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Abbreviations:

DRG, dorsal root ganglion; E, embryonic day; EDN, endothelin; ENS, enteric nervous system; HSCR, Hirschsprung disease; MEN, multiple endocrine neoplasia; NC, neural crest; PNS, peripheral nervous system; QCPN, Quail non Chick Peri-Nuclear; r, rhombomere; SCP, Schwann cell precursor; WS, Waardenburg syndrome.

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Abstract

We review here some of the historical highlights in exploratory studies of the vertebrate embryonic structure known as the neural crest. The study of the molecular properties of the cells that it produces, their migratory capacities and plasticity, and the still-growing list of tissues that depend on their presence for form and function, continue to enrich our understanding of congenital malformations, pediatric cancers and evolutionary biology. Developmental biology has been key to our understanding of the neural crest, starting with the early days of experimental embryology and through to today, where increasingly powerful technologies contribute to further insight into this fascinating vertebrate cell population.

Introduction

Wilhelm His is credited with the first description of the neural crest (NC), from careful observations of chick embryos under the microscopes of the era. He had noticed, on the dorsal side of the closing neural tube, the formation of a *cord* of cells lying *between* the superficial ectoderm and the underlying neural tube, which he called *Zwischenstrang* (intermediate cord) (His, 1868). His described cells emerging from it, gliding over the surface of the neural tube, aggregating laterally and forming the spinal ganglia. The initial area where the lateral edges of the neural plate folded and adjoined was therefore first called the "ganglionic crest". Later, Arthur Milnes Marshall introduced the term "neural crest" as "a continuous longitudinal band, which grows out along the mid-dorsal line of the spinal cord" (Marshall, 1878). His' discovery, although not readily accepted at that time by his colleagues, was of great significance, as will be shown in this overview of what turned out to be a major component of the vertebrate embryo.

The reason why the NC had been mostly ignored by most embryologists for the half century prior to His was its cardinal characteristic, which is to be transitory. The NC population forms, depending on the species, approximately when the lateral borders of the neural plate (also called the "neural folds") join dorsally to close the neural tube. Thereafter, NC cells undergo an epithelial-to-mesenchymal transition, migrate away from their source to invade the entire developing embryo, and settle in selected sites, where they develop into a large variety of cell types. As they disperse, NC cells are usually indistinguishable from those of the embryonic tissues through which they move. Our knowledge about the fate and contributions of NC cells to the vertebrate body was acquired through various experimental strategies – in numerous model systems - that complemented earlier observational studies initiated by His.

This review will first provide a historical overview of the progressive acquisition of our knowledge about the NC, starting with how the NC came to be recognized as unique structure in the vertebrate embryo. We then cover ablation and cell tracking experiments that gave the initial insights into NC cell fate and contributions.

From the late 1960s onward, the avian model was the subject of intensive studies through the construction of chimeric embryos, but the NC was also extensively studied in other vertebrates, in particular, fish and amphibians. We describe how the highly diversified derivatives of the NC spurred investigation into the multipotency of individual NC cells, and how *in vitro* analyses revealed the existence of NC stem cells. Remarkable genetic advances in mice then made it possible to tackle the problem of NC fates and migration routes in mammals. Together with the fundamental knowledge that emerged from the studies on non-mammalian species, this more recent approach is having a remarkable impact on our interpretation and understanding of many human pathologies attributable to abnormal development or function of NC derivatives. This question will be discussed in the last part of this review.

In choosing such a story, we necessarily omit much important work. Functional genetics and *in vivo* live imaging in *Xenopus*, zebrafish and chick were instrumental in deciphering the cellular and molecular mechanisms of NC cell delamination and migration; we refer readers to other reviews for further information (Ahlstrom and Erickson, 2009; McKinney et al., 2013; McLennan et al., 2015; Theveneau and Mayor, 2012; and references therein). A large body of work has unveiled how signals and regulatory genes control the acquisition of the distinct fates of NC cells (recently reviewed in Baster et al., 2018; Bhatt et al., 2013; Uesaka et al., 2016). Finally, an important breakthrough in NC biology was the molecular analysis, mainly in chick and *Xenopus*, of the gene regulatory networks involved in the individualization of this neural structure, which took place at the turn of the 21st century (Bae et al., 2014; Buitrago-Delgado et al., 2015; Martik and Bronner, 2017; Milet et al., 2013; Plouhinec et al., 2013). We hope readers find these articles complementary to our overview.

Recognition of the neural crest as a distinct embryonic structure

Due to the transitory nature of the NC and the extensive migration of its constituent cells, the existence of the NC as a distinct embryonic structure was for many decades the subject of active controversy. It took over eighty years to settle the debate, when Sven Hörstadius stated in his landmark monograph that “it seems clearly established today [in 1950] that the neural crest forms a special rudiment already present in the open neural plate stage” (Hörstadius, 1950).

As mentioned above, His’ views were not widely adopted by the embryologists of that time. Among others, Francis Maitland Balfour (1881; Balfour and Foster, 1876) and Nikolai Feofanovich Kastschenko (1888) thought that spinal nerves were outgrowths of the spinal cord and not produced by the NC; moreover, the spinal ganglia were assumed to originate from the myotomes (and hence be mesodermal in origin). Given this, it is curious to note that the capacity of this ectodermal structure to yield mesenchymal cells was one of the first NC derivatives to be readily identified, using a fish model. The same Kastschenko, working on selachian development, concluded that some of the head mesenchyme originated from the cephalic NC,

while Nikolaus Goronowitsch (1892; 1893) recognized, from his studies on teleost embryos, that the cephalic NC provided the head with skeletogenic mesenchyme. However, this author denied a NC origin for the spinal ganglia.

The intense debates generated by these discrepancies could have been resolved by the work of Julia Platt (1893; 1897). She found that, as well as cranial ganglia and nerves, the NC gave rise to mesenchymal cells, including the cells contributing to the bones and cartilage of the visceral arches and producing the dentine of the teeth. She even created the term *mesectoderm* for the mesenchyme of ectodermal origin that she distinguished from the *mesentoderm* derived from the mesodermal germ layer. These conclusions, drawn by one of the rare women involved in science at that time, were hardly recognized, and further investigations in other vertebrate species by different authors did not help to clarify the debate: some supported, while others refuted, that mesenchymal cells could be produced by the ectoderm (eg. Holmdahl, 1928; Landacre, 1921; Stone, 1922).

The roots of this controversy grew out of Karl Ernst von Baer's "germ layer theory" (1828), according to which homologous structures within the same animal and across different animals must be derived from material belonging to the same germ layer (ectoderm, mesoderm or endoderm). Mesenchymal (loose) cells, and specifically the skeletal tissues that differentiate from them, could thus arise only from mesoderm. It was only in the 1920s that observations associated with experimental work in amphibian embryos (Landacre, 1921; Stone, 1922; Stone, 1926) convinced the scientific community that a proportion of mesenchymal cells of the vertebrate embryo are ectodermally derived *via* the NC.

Following the migration of neural crest cells and discovering their fate

Destruction or microsurgical removal of the neural tube or neural folds *in situ* was widely used in the early twentieth century and provided some fundamental knowledge for the identification of some NC derivatives and their origin along the neural axis (Dushane, 1938). However, extirpation of embryonic territories at early stages can sometimes trigger regenerative responses, which are able to compensate for certain types of ablations. Thus, this approach can establish that a primordium is necessary for the formation of a given structure, but not that it contributes directly to its tissues.

Labelling distinct regions of the neural primordium *in situ* with vital dyes (such as Nile Blue or Nile Red, that coloured but did not poison cells) enabled and confirmed important discoveries initiated by ablation approaches. For example, in addition to the spinal ganglia, NC cells were shown to be involved in the production of pigment cells and the cells that line nerves throughout the entire PNS, as well as contributing to the teeth and to the dorsal fin in fish (Dushane, 1938; Twitty and Bodenstein, 1941). This technique was not entirely reliable due to dye diffusion over time, but there were good examples of its efficacy, such as the thorough studies performed by Hörstadius and Sellman (1941; 1946) on the contribution of NC cells to

amphibian pharyngeal arch cartilage.

Another means of tracking cells through development was by constructing embryonic chimeras *via* cell or tissue transplantation between two distinct embryos; the great potential of this technique was unravelled in amphibian species. Christiaan Pieter Raven (1931) was the first to trace the fate of NC cells by exchanging defined fragments of the neural folds between two distinct species: *Triturus* (a salamander) and *Ambystoma* (the axolotl). The identification of host *versus* donor cells, based solely on differences in the size of their nucleus, was highly labour-intensive and imprecise (single-cell resolution was not possible). Nonetheless, this method permitted recognition of the origin of populations of cells in chimeric tissues, and thanks to it the NC origin of dental papillae – the mesenchyme below each tooth bud that gives rise to dentine and pulp – was discovered in amphibians (Raven, 1935) nearly 70 years before its confirmation in mammals.

Lineage tracing made significant progress through the use of autoradiography to detect tritiated (^{3}H)-thymidine incorporated into dividing neuroepithelial cells after injection into the brain (Sauer and Walker, 1959, in chick; Sidman et al., 1959, in mouse). This technique allowed grafts of defined neural territories labelled in a donor embryo to be implanted in an unlabelled recipient of the same species. Jim Weston (1963) and later Malcolm (Mac) Johnston (1966) and Drew Noden (1975) used the approach to follow the migration of chicken NC cells, while Pierre Chibon (1964; 1967) applied it in *Pleurodeles*, a salamander. While such labelling suffers a few major limitations, including instability and toxicity, several important results can be credited to it, such as the confirmation of the NC origin of spinal and sympathetic ganglia and of pigment cells (Weston, 1963). The derivation of Schwann cells from a mesodermal source was definitively excluded, and Schwann cells were recognized as NC-derived. By implanting ^{3}H-thymidine-labelled *Pleurodeles* NC cells into an unlabelled host, Chibon (1967) confirmed the NC origin of major components of the head skeleton, as did Johnston (1966) in the chick.

Quail-chick chimeras led to a comprehensive avian neural crest fate map

The quail-chick marking system provided a means to study the development of the NC in amniotes, which had not been used as often in experimental embryology as amphibians or fish. It was designed by one of us (Le Douarin, 1969) following the observation that cells of the quail (*Coturnix coturnix japonica*) could be readily distinguished from those of the chick by the structure of their nucleus. Quail nuclei at all life stages have a large nucleolus, caused by a mass of heterochromatin associated with the nucleolus. This particularity is rare in the animal kingdom (Le Douarin, 1971a) and does not exist in the chick (Le Douarin, 1969; Le Douarin, 1971b). When quail cells are transplanted into a chick embryo or associated with chick tissues *in vitro*, the cells of each species retain their nuclear characteristics and can be readily distinguished at the single-cell level in chimeric tissues using the Feulgen-Rossenbeck histological nuclear stain, provided

that the section includes the nucleolus (Le Douarin, 1973). A significant improvement in cell identification in these chimeras occurred with the production of monoclonal antibodies that recognized species-specific antigens. The antibody against a peri-nuclear antigen, QCPN (for Quail non-Chick Peri-Nuclear), prepared by Bruce and Jean Carlson, turned out to be particularly useful from the mid-1990s onward.

Avian NC cell fates were systematically investigated by constructing chimeras in which a fragment of the neural tube (or neural fold at the head level) of a chick embryo was removed prior the onset of NC cell emigration, and replaced by its exact staged counterpart from a quail embryo (reciprocal chick-to-quail grafts were initially performed to validate the approach). The stability of cell labelling allowed migration and fate of NC cells to be followed during the entire incubation period and even for a time after birth (Teillet and Le Douarin, 1970; Kinutani and Le Douarin, 1985). Such experiments were effective in determining which cell types came from the avian NC, and from which axial levels of the neural primordium (for references, Le Douarin, 1982; Le Douarin and Kalcheim, 1999) (**Table 1)**. Together, they provided a fate map of NC cells regarding production of mesenchymal cells, pigment cells and the sensory, autonomic and enteric components of the PNS (**Figure 1**).

The contribution of the NC to cephalic structures was, for the first time, fully demonstrated (Le Lièvre and Le Douarin, 1975; reviewed in Le Douarin and Kalcheim, 1999), and new endocrine derivatives, such as the carotid body and the calcitonin-producing cells of the ultimobranchial body, were identified (Le Douarin and Le Lièvre, 1970; Le Douarin et al., 1972; Le Douarin et al., 1974; Pearse et al., 1973; Polak et al., 1974). Regarding the origin of the PNS, defined regions of the spinal cord and posterior hindbrain were found to be dedicated to producing the sympathetic, parasympathetic or enteric ganglia (**Figure 1**). For example, the so-called “vagal” level of the NC, corresponding to the emergence of the vagus nerve between the axial levels of somites 1 to 7, provides the entire gut with the two plexuses of the enteric nervous system (ENS) whose neurons become cholinergic (Le Douarin and Teillet, 1973). Some of the vagal NC cells (from pre-somitic levels to somite 3) also contribute to the perivascular walls of the posterior pharyngeal arteries and septation of the cardiac outflow tract (Arima et al., 2012; Kirby et al., 1983; Le Lièvre and Le Douarin, 1975) (**Figure 2**). Such work also showed that both pre- and post-ganglionic neurons of the ENS originate from the same level of the neural axis, while the lumbo-sacral NC provides an additional contribution to the posterior ENS (Burns and Le Douarin, 1998; Le Douarin and Teillet, 1973; Yntema and Hammond, 1955) (**Figure 1**). The adreno-medullary NC, located between somites 18 and 24 in the chick embryo (at the level of and just posterior to the forelimb), produces the adrenaline- and noradrenaline-producing cells of the adrenal gland, together with the sympathetic ganglia corresponding to this axial level, but makes no contribution to the ENS (Le Douarin and Teillet, 1974).

These observations raised the question of whether neuronal precursors were pre-committed along the neural axis (*i.e.,* vagal to cholinergic neurons and adreno-medullary to adrenergic neurons), or whether they received inductive signals on their journey and/or at their site of arrest. When vagal NC was transplanted

posteriorly to the adreno-medullary level (and vice versa), the PNS developed normally, showing that neuronal precursors were not pre-committed. Rather, their final phenotype is regulated by external cues acting at their site of arrest and possibly also during the course of their migration. This experiment was the first to demonstrate that the differentiation of neurons is dependent upon signals received from their environment (Le Douarin and Teillet, 1974; Le Douarin et al., 1975), one example out of many where specific insights about the NC have had broad implications for our understanding of how development works.

Decisive contribution of mouse genetics to the knowledge of the mammalian neural crest

Embryonic manipulation is technically challenging in mammals, and access to rat and mouse embryos of post-implantation stages required the development of whole-embryo culture techniques. Thereafter, focal dye labelling, cell microinjection and perturbation experiments all provided insightful information on the migration of mammalian NC cells (Osumi-Yamashita et al., 1994; Serbedzija et al., 1990; Serbedzija et al., 1992; Takakubo et al., 1986; Tan and Morriss-Kay, 1986). These data, however, were limited to early NC developmental stages due to the short period of survival of cultured embryos.

More recently, genetic approaches in mice that rely on the specificity of regulatory elements to drive gene expression in a given tissue and a given moment of development have facilitated long-term mammalian lineage tracing. Conditional site-specific recombination was as beneficial to the study of the NC as it was to developmental biology as a whole. This was first achieved by combining an allele driving the expression of a bacteriophage-derived *Cre* recombinase with an allele containing a *loxP*-flanked (floxed) sequence to be excised in the target cell (Sauer and Henderson, 1988). A particularly influential transgene construction, devised by Andy McMahon and colleagues, contained enhancers driving *Wnt1* gene expression in the murine dorsal neural tube and, thereby, in the premigratory NC (Danielian et al., 1998). One mouse line developed from this construct (commonly known as *Wnt1-Cre*) was particularly useful for the recognition of mammalian NC derivatives. Although some species-specific differences were observed, most derivatives of the NC were similar in mouse and avian species. *Wnt1-Cre*-labelled NC cells contributed to head skeletal elements, ocular and periocular tissues, teeth and the palate (Chai et al., 2000; Gage et al., 2005; Jiang et al., 2002; Matsuoka et al., 2005); the cardiac outflow tract, tricuspid valves and surrounding tissues (eg., El Robrini et al., 2016; Jiang et al., 2000); and the skin, at the level of innervated hair follicles and glands (Wong et al., 2006). An advantage of *Wnt1-Cre*-driven recombination was that it enabled permanent expression of traceable genetic markers such as *LacZ*, allowing new (often late) sites of NC colonization to be identified, such as the marrow of adult long bones (Nagoshi et al., 2008).

However, this genetic system has drawbacks: the *Wnt1-Cre* allele also activates ectopic Wnt signalling in

the ventral midbrain, perhaps because of the confounding genomic rearrangements induced by insertion of the transgene (Goodwin et al., 2017; Lewis et al., 2013). Other *Cre* lines have been developed to target some or most NC cells and examine distinct aspects of their development: *Sox10-Cre*, *htPA-Cre* and *P0-Cre* label migratory rather than premigratory NC cells, for instance (Matsuoka et al., 2005; Pietri et al., 2003; Yamauchi et al., 1999). Additional advantages of the Cre-lox system, largely exploited over the last fifteen years, have been to combine such analyses of lineage tracing with the responses of the same cells to genetic gain- or loss-of-function or to modulate the timing of Cre expression, but we will leave the exploration of these many recent studies to our readers' diligence.

The neural crest and the vertebrate head

The above-described experimental approaches illuminated the remarkable range of NC-derived tissues and organs in the head. The role of the mesectoderm was found to be quantitatively far more important for head development than previously thought before the 1970s (**Figure 2**). Tissues of NC origin in the head are not only skeletogenic for the entire facial skull, inner ear and rostral brain case, but also include soft tissues such as the dermis, fascia and tendons of the facial and ocular muscles, interstitial cells in all head and neck glands examined, and adipocytes of the face and neck (Billon et al., 2007; Couly et al., 1993; Grenier et al., 2009; Le Lièvre and Le Douarin, 1973; Le Lièvre and Le Douarin, 1975; Noden, 1978) (**Table 1**). Strikingly, the striated myocytes of avian iris muscles (Nakano and Nakamura, 1985), but also the ciliary bodies and the corneal endothelium and stroma, are all specialized derivatives of the cephalic NC in both chicken (Creuzet et al., 2005; Johnston et al., 1979) and mouse (Gage et al., 2005).

Cephalic NC cells are also the source of vascular smooth muscle cells and pericytes in a restricted area of the circulatory system (**Figure 2**). This "branchial" sector extends distally from the cardiac outflow tract, whose septation depends on the presence of NC cells (Kirby et al., 1983; Le Lièvre and Le Douarin, 1975; Waldo et al., 1998) along arteries and jugular veins to the distal capillary beds and choroid plexuses of the face and forebrain (Bergwerff et al., 1998; Couly and Le Douarin, 1987; Etchevers et al., 1999; Etchevers et al., 2001) In the forebrain, NC cells give rise to pericytes and to meninges (dura mater and pia mater), although not to associated vascular endothelium (Couly and Le Douarin, 1987; Etchevers et al., 1999; Le Lièvre and Le Douarin, 1975), an observation later borne out in mice (Jiang et al., 2002). In contrast, both meninges and pericytes are of mesodermal origin in all other parts of the brain and spinal cord (Couly et al., 1992; Couly et al., 1995; Pouget et al., 2008).

The embryological results uncovering the paramount role of the NC cells in building various head tissues, together with other considerations, led Carl Gans and R. Glenn Northcutt (1983) to develop the concept of the vertebrate "New Head", according to which properties of placodal sensory organs and the newly acquired NC enabled the evolutionary transition from protochordates to vertebrates. The development of the head as we know it – with protection by the skull, prominent jaws and highly developed sensory organs

and brain – coincided with changes in animal lifestyle, from filter-feeding animals to vertebrates endowed with active predation and locomotion and sophisticated sensory-motor modalities. This theory was updated to account for important experimental embryological findings in the interim (Northcutt, 2005) and has been largely accepted. Thus, the NC is a crucial innovation underlying vertebrate success and diversification. This aspect of the NC provides a powerful demonstration of how embryology can inform evolutionary questions.

The contribution of the NC to the vertebrate head extends to the brain, even though NC cells do not directly produce brain tissue (Creuzet et al., 2004; Etchevers et al., 1999). Experiments performed on avian embryos revealed that both fore- and midbrain development depends upon the production of BMP-antagonizing factors by the region of the cephalic NC fated to construct the facial skeleton ("Facial Skeletogenic Neural Crest" or FSNC: NC from the posterior half of the diencephalon down to the level of rhombomeres 1 and 2 included). Excision of the FSNC results in the absence of facial structures, but the brain also shows major defects: failure of the development of telencephalon, thalamus and optic tectum, those brain regions derived from the lateral territories of the rostral neural plate (Couly and Le Douarin, 1987). Production of fibroblast growth factor 8 (Fgf8) by two secondary brain organisers, the anterior neural ridge (ANR) and the isthmus between midbrain and hindbrain, is crucial for the development of the encephalic vesicles. One of the early consequences of FSNC removal is a strong decrease in the production of Fgf8 by these two areas, and exogenous Fgf8 was able to rescue the deleterious effects of FSNC excision on brain development (Creuzet et al., 2004). FSNCs achieve this effect by producing anti-Bmp4 secreted molecules like Noggin and Gremlin. Bmp4 is a strong antagonist of Fgf8 production (Ohkubo et al., 2002), and NC cells therefore play a regulatory role in the production of Fgf8 during the early stages of neurogenesis (Creuzet, 2009; reviewed in Le Douarin et al., 2012).

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. It also acts as an organizing centre, able to regulate the activity of both the ANR and the isthmus in brain patterning.

Multipotency and stemness of neural crest cells

In the late 1980s, labelling with fluorescent vital dyes further advanced our knowledge of NC fate. A painstaking but efficient method pioneered by Marianne Bronner and Scott Fraser involved the microinjection into single NC cells of a large fluorescent carbohydrate, which avoided issues of membrane-to-membrane diffusion (Bronner-Fraser and Fraser, 1988). Following single cell progeny (until the dilution of the dye by cell divisions) gave insight into their early potential and migratory behaviour; it allowed demonstrating for the first time *in vivo* multipotency of individual premigratory NC cells in the chick (Bronner-Fraser and Fraser, 1988; Bronner-Fraser and Fraser, 1989; Fraser and Bronner-Fraser, 1991), a finding that was later fully confirmed in mammals through NC in vivo lineage tracing using the R26R-Confetti mouse model (Baggiolini et al., 2015). Microinjection of a fluorescent dye into individual NC cells

was subsequently applied to *Xenopus* (Collazo et al., 1993), zebrafish (Raible and Eisen, 1994; Schilling and Kimmel, 1994) and whole embryo mouse cultures (Serbedzija et al., 1994). In a complementary approach, *LacZ*-expressing retroviruses were injected at low dilution into the neural tube, and led to the *in vivo* identification of bipotent neuron/glial NC precursors forming the dorsal root ganglia (Frank and Sanes, 1991).

The wide diversity of cell types arising from the NC and their extensive migration within the body are features they share with hematopoietic stem cells, which give rise to all blood cell lineages throughout life and were the first tissue-specific stem cells identified in higher vertebrates (Till and McCulloch, 1961; Bradley and Metcalf, 1966; reviewed by Metcalf, 2007). Inspired by *in vitro* colony assays developed for hematopoietic progenitors, the developmental potential of single NC cells began to be studied *in vitro* in the late 1980s (see **Timeline**). In some of the first experiments, mesencephalic quail NC cells explanted at an early migratory stage were found to give rise to various combinations and numbers of glial cells, adrenergic neurons and melanocytes, together with less frequent cartilage cells (Baroffio et al., 1988; Baroffio et al., 1991; Dupin et al., 1990). Around this time, and using a similar *in vitro* clonal assay, stem cells producing both neurons and glia in the mammalian embryonic CNS were also discovered (Temple, 1989). Further investigations showed that single quail cephalic NC cells are highly multipotent *in vitro*, giving rise to peripheral neurons and glia, melanocytes, chondrocytes, osteocytes and fat cells (Calloni et al., 2007; Calloni et al., 2009; da Costa et al., 2018). These data support a common cellular origin for components of the cranial PNS and craniofacial mesenchymal tissues in vertebrates, suggesting a model of NC cell diversification involving multipotent progenitors rather than a collection of distinctly committed cells (Dupin et al., 2010; Dupin et al., 2018; Trentin et al., 2004).

In the 1970s, neural tubes were explanted and NC cells allowed to migrating away from the explants before being cultured in order to determine their potency (Cohen and Konigsberg, 1975; Sieber-Blum and Cohen, 1980). Variations on this simple and efficient technique of NC cell isolation were later employed for many *in vitro* studies of avian and mammalian (including human) NC cells (Stemple and Anderson, 1992; Thomas et al., 2008; reviewed in Etchevers, 2011; Dupin and Coelho-Aguiar, 2013). Together with improvements in culture conditions and availability of antibodies against a wider range of phenotype-specific antigens, a more comprehensive picture of the developmental repertoire of NC cells has emerged (reviewed in Dupin and Sommer, 2012; Dupin et al., 2018). It transpired that while some avian trunk NC cells were multipotent progenitors (for glial cells, autonomic neurons, melanocytes and smooth muscle cells) others were only bipotent (Lahav et al., 1998; Trentin et al., 2004).

The cardinal stem cell property of self-renewal was first demonstrated in multipotent rat NC cells in culture (Stemple and Anderson, 1992), and similar NC stem cells were also found in the fetal sciatic nerve (Morrison et al., 1999). Self-renewal was subsequently observed in oligopotent NC cells *in vitro* across

species: bipotent progenitors for melanocytes/glial cells (Trentin et al., 2004) and glial/smooth muscle cells (Bittencourt et al., 2013) in the quail; autonomic/sensory neuron progenitors of the early murine NC (Kléber et al., 2005); and premigratory chick and human cranial NC cells maintained as multipotent floating spheres (Kerosuo et al., 2015). Self-renewal is thus a wide-ranging feature of NC cells.

Interestingly, *in vitro* work has revealed that some NC potentials are not fulfilled *in vivo*. For instance, although incapable of becoming mesenchymal derivatives *in vivo*, amniote trunk NC cells could differentiate *in vitro* into cartilage, bone and fat cells (Abzhanov et al., 2003; Billon et al., 2007; Calloni et al., 2007; Coelho-Aguiar et al., 2013; Ido and Ito, 2006; McGonnell and Graham, 2002). Notably, these mesenchymal cell types were found together with neural cells and/or melanocytes in the clonal progeny of NC cells isolated from trunk and cranial axial levels (Coelho-Aguiar et al., 2013; da Costa et al., 2018). Multipotent neural-mesenchymal NC progenitors are therefore present all along the neural axis.

Remarkably, the multipotency of NC-derived progenitors extends into postnatal and adult stages. Genetic lineage tracing in mice has shown that not only the PNS but also hair follicles, cornea and iris, carotid body, dental pulp, bone marrow and oral mucosa contain oligo- or multi-potent NC cells with self-renewal ability in culture (reviewed in Dupin and Coelho-Aguiar, 2013; Motohashi and Kunisada, 2015). Such widespread tissue distribution of multipotent NC cells may be maintained through association with the pervasive network of PNS nerves and their terminals. While located in their “niche” along nerves, NC cells assume a Schwann cell precursor (SCP) identity; however, after detaching from it, they can adopt a non-glial fate and produce melanocytes both during development and lifelong regenerative turnover in hair follicles (Adameyko et al., 2009). More generally, SCPs appear to serve as a reservoir of NC progenitors with diversified fates and ensure the production of some distant or late-forming NC derivatives such as ventral pigment cells, cranial parasympathetic neurons, endocrine chromaffin cells and subpopulations of enteric neurons (reviewed in Furlan and Adameyko, 2018). In the adult, SCPs can assume a stem cell function for osteoblast and dental pulp cell replacement in the continuously growing mouse incisor (Kaukua et al., 2014); they are also likely to be a source for resident NC-derived mesenchymal stem cells in the bone marrow and dermis (Fernandes et al., 2004; Isern et al., 2014; Nagoshi et al., 2008).

Relevance of experimental embryology to human health

Identification of a common NC contribution to very disparate tissues provided an explanation of clinical associations of symptoms that had not been previously understood. Such diseases that could be ultimately traced back to a problem in NC development were called “neurocristopathies” (Bolande, 1974; Bolande, 1997) (see **Timeline**); (reviewed among many others by Etchevers et al., 2006; Watt and Trainor, 2014; Vega-Lopez et al., 2018). Parallel technical advances in molecular embryology and human genetics at the end of the 20th century then guided the first discoveries of genes causing many of these conditions, and in

return, vertebrate embryo models revealed how these genes worked in NC development.

Many birth defects are imputable to neural crest pathology

The neurocristopathy concept first emerged from embryological lineage tracing. While derivatives of a sole NC cell lineage can be affected by a given cancer or malformation, the original insight was that NC derivatives in multiple but distant organs, such as the skin and the intestine, can be simultaneously afflicted in the same disease. Thus, neurocristopathies may occur in isolation or in syndromic combination with other affected NC derivatives. Together, craniofacial and cardiac outflow tract anomalies, affecting areas heavily dependent on NC cell influx and remodelling, are among the most common and frequently associated human birth defects (Beligere et al., 1977). They are characteristic signs of common genetic conditions such as chromosome 21 trisomy or 22q11.2 microdeletion, but also those induced by the environment during pregnancy (for instance, foetal alcohol spectrum disorders or vitamin deficiencies), with a heavy burden in terms of human morbidity and mortality.

For example, piebaldism (the congenital absence of melanocytes from areas of the skin) can lead to a white forelock on the scalp, but it can also be associated with deafness caused by a lack of melanocytes in the cochlea. Many domesticated animals with white ears, including cows, cats and horses, are also deaf for the same reason (Steel and Barkway, 1989). In the congenital Waardenburg-Shah (WS) syndrome type 4 (WS4), piebaldism and/or hearing loss occur in conjunction with a distinct neurocristopathy known as Hirschsprung disease (HSCR), the lack of distal development of the ENS at the level of the rectum and colon, preventing effective defecation. Ablation experiments in chick embryos carried out by Ernest van Campenhout in the 1930s and by Chester Yntema and Warner Hammond in the 1950s laid the groundwork for understanding that HSCR might stem from a genetic problem to which migratory vagal NC would be particularly susceptible (reviewed by Le Douarin and Kalcheim, 1999). Subsequent lineage-labelling experiments (including quail-chick chimeras) showed that NC cells from the posterior hindbrain migrate to the inner ear, the skin, the ENS, and the cardiac outflow tract. A defined NC region could thus yield progenitors in a wide range of tissues, helping to explain how some children with WS4 exhibit head pigmentation defects in combination with cardiac outflow tract malformations and dysmorphic facial features. However, it should be noted that congenital heart defects and cleft palate are also often found together, without HSCR or deafness, illustrating the variety of neurocristopathy associations.

As reviewed earlier, NC cells from specific origins along the anterior-posterior axis can yield unique types of derivatives in the body, such as those from the FSNC that alone give rise to craniofacial bones, the vagal NC that alone contribute to the aortic valve, or the adreno-medullary NC that alone populates the adrenal gland. The regulation of such regional differences involves *Hox* transcription factor genes, full repression of which was shown to be necessary for skull formation in mouse (Gendron-Maguire et al., 1993; Rijli et al., 1993) and chick (Couly et al., 1998). Lineage-specific *Hoxb1* gain-of-function also interferes with vagal NC identity in mice and induces cardiac outflow tract malformations (Zaffran et al., 2018). In the ocular,

cerebral and facial territories reproducibly affected by Sturge-Weber and cerebrofacial arteriovenous metameric vascular malformation syndromes, there are often malformations of adjacent nerves and NC-derived bones (Krings et al., 2007; Nakashima et al., 2014). Quail-chick xenografts have shown that endothelial cells can differentiate inappropriately in response to signals specific to these distinct sectors of NC-derived perivascular or SCP cells (Othman-Hassan et al., 2001); recently, causal somatic mutations in an intracellular signalling effector have been found within endothelial cells of those facial areas affected in Sturge-Weber syndrome (Huang et al., 2017). This implies that there remains a large class of human neurocristopathies to be identified as such, which are not only due to direct or indirect impairment of cell-autonomous NC differentiation, but also to their non-cell-autonomous inductive functions.

Indeed, the definition of "neurocristopathy" has become more flexible, following experiments showing that NC cells can be instructive during organogenesis but sometimes only leave a minor final contribution to the adult organ. For example, destruction or microsurgical removal of the neural tube or neural folds *in situ*, used for decades to identify potential NC contributions to tissues in animal models (Dushane, 1938), helped to interpret the common origin of thymic and cardiac outflow tract malformations in DiGeorge syndrome. In chicken embryos, such malformations can be reproduced in large part by removing NC at the level of the posterior hindbrain (Bockman and Kirby, 1984). Likewise, forebrain, eye and pituitary growth all depend on the modulation of local inductive signals by the FSNC, as mentioned earlier (Creuzet, 2009; Etchevers et al., 1999; Evans and Gage, 2005). These tissues are sensitive to gene mutations that induce holoprosencephaly, which may be associated with cleft palate or outflow tract malformations (reviewed in Cavodeassi et al., 2018).

NC derivatives are preferentially but almost never exclusively affected in most heritable genetic syndromic "neurocristopathies" because the developmental genes involved usually demonstrate pleiotropy. In the 1990s, the first mutations in genes whose normal function is necessary for NC migration into the developing gut were identified in human HSCR patients and several mouse models (reviewed by Bondurand and Southard-Smith, 2016; see **Timeline**). Mutations in *RET*, which encodes a receptor for glial cell-derived neurotrophic factor (GDNF; Edery et al., 1994), or in the genes encoding the cytokine endothelin-3 (*EDN3*) and its type-B receptor (*EDNRB*) (Edery et al., 1996; Hofstra et al., 1996; Hosoda et al., 1994; Puffenberger et al., 1994), all can cause HSCR. In fact, an overlapping range of WS endophenotypes, including pigmentary anomalies, deafness or HSCR, but also limb malformations, can result from mutations in any one of a handful of individual genes (reviewed in Pingault et al., 2010), including the transcription factors *SOX10*, *PAX3*, *MITF* and *SNAI2*. Piebaldism alone can be caused by interstitial deletions of *SNAI2* (Sanchez-Martin et al., 2002), but also by inactivating mutations of *KIT* encoding the receptor for stem cell factor (SCF) (Giebel and Spritz, 1991), which does not cause WS. Importantly, all the aforementioned transcription factors have been characterized in animal models as key players in gene regulatory networks that control the formation and migration of NC cells (eg., Sauka-Spengler and Bronner-Fraser, 2008; Simoes-Costa and Bronner, 2015) but also other processes (hematopoiesis; muscle, eye, brain, or gonad formation). The similarly pleiotropic

growth factors and their receptors (eg., GDNF/RET, EDN3/EDNRB and SCF/KIT) regulate normal NC maturation and the migration of its derivatives –the ENS and melanocyte**s** – that must disperse furthest from their point of origin and throughout large surfaces of the body. However, all of these and others can also be mutated in multiple types of human cancer, and are collectively known as oncogenes.

Neural crest cell susceptibility to cancer

Quail-chick chimeras identified the NC origin of the sympatho-adrenal lineage (Le Douarin and Teillet, 1971), which later segregates into sympathetic ganglionic and adreno-medullary cells in both avian and mouse embryos (Anderson and Axel, 1986; Furlan et al., 2017). This lineage is particularly susceptible to cancers such as neuroblastoma and pheochromocytoma (a secretory tumour of the adrenal gland). It is also the site of a heritable genetic predisposition throughout life to multiple endocrine neoplasias of type II (MEN2)**,** including pheochromocytoma and tumours of the thyroid medulla and carotid bodies, and other paragangliomas that target NC-derived cells (reviewed in Maguire et al., 2015). Interestingly, mutations in the same genes can cause distinct malformations or cancers in NC-derived tissues. Loss-of-function *RET* mutations can cause HSCR but gain-of-function mutations can cause MEN2 (Takahashi et al., 1999); loss-of-function *KIT* mutations in melanocytes can cause piebaldism but gain-of-function mutations can cause melanoma (Willmore-Payne et al., 2005; Curtin et al., 2006). Gene mutations in the *PHOX2B* transcription factor cause life-threatening hindbrain malformations; as they can also affect vagal or sympatho-adrenal NC derivatives, these can be associated with either HSCR or neuroblastoma (Amiel et al., 2003). Indeed, *PHOX2B* is a driver gene and part of the core regulatory circuitry for isolated neuroblastoma (Boeva et al., 2017), the most common cancer in the first year of life.

For many common cancers of the lung, colon or breast, activating mutations in oncogenes are usually acquired in the affected tissues well after fertilization or birth. However, the very same somatic mutations can cause syndromic birth defects in the neurocristopathy spectrum. For instance, post-zygotic mutations in a handful of genes encoding effectors of receptor-activated signalling pathways cause giant congenital melanocytic nevus (eg., Etchevers et al., 2018; Silva et al., 2018), whereas germline mutations of those genes cause cardio-facial-cutaneous, Noonan or autoimmune lymphoproliferative syndromes, among others (eg., Champion et al., 2011; Oliveira et al., 2007). Susceptibility to neuroblastoma, neurofibroma or melanoma is a feature of these and other constitutional neurocristopathy syndromes.

Finally, experimental embryology using mouse (eg., Rinon et al., 2011; reviewed in Ahsan et al., 2017) and zebrafish (eg., White et al., 2011; reviewed in van Rooijen et al., 2017) has introduced the concept that cancers prone to metastasis reactivate the molecular programs favouring migration and tissue invasion normally used and needed by multipotent NC cells. Thus developmental biology has informed not only the direct and indirect roles of neurocristopathies in birth defects but also in cancer throughout life, although neurocristopathies overall disproportionately affect children.

Stem cell-based therapeutic approaches

Since ethical and scientific challenges make it difficult to isolate and study embryonic human NC cells (Thomas et al., 2008), several protocols have been developed to derive NC-like cells from pluripotent human embryonic stem (ES) cell lines (Kerosuo et al., 2015; Lee et al., 2007), induced pluripotent stem (iPS) cells (Avery and Dalton, 2015; Menendez et al., 2012), or by direct reprogramming of pediatric fibroblasts (Kim et al., 2014). Notably, such protocols could not have been generated without developmental biology studies revealing NC molecular markers and the extrinsic and intrinsic molecules needed for NC cell maintenance and directed cell differentiation; indeed, all efforts to derive specific cell types from stem cells (or by reprogramming) have been guided by lessons learned from the embryo. Applying genome-wide analysis to these cellular models can help define human NC cell differentiation mechanisms during normal development, such as sequential enhancer activation and epigenetic annotations (Bajpai et al., 2010; Rada-Iglesias et al., 2012).

Although translational applications have not yet appeared, patient-derived NC-like cells promise to provide a cellular platform for disease modelling and drug screening, as exemplified for familial dysautonomia (Kim et al., 2014; Zeltner et al., 2016), and to permit future innovative therapies based on autologous cell grafts and tissue engineering (Liu and Cheung, 2016). As a proof of principle, NC-derived dental pulp stem cells, first identified from murine lineage tracing (Chai et al., 2000), emerge from grafts to mineralize and repair experimentally induced calvarial defects (Lacerda-Pinheiro et al., 2012). Xenografts of patient-derived iPS cells differentiated into NC-like cells and then grafted into early mouse embryos, can differentiate into functional pigment cells, which may ultimately facilitate the development of better models for understanding and treating complex neurocristopathies (Cohen et al., 2016). We further predict that gene editing of autologous NC-like cells *ex vivo* may enable them to not only differentiate appropriately for therapeutic implantation back to the donor and replenishment of the affected tissue, but potentially to become migrating medication delivery devices, able to home to remote sites predicted from experimental and molecular embryology.

Concluding remarks

The aim of this review was to highlight the remarkable progress accomplished during the 150 years following the discovery of the NC. The capacity of NC cells to migrate all over the developing embryo and to settle in defined sites is unique in the vertebrate embryo. For more than a hundred years, the main purpose of researchers interested in this structure was to decipher the fate and migration routes of these cells leaving the dorsal neural tube to colonize the embryo. This early and indispensable line of research revealed the original characteristics that had remained elusive for so long: NC cells turned out to be a population of highly multipotent, flexible, proliferative cells that migrate to various sites and are largely influenced in their terminal differentiation by environmental cues.

The multipotentiality of NC cells was revealed by complementary evidence from both single cell *in vitro*

cultures and *in vivo* labelling. Multipotent NC progenitors and stem cells are not only present at early stages of migration and during formation of NC derivatives, but they later persist in an astonishing number of tissues and organs of the adult. Crucial to this wide distribution of NC-derived progenitors in time and space is the role of the vast network of PNS nerves, which convey and store multipotent NC cells of pre-glial identity (the SCPs). Whether these progenitors distributed in many tissues can be mobilized for broader regenerative events or injury repair remains to be explored in appropriate animal injury models. The recently discovered broad distribution and versatility of NC-derived stem cells will spur future exploration of their molecular properties and responses to environmental signals. Multi-omics approaches, particularly those recently developed at single cell resolution, will likely afford important novel insights into these questions in the next future.

After they have settled in their destination, NC cells fulfil multiple functions. By providing the body with the cells of the PNS, they play a major role not only in the coordination of organ functions, but also in adaptation of the organism to environmental conditions. One example of the role of the PNS is the vasoactive reactions to outside temperature; another is the efficient screen against UV radiation provided by melanocytes, a cell type of NC origin, which expands largely in the skin. Moreover, NC-derived neuro-endocrine cells, such as those in the adrenal medulla and carotid body, enable response to environmental stress and further contribute to the maintenance of physiological homeostasis.

One of the most profound discoveries in NC research was that of its paramount role in building the vertebrate head. Among multi-cellular organisms, it is in vertebrates and particularly, in humans, that the brain has reached its most complex differentiation and acquired higher-level capacities. This has coincided with the development of sense organs and the elaboration of complex facial structures to enable and protect them, a function accomplished by the cells of the NC. Plasticity and diversity in cephalic forms conferred by NC cells has also enabled evolutionary success for the vertebrates. The development of cardiopharyngeal structures is particularly critical and, as a consequence, is subject to multiple kinds of interference that engender human variations in cardiac great vessel and oral anatomy ranging from sub-morbid to lethal. Knowledge acquired about cranial NC development in embryonic animal models has also enriched our appreciation of the pathophysiology of dozens of rare diseases, of which only a sample is described in the present review, and which collectively represent another large proportion of congenital malformations. Continued investments in primary research on the many fronts that have opened in NC biology, particularly in stem cell engineering, will have increasingly immediate repercussions on the management of human disease.

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Table 1: Derivatives of the NC	Cell types			
	Neurons and Glial cells	**Pigment cells**	**Endocrine cells**	**Mesenchymal cells**
CEPHALIC NC	Sensory cranial ganglionic neurons Parasympathetic (eg., ciliary) ganglionic neurons Enteric ganglionic neurons Schwann cells along PNS nerves Satellite glial cells in PNS ganglia Enteric glia Olfactory ensheathing cells	Skin melanocytes Inner ear and choroid melanocytes Other extracutaneous melanocytes (in gums, meninges, heart…)	Carotid body glomus cells Calcitonin-producing cells of the ultimo-branchial body	Cranio-facial skeleton : *Dermal bone osteocytes* *Endochondral osteocytes* *Chondrocytes* Other mesectodermal head and neck derivatives : *Odontoblasts, cells of periodontal ligament and tooth papillae* *Connective cells of glands, muscles and tendons* *Facial dermis and adipocytes* *Myofibroblasts, smooth muscle cells and pericytes (cardiac outflow tract and pharyngeal arch-derived blood vessels)* *Meninges (forebrain)* *Cornea endothelial and stromal cells* *Ciliary muscles/ anterior segment of the eye*
TRUNK NC	Sensory (dorsal root) ganglionic neurons Sympathetic ganglionic neurons Parasympathetic ganglionic neurons Satellite glial cells in PNS ganglia Schwann cells along PNS nerves	Skin melanocytes	Adrenal medullary cells	

Timeline

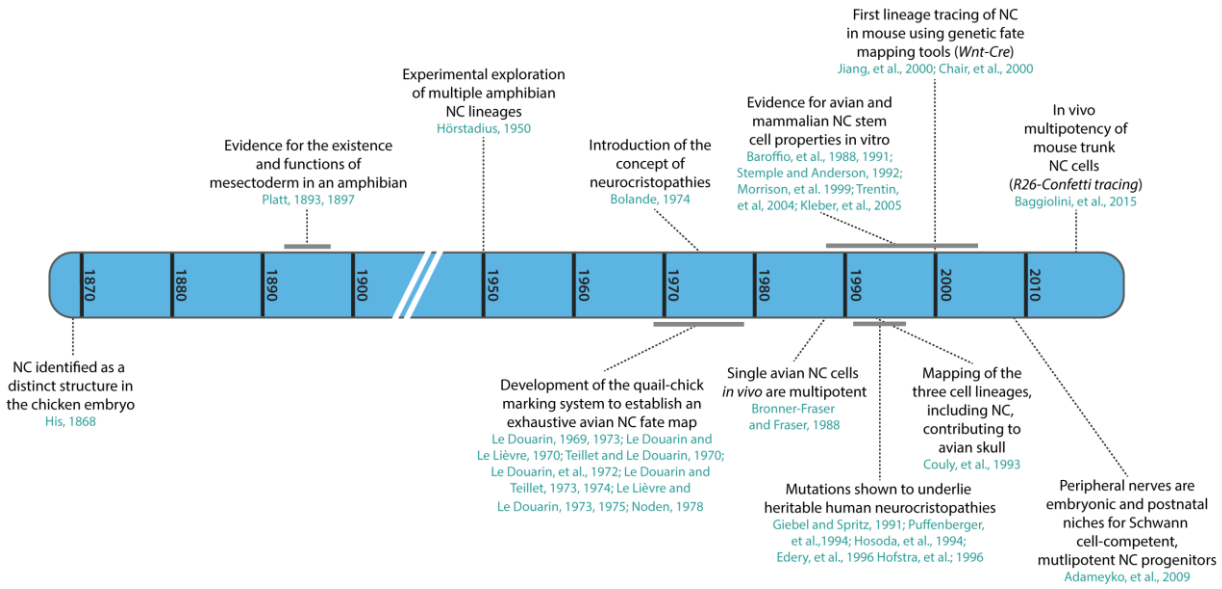

Figure 1: Fate map of NC derivatives

Representations of chicken embryos at 7 and 28 somite pairs (S), with neural folds capable of generating NC cells in gray, indicating in colours the axial levels from which different classes of NC derivatives originate. Adapted from Le Douarin et al., 2004.

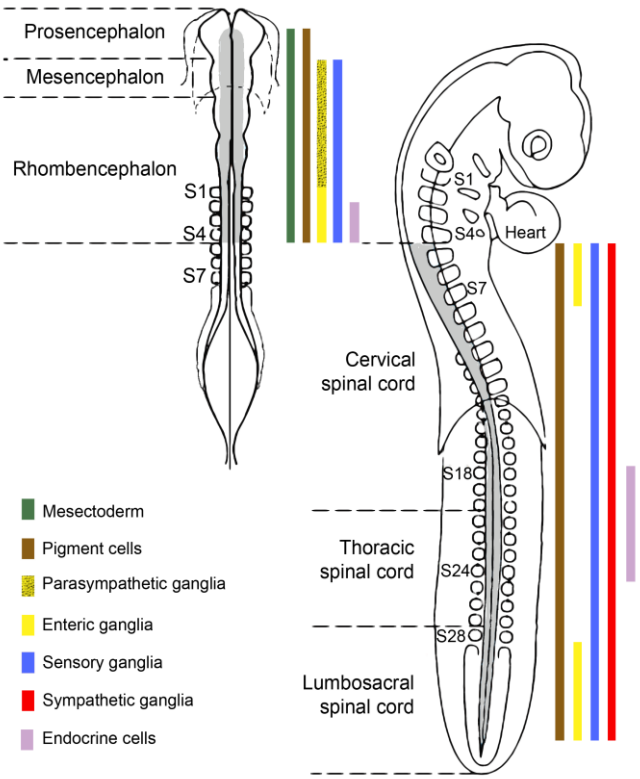

Figure 2: Migration of the cephalic NC cells to the craniofacial and cardiac regions

A. Schematic dorsal view of chicken embryo with 5 somite pairs, at about 30h incubation. Colours represent original levels of NC cells in the neural folds before migration. Adapted from Couly et al., 1996.

B. Stylized right view of chicken embryo at embryonic day (E) 3.5, showing NC cell migration pathways in the craniofacial and branchial arch mesenchyme. Adapted from (Couly et al., 1998).

C. Distribution at E8 of NC cells from rhombomeres 6-8 in cardiac outflow tract, including the tricuspid valves of aortic and pulmonary trunks, as well as pericytes and smooth muscle of blood vessels derived from pharyngeal arches 3-6. Adapted from Etchevers et al., 2001.

Abbreviations: AMes, anterior mesencephalon; Ao, aorta; CC, common carotid arteries; Di, diencephalon; FN, frontonasal bud; LA, left atrium; LV, left ventricle; NC, neural crest; op, optic vesicle; ot, otic vesicle; PMes, posterior mesencephalon ; PA1-6, pharyngeal arches 1-6 (by convention, there is no PA5 in amniotes); PT, pulmonary trunk; r1- 8, rhombomeres 1-8; RA, right atrium; RV, right ventricle; SV, sinus venosus.