood vessels, which plays a major role in thermal regulation, is controlled by the PNS, and the protective screen against the deleterious effects of UV radiations is provided by NC-derived pigment cells that colonize the skin. Moreover, the NC participates in the construction of the cardiovascular system, and, as described above in this chapter, the cephalic NC plays a major role in the development of the most recent structures of the brain, namely, forebrain and midbrain.

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## ***Figure legends***

**Figure 1:** Quail-chick chimeras for investigating the fate of avian NCC.

(A) Schematic of the construction of quail-chick chimeras of the neural tube at “adrenomedullary” trunk level (somites 18 to 24). The neural tube was removed from chick host (1a, 1b) and replaced by its equivalent (1c), previously taken at the same level from a quail donor of the same developmental stage (2). (B, C) Transversal sections of chick host embryo (as indicated in A) stained with Feulgen, after removal of the neural tube (B) and when removal was followed by quail neural tube grafting (B). (C) Resulting chimeric birds at post-hatching stage (C) and at three months old (D), which exhibit quail-specific pattern of brown pigmentation at brachial level, due to colonization of the host skin by the grafted NC-derived melanocytes.

**Figure 2:** Fate map of NC derivatives in the avian embryo as determined by quail-chick chimeras.

(A) Fate map of neural and non-neural derivatives of the NC along the neural axis, represented in the cephalic NC (left, 7-somite stage embryo) and trunk NC (right, 28-somite stage embryo). The rostro-caudal levels of origin of the various NC phenotypes are shown with color-coded vertical bars. While the whole (cephalic and trunk) NC gives rise to pigment cells (grey bars), the origin of mesenchymal derivatives (including skeletal and connective tissues) (green bar) is confined to the cephalic NC from mid-diencephalon down to r8, corresponding to the level of somite 4 (S4). Definite regions of the NC yield PNS derivatives, including sensory (blue bars), parasympathetic (ciliary ganglion) (yellow bar), sympathetic (red bar) and enteric (orange bars) ganglia.

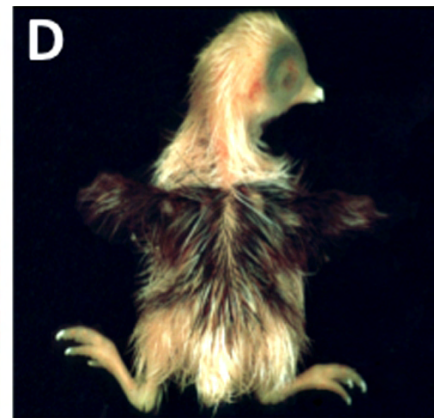
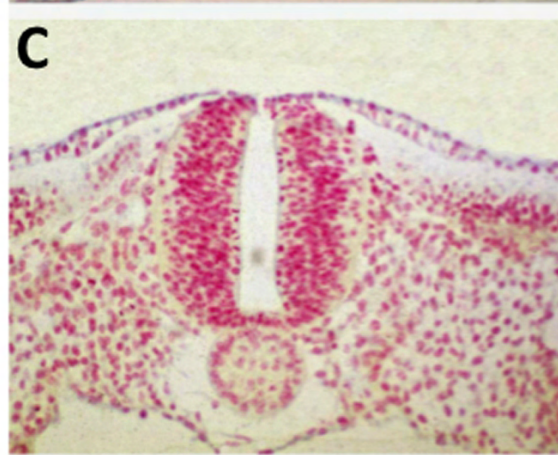
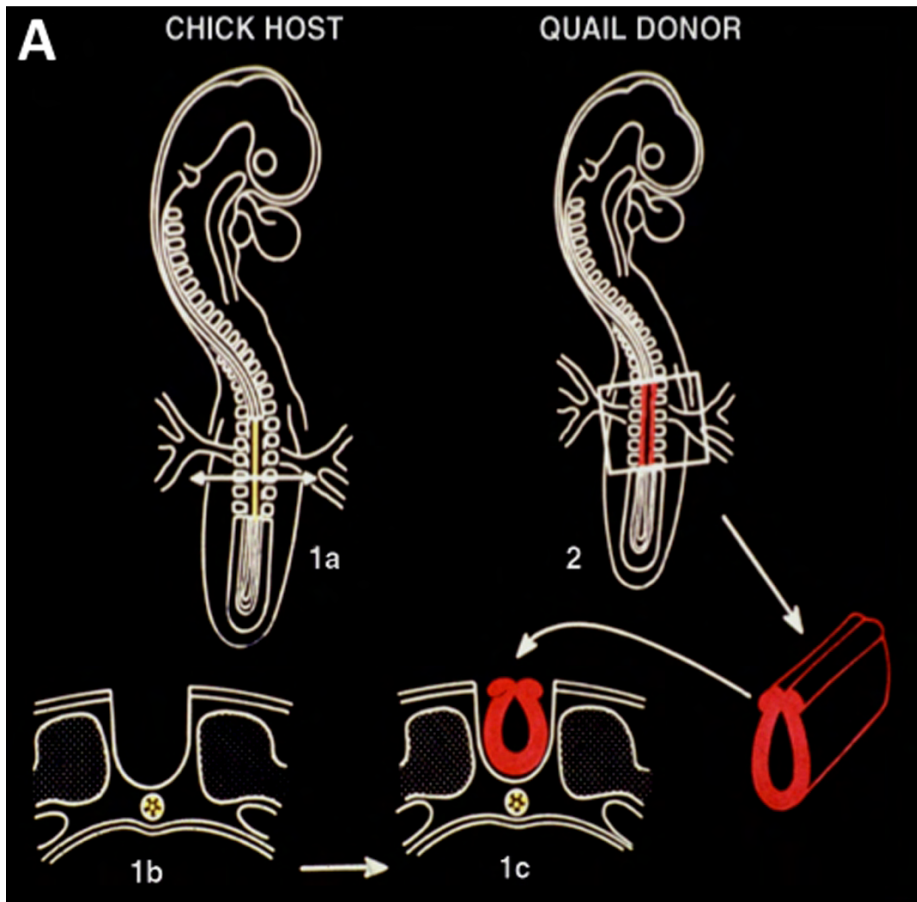
Endocrine (adrenomedullary) cells originate from the trunk NC between somites 18 and 24 (purple bar). (B) Schematic representation of a chick embryo of 28 somite-stage, showing various regions of the NC along the neural axis and their respective derivatives (same color-coding as in A) in ENS plexuses (orange), sympathetic ganglia including the superior cervical ganglion (SCG) (red), parasympathetic ciliary ganglion (CG), and in the medulla of the adrenal gland (AD.GL.).

**Figure 3:** Respective contribution of the NC, cephalic mesoderm and rostral somites to the head skeleton.

Schematics of the lateral right (A) and basal (B) views of the avian chondrocranium at E10, showing the triple origin of the head skeleton, from the NC (red), cephalic paraxial mesoderm (blue) and anterior somitic mesoderm (green). (A) All the facial bones and cartilages and part of the skull derive from the NC whereas the posterior and occipital skeleton is mostly of mesodermal origin. (B) The basisphenoid bone including the sella turcica has a dual origin, from both mesoderm and NC. Annotated bones: 1, angular; 2, basibranchial; 3, basihyal; 4, ceratobranchial; 5, columella (a) and otic capsule (b); 6, dentary; 7, epibranchial; 8, entoglossum; 9, ethmoid; 10, exoccipital; 11, frontal; 12, interorbital septum; 13, jugal; 14, maxilla; 15, Meckel's cartilage; 16, nasal capsule; 17, nasal; 18, occipital (basi); 19, postorbital; 20, quadrate; 21, palatine; 22, parietal; 23, premaxilla; 24, pterygoid; 25, quadratojugal; 26, scleral ossicles; 27, sphenoid (a, basipost-sphenoid; b, basipre-sphenoid); 28, supraoccipital; 29, squamosal; 30, temporal; 31, vomer. (Adapted from Couly et al., 1993).

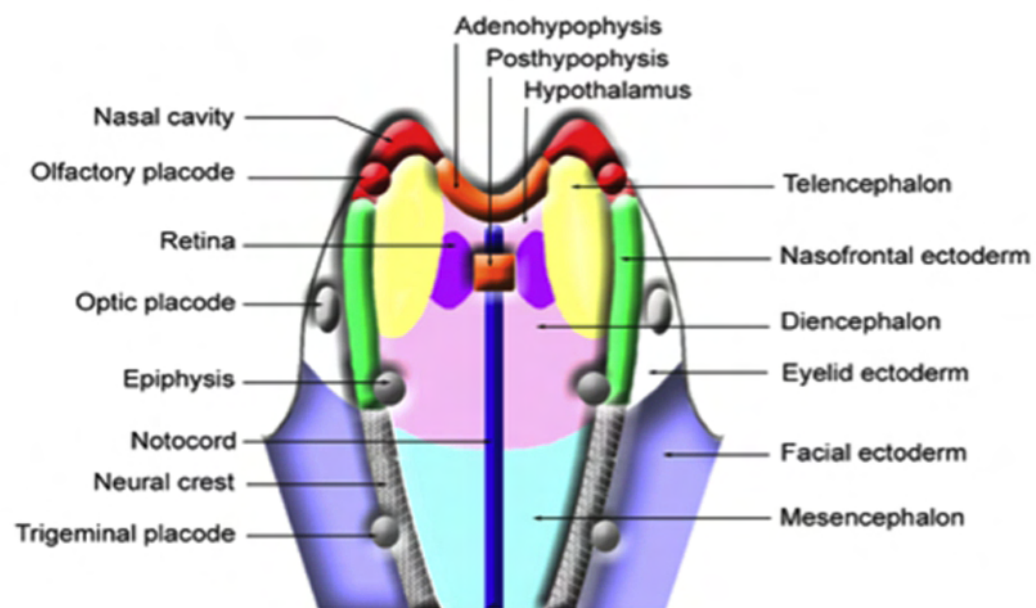
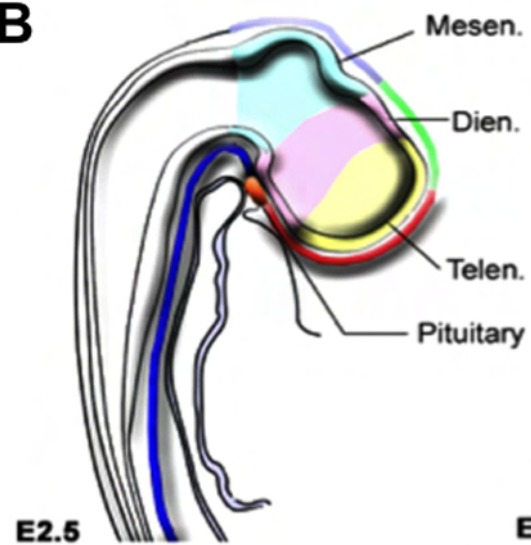
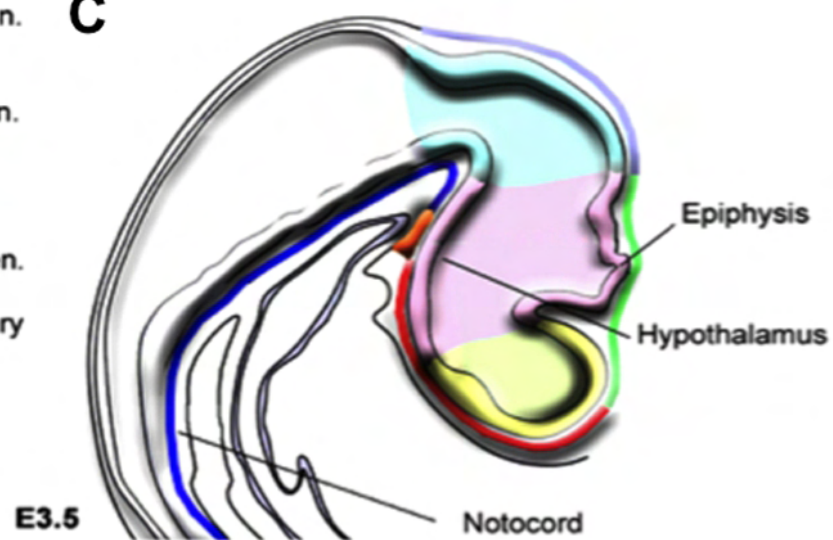
**Figure 4:** Fate map of the early neural plate in the avian embryo.

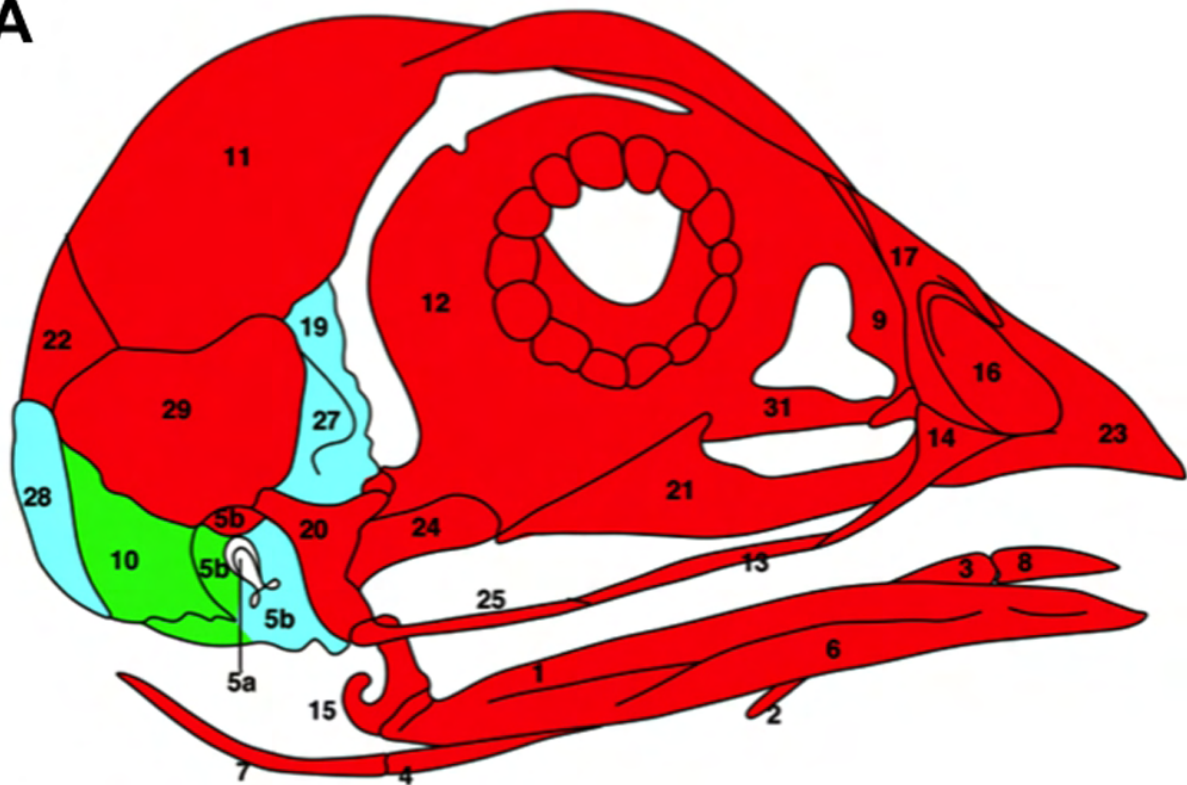
(A) The fate of neural plate territories was determined in the avian embryo at 3-somite-stage (E1.5) by using quail-chick transplantations (Couly and Le Douarin, 1985, 1987). The NC anterior limit is located in the diencephalic neural fold at the level of the epiphysis anlagen; rostral to this limit, the neural fold gives rise to the nasofrontal ectoderm. (B, C) Diagrams of sagittal views at E2.5 (B) and E3.5 (C) show subsequent growth and morphogenesis of neural plate territories (same color-coding as in A). Telencephalic presumptive territories (A, yellow) have joined in the midline and dramatically expanded (B, C, yellow) rostrally to the tip of notocord (in blue). Dien, diencephalon; Mesen, mesencephalon; Telen, telencephalon. (Adapted from Le Douarin and Kalcheim, 1999).







**A****B****C**

**A****B**