Review

The neural crest and neural crest cells: discovery and significance for theories of embryonic organization

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The neural crest has long fascinated developmental biologists, and, increasingly over the past decades, evolutionary and evolutionary developmental biologists. The neural crest is the name given to the fold of ectoderm at the junction between neural and epidermal ectoderm in neurula-stage vertebrate embryos. In this sense, the neural crest is a morphological term akin to head fold or limb bud. This region of the dorsal neural tube consists of neural crest cells, a special population(s) of cell, that give rise to an astonishing number of cell types and to an equally astonishing number of tissues and organs. Neural crest cell contributions may be direct — providing cells — or indirect — providing a necessary, often inductive, environment in which other cells develop. The enormous range of cell types produced provides an important source of evidence of the neural crest as a germ layer, bringing the number of germ layers to four — ectoderm, endoderm, mesoderm, and neural crest. In this paper I provide a brief overview of the major phases of investigation into the neural crest and the major players involved, discuss how the origin of the neural crest relates to the origin of the nervous system in vertebrate embryos, discuss the impact on the germ-layer theory of the discovery of the neural crest and of secondary neurulation, and present evidence of the neural crest as the fourth germ layer. A companion paper (Hall, *Evol. Biol.* 2008) deals with the evolutionary origins of the neural crest and neural crest cells.

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1. Discovery of the neural crest

The neural crest is the name given to the fold of neural ectoderm at the junction between neural and epidermal ectoderm in neurula-stage vertebrate embryos, shown diagrammatically for a chicken embryo in figure 1.

In 1868 Wilhelm His identified a band of cells sandwiched between the developing neural tube and the future dorsal epidermal ectoderm as the source of spinal and cranial ganglia (figure 2) in chicken embryos. He named this band *Zwischenstrang*, the intermediate cord. In 1874, His included *Zwischenstrang* — neural crest cells as we now know them — as one of the organ-forming germinal regions, assuming from their position that these cells arose from the dorsal neural tube. Professor of Anatomy and Physiology in Basel, Switzerland, His was the first to provide a causal explanation for embryonic development, basing his explanation on mechanics (embryos as rubber tubes), developmental physiology, and predetermined organ-forming germinal regions, each of which contained cells with specified fates.

His identified cells that already had migrated to lie above the neural tube. Hence he identified *neural crest cells* rather than the *neural crest* (figure 1). Figure 2 shows the difference between migrating neural crest cells (A and B in figure 2) and the band of cells dorsal to the neural tube (C in figure 2)

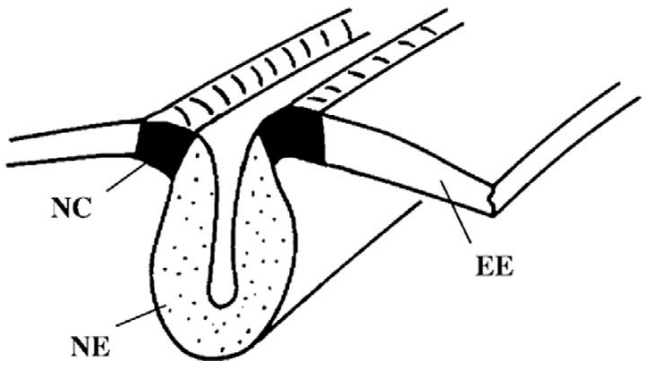


Figure 1. A reconstruction of the neural tube and adjacent ectoderm in an early chicken embryo to show the relationship between the neural ectoderm (NE) which forms the neural tube, the epidermal ectoderm (EE) from which the outer layer of the skin will arise, and the neural crest (NC) located in the fold between neural and epidermal ectoderms.

Keywords. Cell types; discovery; germ layers; natural crest

as depicted in a standard embryology textbook published in 1919. The term 'neural crest' was first used in a paper on the development of the olfactory organ published in 1879. The author, Arthur Milnes Marshall (1852–1893), was Professor of Zoology at Owens College in Manchester where he remained until his death at age 41 in a fall from Scafell Pike, England's highest peak. In a paper on the development of the cranial nerves in chicken embryos, Marshall (1878) used the term neural ridge for the cells that give rise to cranial and spinal ganglia. Realizing that this term was less descriptive than was desirable, a year later he replaced neural ridge with neural crest. As told in his words:

> "I take this opportunity to make a slight alteration in the nomenclature adopted in my former paper. I have there suggested the term neural ridge for the longitudinal ridge of cells which grows out from the reentering angle between the external epiblast and the neural canal, and from which the nerves, whether cranial or spinal arise. Since this ridge appears before closure of the neural canal is effected, there are manifestly two neural ridges, one on either side; but I have also applied the same term, neural ridge, to the single outgrowth formed by the fusion of the neural ridges of the two sides after complete closure of the neural canal is effected, and after the external epiblast has become completely separated from the neural canal. I propose in future to speak of this single median outgrowth as the neural crest, limiting the term neural ridge to the former acceptation. (Marshall 1879, p. 305, n.2)".

In this paper I provide a brief overview of the major phases of investigation into the neural crest and the major players involved, discuss how the origin of the neural crest relates

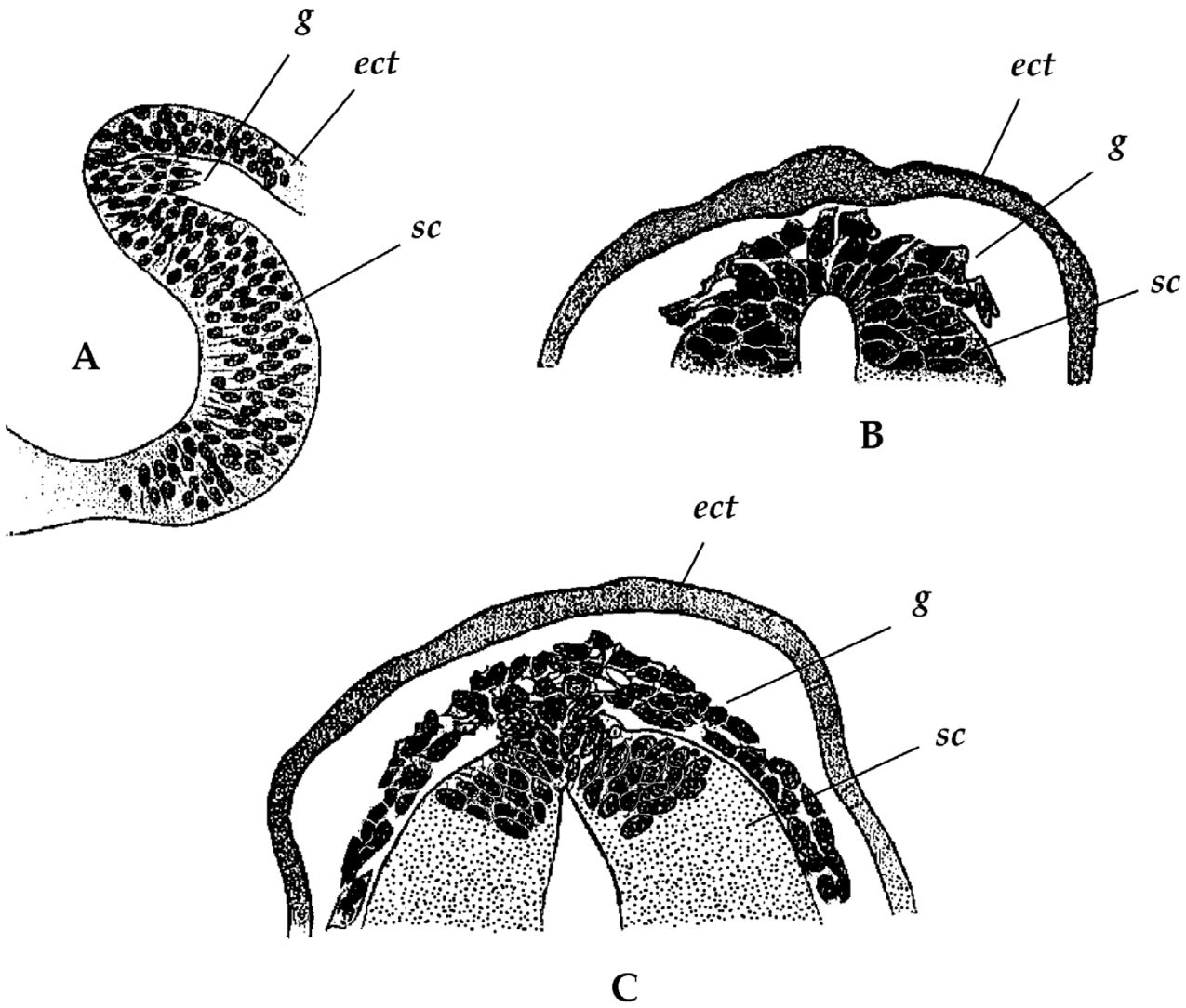


Figure 2. Cross sections of the developing spinal cord from an embryonic chicken (**A**) and a cartilaginous fish (**B**, **C**) to show early migrating neural crest cells (**A**, **B**) and a band of neural crest cells that has accumulated above the neural tube (**C**). ect, epidermal ectoderm; g, neural crest cells that will form the spinal ganglia; sc, spinal cord. Modified from Volume II of *Text-Book of Embryology* (Kerr 1919).

to the origin of the nervous system in vertebrate embryos, discuss the impact on the germ-layer theory of the discovery of the neural crest and of secondary neurulation, and present evidence of the neural crest as the fourth germ layer. A companion paper (Hall, 2008b) deals with the evolutionary origins of the neural crest and neural crest cells.

2. A brief overview of investigations over the past 120 Years

2.1 *1890–1950s*

His and Marshall independently identified the neural crest as the origin of cranial and spinal ganglia and neurons, an origin that was easy for others to accept because of the relationship of these cell types and of the neural crest to the dorsal neural tube, the source of the dorsal nervous system (figure 2).

In the 1890s, however, Julia Platt claimed that the cartilages of the craniofacial and pharyngeal arch skeletons and the dentine-forming cells (odontoblasts) of the teeth of the mudpuppy, *Necturus maculosus*, arose from the ectoderm adjacent to the neural tube (Platt 1893, 1894, 1897). Although supported by several contemporary researchers, Platt's conclusion was not accepted. In fact, her proposal of an ectodermal origin of the pharyngeal arch skeletons raised major controversies.

Why? Because her conclusions ran completely counter to the entrenched germ-layer theory, according to which skeletal tissues arose from mesoderm, not ectoderm (*see* the section on germ layer theory below).

Because a neural crest origin for skeletal tissues was so contentious, there was a 40-year gap between Platt's papers and independent studies in the 1920s and '30s by Landacre (1921), Stone (1926, 1929) and Raven (1931, 1936) demonstrating the neural crest as a major source of mesenchyme, connective tissue and cartilage .Even more detailed reports by Hörstadius and Sellman (1941, 1946) and de Beer (1947) were published in the 1940s. de Beer (1947) also thought it probable that neural crest cells differentiated into the osteoblasts of dermal bones in *Ambystoma*, although the evidence was less convincing than was his evidence of the neural crest origin of cartilage and teeth. Nowadays, not only has the skeletogenic capability of the cranial neural crest been documented in all classes of vertebrates, but the neural crest and its cells occupy a central position in studies of vertebrate development and evolution, beginning with the pioneering study by Gans and Northcutt (1983); *see* Hall (2008a,b) for detailed evaluations.

Despite these studies on the skeletogenic neural crest but because of the germ-layer theory, the focus of interest until the 1940s and 1950s was the neural crest as a source of pigment cells (chromatophores) and neural elements such as spinal ganglia. Amphibian embryos were the embryos of choice.[◊] Neural crest cells give rise to an astonishing number of cell types and to an equally astonishing number of tissues and organs (table 1). The derivatives of neural crest cells can be grouped in various ways. Figure 3 details the relationships of human neural crest cells as determined using standard histological criteria.

Standing as a milestone on the road to understanding the neural crest is *The Neural Crest*: *Its properties and derivatives in the light of experimental research*, a monograph by Sven Hörstadius. Published in 1950, 82 years after the discovery of the neural crest, it was reprinted in 1969 and again in 1988. "Hörstadius," as the monograph is known, was based on a series of lectures delivered during 1947 at the University of London at the invitation of Professor (later Sir) Gavin de Beer, then Head of the Department of Embryology at University College and later Director of the British Museum (Natural History). Hörstadius and Sellman (1941, 1946) had published two large papers devoted to an extensive experimental analysis of the development of the NC-derived cartilaginous skeleton of *Ambystoma*, an experimental verification of Platt's observations on the mudpuppy; see Ebendal (1995) and O Jacobson (2000) for details of his scientific work, and Olsson (2000) for a bibliography.

2.2 *1960s–1970s*

The 1960s ushered in investigations of mechanisms of neural crest cell migration and a move away from amphibian and toward avian embryos as the organisms of choice. The floodgate was opened by the seminal studies of Weston (1963) and Johnston (1966) on neural crest cells migrating from the brain and the spinal cord – known as trunk and cranial neural crest cells (TNCCs, CNCCs), respectively in chicken embryos; by Chibon (1964) with his studies on the skeletogenic neural crest in the Spanish ribbed newt (*Pleurodeles waltl*) by the discovery and exploitation of the quail nuclear marker by Le Douarin (1974); and by an influential review by Weston (1970) on the migration and differentiation of TNCCs.

Detailed maps of the fate of neural crest cells appeared during the 1970s. The microenvironment encountered by these cells was revealed as a major determinant of their migration, differentiation and morphogenesis in normal embryos and in embryos with abnormalities resulting from

[◊]Although studies on anuran (frogs, toads) and urodele (newt, salamander) embryos are often discussed together, bear in mind that relationships between these two groups of amphibians are not fully resolved. Indeed, use of the term amphibian at all is contentious; in current terminology amphibian would imply a monophyletic group, whereas anurans, urodeles and caecilians are independent evolutionary lineages (Hall and Hallgrímsson 2008).

Table 1. A list of the cell types derived from the neural crest and of the tissues and organs that are entirely neural crest or that contain cells derived from the neural crest

Cell Types	
Sensory neurons	Cholinergic neurons
Adrenergic neurons	Rohon-Béard cells
Satellite cells	Schwann cells
Glial cells	Chromaffin cells
Parafollicular cells	Calcitonin-producing (C) cells
Melanocytes	Chondroblasts, chondrocytes
Osteoblasts, osteocytes	Odontoblasts
Fibroblasts (mesenchyme)	Cardiac mesenchyme
Striated myoblasts	Smooth myoblasts
Mesenchymal cells	Angioblasts
Merkel cells	
Tissues or organs	
Spinal ganglia	Parasympathetic nervous system
Sympathetic nervous system	Peripheral nervous system
Thyroid and Parathyroid glands	Ultimobranchial body
Adrenal gland	Craniofacial skeleton
Teeth	Dentine
Connective tissue	Adipose tissue[a]
Smooth muscles	Striated muscles
Cardiac septa, valves, aortic arches	Dermis
Eye	Cornea
Endothelia	Blood vessels
Heart	Dorsal fin
Brain	Connective tissue of thyroid, parathyroid, thymus, pituitary, and lacrimal glands

[a] A recent investigation using both murine and Japanese quail embryos has demonstrated the origin of a population of adipocytes (fat cells) from cranial neural crest (Billon *et al* 2007). Adipocytes have long been thought to arise from cells within mesodermal lineages; indeed there is a vast literature on the origin of adipocytes, chondro- and osteoblasts from clonal cell lines derived from periosteal or bone marrow cells (Hall 2005). Billon and colleagues demonstrated that cultured mouse neural crest cells form adipocytes, that cultures of quail neural crest cells can be induced to differentiate as adipocytes (indicative of their multipotency) and that a subset of adipocytes associated with the developing ear — confirmed by visualization of perilipin, a lipid marker — in mouse embryos arise from the neural crest during normal development. In another analysis, Takashima *et al* (2007) demonstrated that mesenchymal stem cells capable of producing adipocytes, chondro- and osteoblasts arise from $Sox1^{+}$-neuroepithelial cells (that is, neural crest cells) as well as from paraxial mesoderm.

mutations or the consequences of exposure to teratogens such as alcohol or drugs (*see* Bolande 1981, Le Douarin and Kalcheim 1999, and Hall 2008a for analyses).

Syndromes involving one or more cell types derived from the neural crest were recognized as separate and identifiable entities and classified as neurocristopathies — neural crest pathologies. Monoclonal antibodies against individual populations or types of neural crest cells were developed in the 1980s and used to analyze cell determination, specification, lineage and bi-, multi- or pluripotentiality.. Even mammalian embryos, which are difficult to study, began to yield the secrets of their neural crest cells to skilled and persistent experimental embryologists (Bolande 1974; Morriss-Kay and Tan 1987; Hall 2008a).

2.3 *1980s-21st century*

Homeotic transformations and the *Hox* gene code that patterns the major axis of most (and all bilaterally symmetrical) animal embryos were discovered and analyzed in some detail during the 1980s and 1990s (box 1). The NC, which had earlier been shown to be divisible into cranial and trunk regions, was further subdivided following the recognition of:

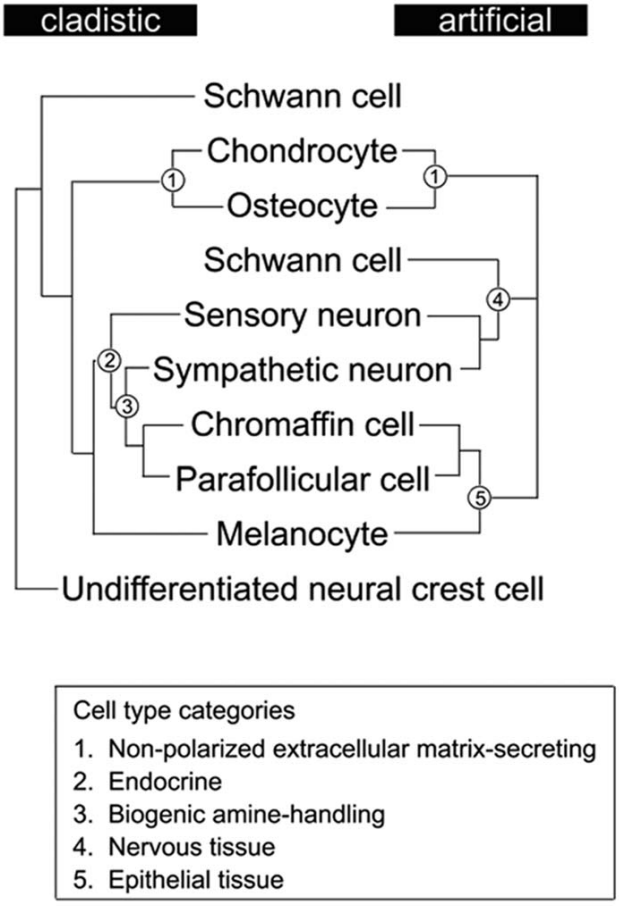


Figure 3. Two classification schemes for human neural crest cells showing the different relationships obtained in a cladistic analysis (cladistic, using PAUP 4.0b10) and in an analysis using standard histological criteria (artificial). Of five cell-type categories (1–5) shown, only the skeletal cells (chondrocyte, osteocyte) separate out in both schemes, although chromaffin cells of the adrenal glands and parafollicular cells of the thyroid glands cluster as a subgroup. Note that neural crest-derived neurons (sensory, sympathetic) do not cluster as an individual (monophyletic) group, reflecting their origins from different neural crest cells populations. See Vickaryous and Hall (2006) for further analysis and for the 19 characters used in the cladistic analysis. From Vickaryous and Hall (2006).

- sub-populations in the hindbrain associated with specific rhombomeres (r) and segmental patterns of expression of *Hox* genes;
- a vagal and a sacral neural crest in the neck and caudal body respectively, from which arise enteric ganglia, and the neurons of the parasympathetic nervous system of the intestine and blood vessels;
- a cardiac neural crest that contributes cells to the valves, septa and major vessels of the heart.

The major derivatives of each of these regions are outlined in table 2. Relationships between these derivatives are shown in figure 3.

Mapping the neural crest became ever more fine-grained as comparative studies were undertaken, some within specific phylogenetic frameworks to test explicit evolutionary hypotheses. Knowledge of the molecular basis of the migration, differentiation and death (apoptosis) of neural crest cells advanced considerably.

Last but by no means least in this brief overview, is the role that studies of the neural crest have played in forging a new synthesis between developmental and evolutionary biology. The neural crest is a vertebrate synapomorphy; that is, a character shared by all vertebrates to the exclusion of all other animals. Evolutionary biologists sought the origin of the vertebrate head in the unique properties of the NC, placing the neural crest at center stage in the vertebrate evolutionary drama. Indeed, the vertebrate head is a 'new head' added to the rostral end of a more ancient invertebrate head (Gans and Northcutt 1983; Hall 1999a, 2008a,b). The vertebrate heart is also new, for it, too, contains neural crest derivatives, the cardiac neural crest.

3. Neural crest and germ-layer theory

The bulk of the discussion in de Beer's 1947 paper evaluated the significance of his own results concerning the neural crest origin of pharyngeal cartilages to germ-layer theory, which had placed evolutionary studies of embryonic development in a straitjacket for almost a century. The germ-layer theory makes three claims:

(i) Early embryos organized as homologous layers: ectoderm and endoderm in diploblastic animals such as sponges and coelenterates; ectoderm, mesoderm and endoderm in triploblastic animals such as sea urchins, flies, fish and humans.

(ii) Embryos (and so larvae and adults) form by differentiation from these germ layers.

(iii) Homologous structures in different animals arise from the same germ layers.

The germ-layer theory exerted a profound influence on those claiming a neural crest — that is, an *ectodermal* — origin for tissues such as mesenchyme and cartilage, traditionally believed (indeed 'known') to arise from mesoderm. Hörstadius (1950, p. 7) commented on the "violent controversy" that followed the assertion of a neural crest origin for mesenchyme by Platt, and others such as Brauer, Dohrn, Goronowitsch, Lundborg, Kastschenko and Kupffer, and the opposition to such a heretical idea by Buchs, Corning, Holmdahl, Minot and Rabl — a veritable who's who of comparative morphologists of the day.

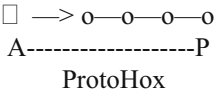
A brief discussion of germ layers and the germ-layer theory follows; see Oppenheimer (1940) and Hall (1998, 1999a, 2008a) for detailed discussion of the discovery of germ layers, their naming, germ-layer theory, and references to early studies.

Box 1 *Hox* genes

Recognition of the importance of *Hox* genes marks a fascinating episode in the history of the search for relationships between animals, the origin of body plans associated with individual phyla, and, with respect to our topic, the origin of chordates and vertebrates. What follows below is intended to establish the primary role of *Hox* genes in patterning in general, not to list all the studies relating *Hox* genes to the neural crest.

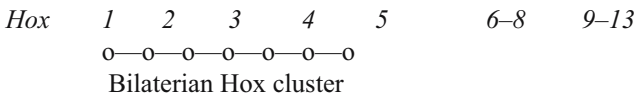
Vertebrate homeobox (Hox) genes with sequence homology to such gene complexes as *Ultrabithorax* and *Antennapedia* in the fruit fly *Drosophila* are orthologues of a series of transcription factors organized as homeobox clusters throughout the animal kingdom (Carroll *et al* 2005). As in *Drosophila*, the order of *Hox* genes within a cluster is paralleled by an anterior-posterior sequence of gene expression. Conservation of the roles of these genes in vertebrates and in *Drosophila* is demonstrated by research showing that, for example, after being transfected into *Drosophila*, the mouse *Hoxb6* gene elicits leg formation in the place of antennae (Halder *et al* 1995).

Considerable information now is available on the evolution of the genes leading to the *Ultrabithorax* and *Antennapedia* gene complexes. The scenario (outlined below[a]) is that a single protoHox gene (□) duplicated to produce a ProtoHox cluster of four genes (o) arranged in an anterior to posterior (A-P) sequence:



Duplication of this ProtoHox cluster produced two clusters of four genes each, four Hox and four ParaHox.

Further tandem duplications of three of the Hox cluster at the origin of the bilaterally symmetrical animals (the Bilateria) produced the Hox clusters found in bilaterians; Hox cluster 3 remained as a single gene in both Hox and ParaHox clusters.[b]



The number of Hox clusters varies among vertebrates: four clusters of 39 genes in mice, three clusters in lampreys, and up to seven in teleost fish. Duplication of the genome at the origin of the chordates is the most likely current explanation for the four clusters; duplication sets up the possibility of future structural and functional divergence and *specialization of function among copies of the genes*.

Four possibilities, which are not mutually exclusive, could explain evolutionary changes in gene function:

- Two involve change in gene number, either (i) the number of Hox gene clusters or (ii) the number of genes per cluster. Duplication of Hox clusters *before* the teleosts arose — perhaps associated with duplication of large portions of chromosomes or entire chromosomes or genomes — would have taken the number from four to eight in teleosts. This, coupled with subsequent loss of one cluster, would explain the seven clusters in zebrafish.
- The other two possibilities involve altered function**,** either (iii) modification of individual *Hox* genes through regulatory or other changes, or (iv) increasing the complexity of interaction between gene networks, either of which could come about by alteration in the upstream and/or downstream regulation of a Hox gene(s).[c]

The *patterning role* carried out by *Hox* genes is demonstrated by studies in which knocking out or knocking in a *Hox* gene to eliminate or enhance its function in mice is followed by the transformation of skull, vertebral or other features into a more anterior element in the sequence. Such a transformation is known as homeotic, a term introduced into biology by William Bateson in the early 20th century. In the tadpoles of some species of frogs, an amputated tail can be made to transform homeotically into the duplicated posterior portion of the body, including hind limbs and a pelvic girdle, rather than regenerating a tail. Meckel's cartilage and the ossicles are duplicated following *Hoxa2* knockout, essentially because the second pharyngeal arch fails to form. Instead, a second set of first arch elements forms more anteriorly than the normal position of the first arch; that is, there is a homeotic transformation of second arch to first arch structures.

[a] Based on the scheme outlined in Shimeld (2008).

[b] See Holland and Graham (1995), Peterson and Davidson (2000), Shimeld and Holland (2000) and Carroll *et al.* (2005) for literature on the major roles of *Hox* genes in bilaterian evolution.

[c] Modes of evolutionary change in gene activity at levels other than structural changes in gene sequences have emerged in recent years. Changes in *cis*-regulation and changes in noncoding RNA molecules such as MicroRNAs (MiRNAs) are two under active investigation. See Hall and Hallgrímsson (2008) for the evolution of gene regulation, Eberhart *et al* (2008) for MiRNA regulation of the *PdgfRa* gene and cleft palate in zebrafish, Heimberg *et al*. (2008) for an overview of MiRNAs, and Miller *et al* (2007) for parallel evolution of *cis*-regulation of the gene for c-kit ligand in the evolution of pigmentation in marine and freshwater species of threespine sticklebacks and in humans.

Table 2. Derivatives of the neural crest in relation to the four major regions of the crest.

Cellular derivative
Cranial neural crest
Mesenchyme (Other contributions: Induction of the thymus and parathyroid glands)
Connective tissue (including muscle sheaths)
Cartilage
Bone
Dentine (odontoblasts)
Parafollicular cell (ultimobranchial bodies) of the thyroid gland.
Cornea
Sclera
Ciliary muscle and muscles for eye attachment
Inner ear (with otic placode)
Sensory ganglia of cranial nerves V, VI, IX and X
Vagal and sacral neural crest
Neurons of parasympathetic nervous system of alimentary canal
Neurons of parasympathetic nervous system of blood vessels
Enteric ganglia
Trunk neural crest
Pigment Merkel cells
Dorsal root ganglia
Neurons and ganglia of the sympathetic nervous system
Chromaffin cells of the adrenal medulla
Epinephrine-producing cells of the adrenal gland
Cardiac neural crest
Connective tissue associated with the great vessels of the heart
Aorticopulmonary septum of the heart
Smooth muscles of the great arteries
Ganglia (celiac, superior and inferior mesenteric, and aortical renal)

3.1 *Germ-layer theory*

Germ-layer theory had its origin in the early nineteenth century.

In the course of a pioneering study on the development of chicken embryos undertaken for his MD thesis, Christian Heinrich Pander (1817) recognized that the blastoderm is organized into the three germ layers we now know as ectoderm, mesoderm and endoderm. Pander referred to an upper 'serous,' a lower 'mucous,' and a rather ill defined middle 'vessel' layer, and coined the terms *Kembla* (germ layer) and *Keimhaut* (blastoderm). Eleven years later, Karl von Baer extended Pander's findings by demonstrating that all vertebrate embryos are built on this three-layered plan.

On the basis of his studies of the organization of coelenterates, Thomas Huxley came to the important conclusion that the outer and inner layers of adult coelenterates are homologous with the outer and inner layers of vertebrate embryos (Huxley 1849). In one fell swoop, Huxley extended the concept of germ layers from vertebrates to invertebrates, from embryos to adults, and from ontogeny to phylogeny. No biologist could ignore germ layers.

In 1853, George J Allman coined the terms ectoderm and endoderm for the outer and inner layers of the hydroid *Cordylophora*. In his encyclopedic treatment of animal development published between 1850 and 1855 the Polish embryologist, histologist, physiologist and neurologist, Robert Remak identified Pander's rather indistinct middle layer as a distinctive germ layer, which Huxley (1871) named mesoderm. In recognizing the cellular basis of the germ layers, Remak related germ layers to the cell theory and established the cell as the fundamental developmental unit. Remak provided the first histological descriptions of each germ layer, to which he gave the names ectoderm, mesoderm and endoderm.

Against the background that not all animals develop from three-layered embryos, in the 1870s the influential English zoologist Sir Edwin Ray Lankester expanded the germ-layer concept from ontogeny into systematics by dividing the animal kingdom into three grades based on numbers of germ layers:

- *Homoblastica* — single-celled organisms
- *Diploblastica* — sponges and coelenterates
- *Triploblastica* — the remainder of the animal kingdom (Lankester 1873, 1877).

Lankester's scheme stood for 125 years until evidence was assembled that vertebrates are tetrablastic not triploblastic, the neural crest constituting a fourth germ layer — the topic of the second last section of this chapter (Hall 1998, 1999a).

Not all embraced the germ-layer theory. Adam Sedgwick, Chair of Zoology at Cambridge, rejected germ-layer and cell theories entirely, claiming that one could not even state what the cell theory is—it is a phantom, he said, and if extended to the germ layers it is the "layer phantom" (Sedgwick 1894, p. 95). For many, the problem was the apparent origin of multiple cell types from a single layer or region of the embryo.

3.2 *Multiple tissues from single layers*

Sedgwick's criticisms arose, in part, from the nature and origins of mesenchyme and peripheral nerve trunks, and of the neural crest itself.

The term mesenchyme was coined in 1882 by Oscar and Richard Hertwig (brothers and brilliant zoologists) for those cells that leave the mesodermal germ layer during formation of the coelom and form elements of connective tissue or blood. The term is now used for meshworks of cells irrespective of their germ layer of origin.[1] A century ago, however, an ectodermal neural crest producing head mesenchyme created major problems for the entrenched germ-layer theory. So too did the observation that mesenchyme, nerves, muscles, connective and vascular tissues all develop from a single layer in the vertebrate head. This was not what was demanded by a rigid germ-layer theory in which:

- ectoderm formed nerves and epidermis,
- mesoderm formed muscle, mesenchyme, connective and vascular tissues, and
- endoderm formed the alimentary canal.

A diversity of cell and tissue types, however, do arise from single germ layers. As Kölliker (1884) demonstrated 125 years ago, epithelial, neuronal and pigmented cells all arise from ectoderm, and under experimental conditions, structures can form from a different germ layer than the one from which they arose embryonically. de Beer (1947) cited asexual reproduction, regeneration and adventitious (ectopic) differentiation as further situations in which structures develop from a different germ layer than the one from which the original structure formed.

Consequently, the germ-layer theory neither speaks to the full developmental potential of individual germ layers nor to determination or cell fate within a germ layer.

De Beer carefully pointed out that the germ-layer theory is a morphological concept that does not speak to developmental potencies or to cell fate, concluding:

> "…that there is no invariable correlation between the germ layers and either the presumptive organ-forming regions or the formed structures. It follows that the germ layers are not determinants of differentiation in development, but embryonic structures, which resemble one another closely in different forms although they may contain materials differing in origin and fate. *The germ-layer theory in its classical form must therefore be abandoned*. (1947, p. 377, emphasis added)".

In the original form of the germ-layer theory— thus also in its strictest application — only those structures that develop from equivalent layers were regarded as homologous. Again, according to de Beer, the 'problem' with fitting a neural crest origin of cartilage into the germ-layer theory is largely due to a misconception of the theory of homology and the misapplication of homology based on adult structures to homology based on developmental origin and developmental processes — the "attempt to provide an embryological criterion of homology" (p. 393). This has important consequences, for we now know that homologous structures need not arise from the same embryonic area or indeed, by the same developmental processes.[2]

Despite his denigration of germ-layer theory, de Beer was reluctant to abandon it entirely, as can be seen in the conclusion to his 1947 paper, a nice example of "the dogged attempt of the human mind to cling to a fixed idea" (Oppenheimer 1940, p. 1):

> "There is just sufficient constancy in the origins and fates of the materials of which the germ layers are composed to endow the ghost of the germ-layer theory with provisional, descriptive, and limited didactic value, in systematizing the description of the results of the chief course of events in the development of many different kinds of animals; provided that it be remembered that such systematization is without bearing on the question of the causal determination of the origin of the structures of an adult organism (p. 394)."

Sedgwick's rejection of, and de Beer's concerns over, germ-layer theory highlight a fundamental feature of vertebrate development, discussed in the following two sections and that is either unknown or underappreciated by most students of development, indeed by most biologists.

4. Heads and tails

Of the three claims of the germ-layer theory,

(i) the first — that early embryos are arranged into equivalent layers — is always true;

[∝]The ganglion of Remak found in birds, which originates in the lumbosacral NC and is the major parasympathetic nervous system element in the hindgut is named after Robert Remak (1815–1865).

[1]*See* Hall (1998, 2005b) for discussions of the use of the term mesenchyme.

[2]For discussion of the development of homologous structures from different embryonic regions or by different developmental processes, and for the concept of latent homology, *see* de Beer (1971), Hall (1994, 1995, 1999a,b, 2003a), Stone and Hall (2004) and Janvier (2007).

(ii) the second — that embryos form by differentiation from these germ layers — is not true for the caudal development of vertebrate embryos (see below); while

(iii) the third — that homologous structures in different animals arise from the same germ layers — need not be true.

Our consideration of these issues begins with Holmdahl (1928), who distinguished two phases of vertebrate embryonic development: (i) primary development for the laying down of germ layers, and (ii) secondary development for the development of the caudal region of the embryo without the specification and segregation of germ layers. Holmdahl's second phase is predicated on a lack of germ-layer involvement in the caudal region and tail buds of vertebrate embryos. Why? Because the mechanisms of neurulation operate differently in the head and body than they do in the tail. Indeed, the opposite ends of the same embryo develop by fundamentally different developmental mechanisms, primary and secondary neurulation**,** where primary neurulation is based on germ layer formation and secondary neurulation on a bud or cellular blastema without formation of germ layers.

4.1 *Secondary neurulation and tail buds*

The cranial region of vertebrate embryos arises by primary neurulation through delamination and migration of the germ layers. The caudal end arises by secondary induction and the transformation of epithelial cells into a mesenchymal tail bud.[3] Secondary neurulation is characteristic of all vertebrates studied; lampreys, fish, amphibians, birds and mammals (including humans). Such a widespread distribution (and conservation) of secondary neurulation across the vertebrates is consistent with secondary neurulation being an ancient developmental process inherited from the common vertebrate ancestor.[4]

Kölliker (1884) was aware of the problem raised for the germ-layer theory by his finding that the most caudal part of the nervous system arises from mesoderm, not ectoderm. Using the method for vital dye staining of cells published by Vogt in 1925, Bijtel confirmed Kölliker's finding by demonstrating that tail somites in amphibian embryos arise from the medullary (neural) plate; that is, that 'mesodermal' cells arise from ectoderm. Studies using ^{3}H-thymidine-labeled grafts and quail/chicken chimeras extended these findings to birds, in which neural, muscular, vascular and skeletal tissues arise from a common tail bud mesenchyme (Griffith *et al* 1992; Hall 2000b).

Muscle, cartilage, neuroepithelial and pigment cells all differentiate in culture from what appears to be homogeneous tail bud mesenchyme. Because these tissue derivatives represent multiple germ layers, Griffith *et al* (1992) concluded that the tail bud consists of three unseparated germ layers, an interpretation that assumes that germ layers have been specified in this embryonic region, which we now know they have not. Kanki and Ho (1997) showed that pluripotent cells within zebrafish tail buds contribute to caudal trunk tissue that lie rostral[⊕] to the anus as well as to the tail bud itself. Davis and Kirschner (2000) used photoactivation of fluorescence labeling of groups of cells to show that the tail bud in *Xenopus* contributes cells to the neural tube, notochord, somites (muscle cells) and other structures. They concluded that the tail bud represents a region in which germ layer decisions are delayed. As the tail bud is of mixed origin, and as there is no separation into germ layers, we do not know whether caudal neural crest are entirely ectodermal in origin. This is a far cry from germ layers laying the foundation for the more rostral embryo.

With primary neurulation from germ layers and secondary neurulation from a tail bud blastema, fundamental distinctions exist between the origins of cranial and caudal neural crest cells. Indeed, we can think or primary and secondary neural crest cells and the neural crest as having a dual origin: cranial (primary) neural crest from neurectoderm; caudal (secondary) neural crest from a tail bud. An additional line of evidence is the origin of the tail bud by embryonic induction and not from germ layer delamination.[5]

[3]For current understanding of epithelial–mesenchymal transitions, see the papers in the special issue of *Acta Anatomica* edited by Newgreen (1995) and see Savagner (2001), Kang and Svoboda (2005) and Morales *et al* (2005).

[4]*See* Schoenwolf and Nichols (1984), Schoenwolf *et al.* (1985) and Le Douarin *et al* (1996) for secondary neurulation in avian embryos; Beck and Slack (1998, 1999) and Davis and Kirschner (2000) for *Xenopus*; Wilson and Wyatt (1988) and Hall (1998, 2000b, 2008a) for murine embryos; Müller and O'Rahilly (2004) and O'Rahilly and Müller (2006, 2007) for human embryos; and Hall (1998, 1999a, 2000b,c, 2007, 2008a) and Handrigan (2003) for further discussions of phases of development and secondary neurulation.

[⊕]Throughout the text, I endeavor to be consistent in using rostral and caudal rather than anterior and posterior if referring to the organization of axial embryonic structures such as the neural tube, neural crest or somites.

[5]*See* Schoenwolf and Nichols (1984) and Schoenwolf *et al.* (1985) for the dual origins of the NC, and Griffith *et al* (1992) and Hall (2000b) for primary and secondary body development. In studies using chicken embryos, Weston *et al* (2004) found PdgfRα-positive mesenchymal cells arose from non-neural ectoderm adjacent to the neural tube. Taking PdgfRα as a neural crest marker, they regard these as neural crest cells that arise from beside the neural tube, a 'metablast' in their terminology.

4.2 *Induction of tail buds*

Epithelial–mesenchymal interactions, which are secondary inductions, initiate or regulate differentiation, growth and/or morphogenesis of most organs in vertebrate embryos.

Limb outgrowth is controlled through epithelial–mesenchymal signaling involving an apical ectodermal ridge (AER). The tail bud is also surmounted by a ridge known as the ventral ectodermal ridge (VER). Is the VER the tail bud equivalent of the AER?

Removing tail ectoderm almost completely eliminates tail growth in embryonic chickens just as removing the AER eliminates limb growth. Limb and tail induction in avian embryos share common mechanisms, as shown by experiments in which limb bud mesenchyme grafted beneath tail ectoderm induces tail ectoderm to form an ectodermal ridge that regulates limb outgrowth, chondrogenesis and skeletal formation.[6]

Mutations that decrease or eliminate the AER slow or stop limb development. Similarly, the VER is missing in *vestigial tail* (*vt*) and *Brachyury* (*T*) mutant mice in which tails fail to form. In *repeated epilation* (*Er*) mutant embryos, the VER and the AER are abnormal, as are tail and limb development. Reduced proliferation in the ventral part of the tail accounts for rostral defects in *curly tail* mutant mice.[7]

As with the development of the limbs, heart, kidney and other organs, tail development occurs by secondary induction and not by primary development from a germ layer (which occurs before epithelial–mesenchymal interactions are initiated), reinforcing the fundamental similarity between the development of the most caudal region of early vertebrate embryos on the one hand, and organ systems arising later in development on the other.

5. Neural crest as the fourth germ layer

The neural crest meets all the criteria used to define and identify a germ layer.

Ectoderm and endoderm are primary germ layers; they ere the first to appear in animal evolution, are the earliest to form embryonically, are present in the unfertilized egg, and are determined by cytoplasmic factors deposited by the mother into the egg by a process known as maternal cytoplasmic control. Mesoderm is a secondary germ layer; it is not preformed in the vertebrate egg but arises by activation of zygotic genes following inductive interactions between ectoderm and endoderm.[8]

Like mesoderm, the neural crest arises early in development and gives rise to divergent cell and tissue types. Like mesoderm, the neural crest arises by secondary induction from a primary germ layer. The neural crest therefore meets the criteria of a secondary germ layer.

As the fourth germ layer, the neural crest is confined to vertebrates, which are therefore tetrablastic not triploblastic.[9] Indeed, possession of a neural crest is a vertebrate synapomorphy. A cladistic analysis of 411 human cell types undertaken by Vickaryous and Hall (2006) and illustrated in figure 3, and an analysis of tissue-specific genes and gene programmes using a bioinformatics approach by Martinez-Morales *et al* (2007) provide independent support for lineage relationships among neural crest cells from developmental, molecular and evolutionary points of view. Clinicians and medical geneticists acknowledge the neural crest as a germ layer by recognizing neurocristopathies, in which the common link among affected tissues and organs is their origin from the NC.

Just as the evolution of mesoderm allowed triploblastic organisms to form new, often novel body parts, so the evolution of the neural crest allowed vertebrates to form new, often novel body parts (Hall 2005a). To quote from a recent analysis of how new features appear in evolution:

> "The evolutionary versatility of the neural crest justifies Hall's claim that it is an emergent, fourth germ layer. Along with duplication of the whole genome in the early vertebrates, and further duplication and differentiation of genes that regulate development and physiology, the neural crest provided powerful experimental tools for emergent evolution. (Reid 2007, p. 216)".

The three germ layers recognized for the past almost 180 years can be replaced by four germ layers, two being primary (ectoderm and endoderm) and two secondary (mesoderm and neural crest). This is a far cry from the days when to claim that mesenchyme arose from ectoderm was biological heresy and professional suicide, as Julia Platt found.

[6]*See* Grüneberg (1956), Hall (2000b,c, 2005b, 2007) and Handrigan (2003) for limb and tail bud development. The ability of some amphibian tadpoles to respecify the tail during regeneration to produce ectopic limbs (Mohanty-Hejmadi *et al* 1992) further reinforces the links between limb and tail development.

[7]*See* Grüneberg (1956), Johnson (1986) and Hall (2005b) for mutations affecting mouse tail-development, and Wilson and Wyatt (1988) for closure of the caudal neuropore in *vL* mutant mice and for suggesting that defective secondary neural crest may contribute to spina bifida in these mutants.

[8]For recent overviews of the origin of the mesoderm in development and evolution, see Sasai and De Robertis (1997), Martindale *et al* (2004) and Putnam *et al* (2007).

[9]*See* Hall (1998, 1999b, 2000d, 2005b, 2008a), Opitz and Clark (2000), Carstens (2004), Stone and Hall (2004) and Vickaryous and Hall (2006) for discussions of the evidence for the neural crest as the fourth germ layer.

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