# Chapter 2 Embryological Origins and the Identification of Neural Crest Cells

# Neural Crest

This chapter seeks to answer a twofold question: When and by what mechanisms do the neural crest (NC) and neural crest cells (NCCs) arise during embryonic development?

In one sense, the embryological origin of the NC is self-evident. The NC is the apex of the neural folds of neurula-stage embryos (Fig. 2.1). The very name neural crest—like the crest of a mountain—is indicative of this location. But NCCs are not the only derivatives of the neural folds; neural and epidermal ectoderms arise from the neural folds. Furthermore, placodal ectoderm (the development and derivatives of which are discussed in Chapter 6) arises from the lateral neural folds or from ectoderm immediately lateral to the neural folds. Indeed, it can be difficult, if not impossible, to label NCCs in the neural folds without labeling placodal ectoderm.

The NC can also be defined as a region that lies at or forms the border between the neural and epidermal ectoderm (Fig. 2.1) or as the region of the embryo from which NCCs arise.

# **Before Neurulation**

Although most evident in neurula-stage embryos, the intimate association between the four presumptive areas—neural crest, neural, epidermal, and placodal ectoderm—does not arise at neurulation: **Specification of the NC begins during gastrulation**, although without special methods, however, neither NC nor other ectodermal cell types can be identified before neurulation.

Three different methods—vital staining, extirpation, and cell labeling—show that in early amphibian blastulae the future NC lies at the border between presumptive epidermal and neural ectoderm (Fig. 2.1). Grafting <sup>3</sup>H-thymidine-labeled regions of chicken epiblasts into unlabeled epiblasts similarly reveals presumptive NC at the epidermal–neural ectodermal border at the blastula stage of embryonic development (Fig. 2.2), although neither epidermal nor neural markers are expressed until after the onset of neurulation.<sup>1</sup>

Nc

Ee

Ne



**Fig. 2.2** Fate map of the epiblast of a chicken embryo showing the location of future neural crest (NC) at the boundary between epidermal (Ee) and neural (Ne) ectoderm. Based on data from Rosenquist (1981) and Garcia-Martinez *et al.* (1993)

Fate maps of the epiblast of murine embryos provide insights into the location of prospective NC before neurulation. Using clonal analysis, Lawson and colleagues (1991) derived a sufficiently detailed fate map of the mouse NC that they could compare it with fate maps of the epiblast in chicken and urodele embryos. Research from Patrick Tam's laboratory provided an insightful comparison into what they termed the 'striking homology' between the fate maps of representative fish, amphibian, avian, and mammalian embryos.<sup>2</sup> The congruence of these fate maps includes the

location of the presumptive neural crest at the border between neural and epidermal ectoderm. One important finding in mouse embryos, consistent with what is known from secondary neuralation (see Chapter 1), is that clonal descendants within the epiblast are not confined to single germ layers and that germ layers are not fully segregated until gastrulation.

Cruz and colleagues (1996) used DiI injection to map the fate of the epiblast in the Australian marsupial 'mouse', *Sminthopsis macroura*. Although they demonstrated that neurectoderm gives rise to epidermal and neural ectoderm, they did not map the NC. Indeed, as noted in Chapter 1, only a few individuals have investigated the NC during marsupial embryonic development, although Hill and Watson, in studies published in 1958 but begun in 1911, documented NCCs and their contribution to cranial mesenchyme and ganglia in a number of Australian marsupials<sup>3</sup> and in American opossums (*Didelphis* spp.); see Box 7.5 for available information on marsupial NCCs.

## **Establishing the Epidermal–Neural Border**

A long-standing interpretation of studies directed at determining the origin of the NC has been that NC arises at the **border** of neural and epidermal ectoderm precisely, because this is where neuralizing and epidermalizing influences meet, the combined action of these influences generating the NC.

In perhaps the first study to raise this interpretation, undertaken using the common European salamander and the alpine (Arctic) newt, Rollhäuser-ter Horst (1980) replaced future NC of neurula-stage embryos with future epidermal ectoderm from gastrula-stage embryos. The grafted ectoderm formed neural folds that, according to the interpretation, responded to the combined neuralizing induction of the notochord and epidermalizing induction of the lateral mesoderm and differentiated into NC.

Do we know which events determine that cells at the presumptive epidermalneural border in such early embryos will form NC? Are NCCs induced or do they self-differentiate? If induced, is their induction separate from, part of, or subsequent to (and/or dependent upon) neural induction?

Some of the answers to these questions come from analyses of the origin of placodal ectoderm, some from the origin of Rohon–Béard neurons, both discussed in Chapter 6. In these studies, as in those outlined below, cellular and/or molecular markers are essential in tracing the origin of the NC and NCCs. Early studies, discussed below, used particular cell types as markers. More recently, and as discussed in the following section, molecular markers have been used almost exclusively to follow the initiation of NCCs.

Although not without their problems (Box 2.1), early studies in which neural folds were isolated and transplanted provided important information on the origin of the NC. Cell/tissue types such as mesenchyme, pigment cells, and cartilage known to arise from NCCs were used as markers to indicate that NC had been induced, although mesenchyme, which arises from NC and from mesoderm, is not a reliable

# Box 2.1 Isolating, extirpating, or grafting premigratory NCCs

Attempts are often made to isolate NCCs from neural folds before the cells have delaminated and migration has begun.

Because neural folds contain neural, epidermal, and perhaps placodal ectoderm in addition to NCCs, it can be difficult to isolate NC from the neural folds without including other cell types, and knowing whether you are isolating (or grafting) neural folds or NC is important. Unless neural folds are isolated carefully, a graft of a neural fold may contain neural and epidermal ectoderm (and perhaps placodal ectoderm; see below) in addition to NC. Drawing conclusions about intrinsic patterning of NCCs or about NC and/or placodal origins or particular cell types can thus be problematic. If epithelial ectoderm is included in the grafts, patterning that appears intrinsic to NCCs may, in fact, be imposed by the epithelial ectoderm. On the other hand, in situations in which ectoderm is required to induce NCCs, grafting NC alone will not reveal the differentiative potential of the grafted NCCs.

Extirpating NC is not totally satisfactory either. Some NCCs may be left behind; others may have delaminated and begun to migrate before the extirpation. Adjacent cells—neural ectoderm, or NC rostral or caudal to the region extirpated (or from the contralateral side if NC is removed from only one side)—may replace the extirpated NC through *regulation*, a topic discussed in Chapter 10. The NCCs removed may normally have played a role in inducing non-NC cells. Absence of a tissue or cell type after NC extirpation, therefore, is not unequivocal proof of NC origin.

All these caution means that the results of studies using extirpation have to be interpreted with caution; replacing extirpated NCCs with a similar population of labeled cells from another embryo provides an essential marker to follow the fate of the transplanted cells. Nevertheless, before such labeling methods were discovered, important (and usually correct) conclusions about NCCs were made.

marker for NC origin if mesoderm is also present. (A recent analysis by Blentic *et al.* (2008) demonstrates that Fgf signaling from pharyngeal arch epithelium is required (but not sufficient) for CNCCs to be directed into differentiating as mesenchyme; see Box 3.4)

However, the differentiation of pigment cells can be an excellent marker for the differentiation of a NC phenotype. As the retina and small populations of dopamineproducing neurons in the substantia nigra in the midbrain are the only other sources of pigment cells in vertebrates, differentiation of pigment cells is often a sufficient marker of NC origin (see Chapter 5). Cartilage also can be a marker of NCCs. Cartilage (and neural tissue) was evoked from early gastrula ectoderm of the European common frog using concanavalin A as the evoking agent. As the starting tissue was the embryonic ectoderm, and as no mesoderm was included, the cartilage that formed was presumed to be NC in origin and the gastrula ectoderm to have produced NCCs.

In a different approach, NC is induced and NC derivatives differentiate in lateral epiblast ectoderm in Japanese quail embryos into which a chicken Hensen's node (the site of the future notochord) is grafted. Chondrocytes form and can be positively identified as NC in origin because they express the Japanese quail nuclear marker; that is, they have been induced from the host epiblast by Hensen's node.<sup>⊕</sup> With the development of further markers, Bronner-Fraser and her colleagues used a similar approach to identify HNK-1-positive or *Snail2*-expressing cells (see below) in association with grafted neural plates.<sup>4</sup>

Arising as they do at the border between future neural and epidermal ectoderm (see Fig. 2.1), NCCs could be epidermal or neuroectodermal. Because they arise from the apical region of the neural folds but more especially because they produce neurons and ganglia and because some lineages can give rise to NCCs *and* CNS neurons, NCCs are regarded as derivatives of neural and not epidermal ectoderm; this is why we call it the NC and not the epidermal crest. In support of this designation, NCCs do not appear when epidermal–ectodermal derivatives arise in the absence of neural derivatives. The experimental induction of neural tissue, however, is accompanied by NC formation, although derivatives of the NC such as pigment cells and mesenchyme *can* arise in the absence of neural derivatives. The molecular markers outlined below provide further evidence of the neural lineage connection.

## NCC Markers and Specification of the NC

Markers of NC are more than convenient labels allowing us to identify NC or NCCs. Many play a role in the formation of the NC and/or in the delamination of NCCs. It is as markers, as active players, and, in some cases, as providing evidence of the connection of the NC to the neural lineage, that they are discussed below. More cellular aspects of NCC delamination are discussed in Chapter 3.

In an insightful series of papers published over the past 5 years, Daniel Meulemans, Marianne Bronner-Fraser, and their colleagues have addressed genes associated with the NC in the context of what they term a 'Neural crest gene regulatory network', with three levels of action:

- (i) **Inductive** (interactive) **signals** (Bmps, Wnts, Fgfs, and Notch/Delta) that establish the neural plate border (see Fig. 2.9) and upregulate **transcription factors** in the *Msx*, *Pax*, and *Zic* families at the neural–epidermal border.
- (ii) In turn, and after NC induction, these transcription factors upregulate genes of the *Snail*, *SoxE*, *FoxD3*, and other gene families that are specific to NCCs and

<sup>&</sup>lt;sup>⊕</sup> In addition to being the site of the future notochord and, therefore, a major player in the induction of neural ectoderm and NC, Hensen's node in tetrapods and its homolog, Kupffer's vesicle, in fish (see Box 9.1) imposes rostrocaudal patterning onto the NC during primary neuralation.

that are expressed before **NCC epithelial** —> mesenchymal transformation and migration (see Fig. 2.7 for *FoxD3*).

(iii) These transcription factors activate **downstream effector genes** associated with the **migration and differentiative potency of NCCs**.

This is an ancient network<sup>5</sup>—upstream elements (components of (i) and (ii)) are present in the North American sea lamprey—but, as you might expect, downstream or distal elements under (iii) show greater differences between agnathans and gnathostomes.<sup> $\oplus$ </sup>

# *HNK-1 and Pax7* $^{\diamond}$

HNK-1, an antibody against a cell surface sulfoglucuronyl glycolipid labels avian premigratory and *some* postmigratory NCCs, labels odd-numbered rhombomeres in the hindbrain, but does not label NCCs that are more fully differentiated (Fig. 2.3). Nor is HNK-1 required for NCC delamination in chicken embryos.<sup>6</sup> HNK-1 also identifies NCCs in embryonic lampreys, fish, birds, and mammals, but not in amphibians, while HNK-1-positive cells associated with the neural tubes in ascidian embryos provide one class of evidence that ascidians possess precursors of NCCs (see Chapter 4). The retinoid X receptor- $\gamma$  nuclear receptor gene, which is expressed in migrating chicken NCCs as they enter the somites—and at Hamilton– Hamburger (H.H.) stages 24–27 in the peripheral nervous system, dorsal root, and cranial ganglia—may be an earlier marker than HNK-1 for migrating avian TNCCs.

One has to be cautious in using HNK-1 as the sole marker for NCCs, however; the antigen was generated by immunizing a mouse with extracts of human natural killer cells—hence, HNK1—but is present on the surfaces of many cell types. HNK-1-positive cells are present in the avian embryonic gut before it is colonized by NCCs and mesenchymal cells of mesodermal origin can be labeled with HNK-1. During gastrulation in chicken embryos, HNK-1 and *Snail1* (see below) are regulated by *Pax7*, suggesting that *Pax7* could be used as an early marker for NC-fated cells; *Pax7* is broadly expressed in cranial and trunk NCCs in zebrafish (see Chapter 4).<sup>7</sup>

<sup>&</sup>lt;sup>⊕</sup> Living jawless vertebrates (agnathans) were formerly included in the cyclostomes, a group comprised of lampreys (petromyzontids), hagfish (myxinoids), and various groups of extinct jawless vertebrates. Cyclostomes, however, are not a natural (monophyletic) group. Researchers have grappled with whether lampreys and hagfish represent a monophyletic group of vertebrates with a common ancestor, or whether they represent two separate lines of jawless vertebrates (Fig. 1.3; and see Figs. 4.3 and 4.4).

<sup>&</sup>lt;sup>◊</sup> Pax (Paired box) genes, of which there are nine arranged in four groups, are transcription factors linked on the basis of a shared Paired domain. A partial or complete homeodomain also may be present. Each of the nine *Pax* genes acts within a specific tissue. Eight of the nine are discussed in this book. The only one not discussed, *Pax4*, functions in the β cells of the islets of Langerhans in the pancreas. Vertebrates have multiple copies of *Pax* genes resulting from gene duplication (see Box 1.2). Where vertebrates have a single gene, amphioxus has a single copy of the orthologous gene: *Pax 3* and *Pax7* in vertebrates, *Pax 3/7* in amphioxus (*AmphiPax3/7*); *Pax 1* and *Pax9* in vertebrates, *AmphiPax1/9* in amphioxus.

Fig. 2.3 These two fluorescent micrographs of adjacent thin sections through the trunk of an H.H. stage 18 chicken embryo show the comparative distribution of antibodies against the cell adhesion molecule Cad2 [N-cadherin] (A), and HNK-1 (B) HNK-1 is expressed strongly in migrating neural crest cells, which appear white in **B**. Cad2 is expressed in the lumen of the neural tube (NT), notochord (N) and myotome (M), but not in neural crest cells. Reproduced from Akitaya and Bronner-Fraser (1992), Copyright © (1992), from a figure kindly supplied by Marianne Bronner-Fraser. Reprinted by permission of Wiley-Liss Inc., a subsidiary of John Wiley & Sons, Inc.





# Snail-2, Bmp4, and Cadherins

The zinc-finger transcription factor-encoding gene *Snail2* —previously known as  $Slug^{\oplus}$ —has been used to great advantage as a marker for pre- and postmigratory NCCs.

<sup>&</sup>lt;sup> $\oplus$ </sup> The Human Genome Organization (HUGO) Nomenclature Committee has approved a new terminology for the genes previously known as *Snail* (now *Snail1*) and *Slug* (now *Snail2*). *Snail 1* and *Snail 2* are orthologs of the *Drosophila* genes *Snail homologue 1* and *Snail homologue 2*, respectively.

*Xsnail2* (where *X* stands for *Xenopus*) is expressed within the NC in neurulastage embryos and has been used as a marker for NC in studies in which NC is induced in *Xenopus*. *XSnail2* is downstream of *XSnail1*. As it induces NCC markers, *XSnail2* could play a role in NC induction, a role investigated by overexpressing mutant constructs in *Xenopus*. Early inhibition of *XSnail2* blocks the formation of NC; later inhibition prevents the migration of NCCs.<sup>8</sup> A recent analysis of NCC formation showed that the basic helix–loop–helix (bHLH) transcriptional repressor gene, *Xhairy2*, is localized in presumptive NC before expression of *Snail2* or *FoxD3* (see below and Fig. 2.4). *Xhairy2* appears to maintain presumptive NCCs as proliferative and nondifferentiating.<sup>9</sup>

As introduced in the previous section and discussed more fully in Chapter 3, migrating NCCs express cell adhesion molecules, such as N-CAM (neural cell adhesion molecule), Cad2 (N-cadherin), and Cad6B (Figs. 2.3 and 2.4), molecules that are regulated by *Snail2*. Cadherins are regulated by the genes *Snail2* and *Bmp4*, the latter a member of the TGF $\beta$  family of secreted factors (see Chapter 3). The binding of *Snail2* to regulatory sites for *Snail2* on *Cad6B* represents the first demonstration of a direct target of Snail2. Downregulation of Cad6B is triggered by Bmp4, which acts via an Adam10-dependent mechanism to cleave Cad2 into soluble fragments within the cytoplasm (Fig. 2.5, and see Chapter 3).<sup>10</sup>



From dorsal neural specification to NCC EMT (prior to delamination)

**Fig. 2.4** Major genes and pathways known to regulate the early development of NCCs, shown as four steps: determination of the dorsal neural tube (dorsal determination), segregation and survival of NCCs, and the epithelial —> mesenchymal transformation that allows delamination. Bmp, Wnt, Notch, FGF and retinoic acid (RA) are involved at all stages. Adapted from Morales *et al.* (2005)

Fig. 2.5 A summary of the genetic cascade involved in epithelial -> mesenchymal transformation and the delamination of NCCs. High levels of Noggin in mesoderm adjacent to the neural tube blocks Bmp4, Wnt1, and cyclin-D1 to prevent delamination. Expression of Cad2 (N-cadherin) in the dorsal neural tube (left) along with Adam10 also block cyclin1, preventing delamination. Inhibition of Noggin transcription initiates delamination by activating Cyclin-D1 via the canonical *Wnt* pathway (Bmp4  $\longrightarrow$  Wnt1  $\longrightarrow$ Cyclin-D1) and by cleavage of Cad2 to CTF1 and 2. Adapted from Shoval et al. (2007)



## Sox Genes

**Sox genes** are transcription factors that produce high-mobility group (HMG) proteins with many and varied functions. The name Sox is an acronym for S ry HMG-box transcription factors.

Sox genes are organized into 10 families, SoxA–SoxJ, which are related on the basis of similarity in the sequence of their DNA-binding HMG domain; all share the DNA motif (A/T)(A/T)CAA(A/T)G. Because all Sox genes are activated following interactions with partner molecules, they can exert different roles at different stages in the initiation, differentiation, and/or maintenance of the *same cell type*. Consequently, as important regulators of NCC initiation, development, and maintenance (Fig. 2.4), Sox genes appear over and over again on the pages ahead.

The SoxE subfamily is united on the basis of a shared C-terminal transcriptional activation domain. An important group of three SoxE genes (*Sox8*, *Sox9*, and *Sox10*) involved in NCCs was revealed in 1998 from studies with mice, in which defects in NC-derived ganglia of the colon were traced to a mutation in *Sox10*. The wide-ranging action of *Sox9* in NC and non-NC tissues is seen in *Campomelic dysplasia*, a human condition characterized by craniofacial defects, sex reversal, and malformed endochondral bones, resulting from a mutation in one allele of *Sox9*.

*Sox10*: *Sox10* is a major player in the four major processes responsible for the development of NCCs, processes that underlie the development of many cell types:

- initiation of the neural crest;
- maintenance of the multipotency of NCCs;
- specifying NCCs into particular lineage fates; and
- initiating the differentiation of specified cells.<sup>11</sup>

*Sox10* is expressed prominently in premigratory NCCs along the entire neural axis. Were it not for the fact that it is rapidly downregulated in the earliest stages of the differentiation of many NCCs, *Sox10* would be a good pan-NCC marker. The one exception is glial cells, in which expression of *Sox10* continues in embryos and adults (see Chapter 6).

Involvement of *Sox10* in NC formation is evident in the requirement for the expression of *Sox10* to activate expression of the NCC marker gene *Snail2* as early as blastula or gastrula stages of development. In *Xenopus* and in chicken embryos, *Sox9* induces *Sox10* expression (Fig. 2.4). Consequently, separating the actions of these two members of the SoxE subfamily is difficult, especially when different taxa are compared; NCCs are reduced in number in *Sox10*-mutant *Xenopus* and zebrafish but are present in normal numbers in *Sox10*-mutant mice.

Once formed, NCCs are maintained in a multipotent state by Sox10 under the regulation of Bmp2 and Tgf $\beta$ ; see Fig. 7.7 for Sox10 as a marker for ectopic expansion of NCC in mouse embryos.

*Sox9*: *Sox9* appears in several contexts through the book as an important regulator of various aspects of NCC development (Fig. 2.4, and see Fig. 4.10). Recent analysis implicates a mediator coactivator complex in the interaction between *Sox9* and transcriptional regulation via RNA polymerase II (Rau *et al.*, 2006).

Taxon-specific differences are evident in the role of Sox9. For example,

- (i) The induction of NCCs in *Xenopus* is dependent on *Wnt* signaling, which in turn is dependent on *Sox9*.
- (ii) Sox9 is involved in suppressing the death and so maintaining the survival of NCCs in zebrafish (Fig. 2.4). Zebrafish have two orthologs of Sox9, Sox9a and Sox9b, which function together as the single Sox9 gene functions in tetrapods.
- (iii) Sox9 regulates the expression of FoxD3 in mice, but not in zebrafish or Xenopus (Fig. 2.4).
- (iv) Sox9 is required to induce the otic placode in Xenopus but not in mice.

*Sox8*: The role of *Sox8* is less well understood than are those of *Sox9* and *Sox10*, in part because of surprising differences in apparent function between taxa, and in part because of a combination of overlapping and nonoverlapping functions between the three genes.

*Sox8*-deficient mice show weight loss but no defects that can be traced back to the NC or to NCCs. This is not because NCCs are unaffected. Rather, it is because of functional redundancy with *Sox9* and *Sox10*. Because it functions upstream of *Sox9* and *Sox10* in mouse embryos, *Sox8* can modify *Sox10* function in *Sox10*-mutant mice (Hong and Saint-Jeannet, 2005).

Taxon-specific differences were highlighted in a recent study of the expression and function of *Sox8* in *Xenopus* (Fig. 2.6 [Color Plate 1]). The chief differences from previous studies using chicken and mouse embryos are:



**Fig. 2.6** Expression of *Sox8*, *Sox9*, and *Sox10* in NCCs and in NCC derivatives in *Xenopus* embryos. *Sox8* is expressed around the blastopore in gastrulae (**A**, **B**, **C** and **D**) and then lateral to the neural plate (*arrows* in **B**, **C**, and **D**). In slightly later embryos, *Sox8* (**E**), *Sox9* (**F**), and *Sox10* (**G**) are expressed in the neural folds, the site of the future NC. Panel (**H**) shows *Sox 8* expression in both medial (*arrowheads*) and lateral (*arrows*) NC, shown here in a transverse histological section. (**I** and **J**) slightly later stage in neurulation showing the extent of expression of *Sox8* in the NC and expression in the future cement gland (*arrow* in **J**). With closure of the neural tube — shown dorsally in **K** and laterally in **L** — Sox8 is expressed in migrating CNCCs (*arrows* in **K**) and in premigratory TNCCs (*arrowheads* in **L**). The nine panels in (**M**) compare expression of the three *Sox* genes at two tail bud stages (25 and 35). Note co-expression in CNCCs but down-regulation of *Sox10* (are expressed in the primordium of the pancreas (*arrowheads*). Figure kindly provided by Jean-Pierre Saint-Jeannet (see Color Plate 1)

- *Sox8* is expressed early in *Xenopus* embryos, as early as the mid-gastrula stage, and so is the earliest marker of future NC known.
- Expression of *Sox8* in the NC precedes *Sox9* in *Xenopus* and follows *Sox9* and *Sox10* in chicken and mouse embryos, but *Sox8* is not expressed in the NC of zebrafish embryos.
- The earlier expression of *Sox8* in *Xenopus* embryos has the consequence that for a short time, coinciding with when NCCs are specified, *Sox9* and *Sox10* are not available to compensate for any loss of *Sox8*.
- The timing of the induction of the NC is delayed in *Xenopus* embryos in which *Sox8* is knocked down using a *morpholino*, an effect that can be rescued with restoration of *Sox8* expression.
- In *Xenopus*, *Sox8* regulates the onset of expression but not the maintenance of the marker genes for the NC, *Snail*, and the winged-helix transcription factor, *FoxD3*.
- Migration, but not the proliferation of NCCs, is delayed in *Sox8*-deficient *Xenopus* embryos, resulting in major defects in several NCC lineages, severe loss or reduction of the craniofacial skeleton and dorsal root ganglia in all embryos, and reduction of pigmentation in two-thirds of treated embryos.

*LSox5*: *LSox5* is the long form of *Sox5*; the functions of *Sox5* have not been elucidated, although it seems more associated with glial cells than with NC- or placodederived neurons. *LSox5* was first isolated in a screen of chicken embryos, where it is expressed in premigratory and migratory cranial and more caudal TNCCs. Regulated by *Sox9* (see Fig. 4.10), *LSox5*, *Snail1*, and *Snail2* initiate migration by acting through RhoB, a low-molecular-weight GTPase in the Ras protein family (Fig. 2.4). Overexpressing *Snail2* by gain of function in chicken embryos enhances RhoB expression and increases the number of NCCs (HNK-1-positive cells) that form in the neural tube.<sup>12</sup>

A role in specifying NCCs, revealed after misexpressing *LSox5* in the dorsal neural tube, elicited additional and ectopic NCCs beside the dorsal neural tube. Expression of *LSox5* alone, however, is *not* sufficient to generate NCCs; active *Sox9* is required to generate a full complement of NCC markers and functions (Hong and Saint-Jeannet, 2005\*, and Fig. 2.4). *LSox5* acts cooperatively with *Sox6* and *Sox9* to promote chondrogenesis.

#### Wnt genes

Wnt genes have emerged as important signaling molecules in development, in no small part because they signal through several transduction pathways. The major pathway, the one by which Wnts exert their effects on NCCs, is through stabilization and regulation of the transcriptional role of  $\beta$ -catenin in **the canonical Wnt pathway** (Fig. 2.5). Much remains to be discovered, and Wnt signaling pathways are understood in considerably greater detail than are outlined in Fig. 2.5. Furthermore, cross-regulation between Wnt and Notch signaling pathways ('Wntch signaling')

and roles for Wnt in the specification of cell fate in bipotential cells are emerging, for which see Hayward *et al.* (2008).

*The Canonical* Wnt *Pathway*: The phrase 'canonical Wnt pathway' refers to a cascade initiated by Wnt proteins binding to their cell surface receptors (members of the Frizzled family), resulting in the activation of proteins in the Disheveled (Dsh) protein family that form part of the Wnt receptor complex in cell membranes. Further downstream changes culminate in regulation of the amount of  $\beta$ -catenin reaching the nucleus (Fig. 2.5).  $\beta$ -Catenin interacts with transcription factors of the T-cell specific/lymphoid enhancer binding factor (Tcf/Lef) family, which upregulate specific gene expression.

*Frizzled* genes, which encode Frizzled Wnt receptors, are upregulated in NCCs and in condensing mesenchyme. The protein Kermit interacts with the C-terminus of *Frizzled3* (*Xfz3*) in *Xenopus*; NCC induction is blocked in *Xenopus* if Kermit is knocked out, and expression of *Xfz3* is required for *XWnt1* to be expressed and NC to be formed.<sup>13</sup>

The NonCanonical Wnt Pathway: Noncanonical (planar cell polarity or Wntprotein kinase C–Ca<sup>++</sup>) Wnt signaling is independent of  $\beta$ -catenin, but acts through domains on Dsh proteins to phosphorylate regulatory sites of JNK proteins, which are the products of *mitogen-activated protein kinase* (*MAPK*) genes. Pescadillo, a nuclear protein regulated by the noncanonical Wnt pathway, plays a role in CNCC migration in *Xenopus*; loss of function of Pescadillo leads to cranial cartilage defects.

Although the canonical andnoncanonical pathways are separate, individual *Wnt* genes can operate in tandem to regulate cell specification. For example, when operating via the canonical Wnt pathway, *Wnt1* inhibits the induction of NCCs in chicken embryos. When operating via the noncanonical Wnt pathway, *Wnt6* induces NCCs through specification of the neural plate border (Fig. 2.7 [Color Plate 2]); *Wnt6* can operate through both canonical and noncanonical pathways.<sup>14</sup>

Wnt *Expression and Function*: Of the 21 genes in the *Wnt* family, at least 10 are expressed in 8–9.5-day-old mouse embryos (Table 2.1), three with sharp boundaries of expression in the forebrain immediately before the onset of CNCC migration.<sup>15</sup>

*Wnt1* is involved in the determination of the midbrain–hindbrain boundary—an important organizing center (see below and Box 3.3)—and in patterning the midbrain. Given that *Wnt1* is expressed in the dorsal neural tube throughout most of the body axis, *Wnt* expression cannot be used as a marker for specific populations of NCCs; Wnt1-cre mice were generated to take advantage of the finding that *Wnt* is a marker for all NC derivatives. Indeed, using Wnt-cre as a marker system in mice it was demonstrated that conditionally knocking out *Wnt* results in loss of NC derivatives, while constitutive activation of *Wnt* directs most NCCs into a neuronal cell fate.<sup>16</sup>

*Wnt*-signaling also plays a role in regulating the proliferation of NCCs. Double mouse mutants (*Wnt1<sup>-</sup>/Wnt3a<sup>-</sup>*) display defective NC and deficient dorsal neural tubes. The stapes and hyoid bones—both derivatives of hindbrain NC—are missing and thyroid cartilages abnormal. Mice lacking either *Wnt1* or *Wnt3a* form reduced numbers of TNCCs, resulting in reduced numbers and inhibited differentiation of

		Table 2.1 Ages and Inched stages	or more accerob	
Day of gestation	Theiler stage <sup>b</sup>	Morphological stage	Somite numbers	Neural tube development
∞	12	Late primitive streak	1–8 (3–4)°	Open neural plate with neural groove and neural folds NCCs delaminate from the midbrain and rostral portion of the hindbrain
8.5	13	Rotation of embryo	(5–7) <sup>c</sup> 8–12	NCCs delaminating from all levels of the brain Initial elevation of neural folds
9	14	Anterior neuropore	13-20	Elevation, convergence, and fusion of the neural folds to form the
			(16) <sup>c</sup>	hollow neural tube; formation and closure of anterior neuropore Delamination of CNCCs complete
9.5	15	Forelimb buds appears	21–29	Migrating CNCCs; formation of posterior neuropore
10	16	Hindlimb buds appear	21–30	Neural tube completely fused; closure of posterior neuropore; panolia of cranial nerves as condensations
11	18	lens vesicle detaching ectoderm	36-42	Regions of the brain are distinct. Neural tube is fused from
<sup>a</sup> Given as in a typic <sup>b</sup> As described in T	cal inbred strain. heiler (1972) on	the basis of whole and sectioned er	nbryos.	

Table 2.1 Ages and Theiler stages of mouse development in relation to NCC origins<sup>a</sup>

<sup>c</sup> As determined by Nichols (1987) using transmission electron microscopy.



**Fig. 2.7** *Wnt6* and NC induction depicted in cross-sections of the neural tubes of H.H. stage 18 (3-day) chicken embryos using FoxD3 protein and HNK-1 as NC markers. (**A**) Wnt6 (*brown*) and FoxD3 (*blue*) expression in neural ectoderm (*arrowheads*) in a control embryo. The area marked \* is shown in the insert. (**B**) Reduced Wnt6 (*brown*) expression and absence of FoxD3 (*blue*) expression in a Wnt6 siRNA-treated embryo. (**C** and **D**) Reduced expression of FoxD3 (*blue*) at three rostrocaudal levels of the dorsal neural tube in a Wnt6 siRNA-treated embryo (**D**) when compared with control (**C**). (**E**) FoxD3 (*blue*) expression in the dorsal neural tube of a control embryo (**E**) is reduced significantly in Wnt6 siRNA-treated embryo (**D**, *white arrowhead*). Figure kindly supplied by Imelda McGonnell (see Color Plate 2)

melanocytes. The transcription factors Ap2 $\alpha$  and Ap2 $\gamma$ ,<sup> $\oplus$ </sup> discussed in Chapters 6 and 7, are regulated by *Wnt* genes and, at least in zebrafish, regulate the expression of *Snail2* to play a role in NC induction. In skeletogenic NCCs, *Hoxa2* is a target of *Ap2\alpha*, which in turn is regulated by (and can substitute for) Bmp in NC induction.<sup>17</sup>

<sup>&</sup>lt;sup> $\oplus$ </sup> Ap2 is a family of four transcription factors (Ap2 $\alpha$ , Ap2 $\beta$ , Ap2 $\gamma$ , and Ap2 $\gamma$  $\partial$ ) that share conserved DNA binding and dimerization domains. Ap2 $\alpha$  plays a critical role in NC induction, and in NCC initiation and maintenance. Ap2 $\alpha$  is expressed in amphioxus, so its role in neural tube development preceded the origin of the NC and the vertebrates (Meulemans and Bronner-Fraser, 2002).

## Specification of Ectoderm as Neural or Epidermal

Does the association between NC and neural tissues mean that the NC, like neural ectoderm, arises during or in association with neural induction, or is the NC set aside as a determined layer earlier in development? Given that the NC arises at the border between neural and epidermal ectoderm, we need to take a brief look at neural induction (a topic worthy of a book in its own right) and at how neural and epidermal ectoderm are specified.

According to the classic interpretation of the associations between notochord, neural ectoderm, and NC proposed by Raven and Kloos in 1945, the neural tube is induced by notochord, and NC is induced by lateral mesoderm (Fig. 2.8). The argument went as follows: the presumptive notochord contains more inducers than the lateral mesoderm. The notochord therefore induces neural structures and NC, while the lateral roof induces NC alone (Fig. 2.8). This interpretation rests on the assumption of a lower threshold for induction of NC than for induction of neural tissue, and on a graded distribution of neuralizing inducer with the mesoderm, with the highest concentration in dorsal mesoderm (notochord). Below is an outline of how ectoderm is dorsalized, and neural and epidermal ectoderm are specified, as essential background to discussing the induction of the NC itself. Members of the Bmp family of growth factors play major roles at several stages during neural induction, as outlined below.<sup>18</sup>

- (1) Dorsalization of ectoderm: Following interactions during gastrulation, ectoderm is dorsalized by *Bmp7* from the mesoderm, and ventralized/caudalized by *Hox* genes; in *Xenopus* and chicken embryos, overexpressing *Bmp7* in ventrolateral mesoderm dorsalizes the neural tube and promotes expansion of neural ectoderm.
- (2) **Neural or epidermal ectoderm**: Neural induction flows from interactions between axial mesoderm (presumptive notochord) and the overlying dorsalized ectoderm, establishing the location of the future dorsal nervous system.

Initially, Bmp4 is distributed throughout the neural ectoderm in a gradient that is highest rostrally and decreases caudally. The gradient is established by a reciprocal gradient of the Bmp4 inhibitor, Noggin, a secreted polypeptide. Induction and rostrocaudal patterning of the nervous system both involve cascades of signals that suppress Bmp4 (a growth factor that plays a key role in



Fig. 2.8 A model of induction of neural plate (NP) and neural crest (NC), based on differential strength of induction (shown by the *thickness* of the *arrows*). Notochord (*circle*) is a stronger inducer than is lateral mesoderm. See text for details

NC induction; see below). Bmp4 and Bmp7 must both be inhibited if ectoderm is to become neural. Receptor mediation is part of the mechanism; injecting a dominant negative Bmp4 receptor into *Xenopus* animal cap ectoderm neuralizes the ectoderm, a neural fate that can be reversed after injecting *Bmp4* mRNA. Activin, another growth factor in the Tgf $\beta$  superfamily, inhibits neuralization but does not induce an epidermal cell fate.<sup>19</sup>

Bmp2 also ventralizes the embryonic dorsoventral axis and mesoderm and nervous system and is involved in later organogenesis; in *Xenopus*, neurula-stage embryos, zygotic transcripts of Bmp2 are expressed in the NC, olfactory placodes, pineal gland, and heart primordia.

(3) **Fore- and hindbrain**: Depending on the species, Chordin (a protein involved in the determination of the dorsoventral body axis; see Box 4.1), Noggin, and/or follistatin (a protein that binds to the growth factor, activin, and inhibits Bmp7) bind to Bmp4 to prevent Bmp4–receptor interactions, and so specify the most rostral neural ectoderm associated with fore- and hindbrain. *Noggin* is then downregulated in gradient fashion within the ectoderm, effectively setting the earliest stage when NCCs can delaminate from the neural tube (see Chapter 3).

# **NC Induction**

# **Bmps**, Wnts, and Fgfs

Three major classes of genes have emerged as involved in NC induction at the neural–epidermal border in different vertebrates: **Bmps, Wnts,** and **Msx genes** (Fig. 2.9). In a recent review emphasizing the functions of Bmps and Wnts in the NC, Raible and Ragland (2005\*) discuss several models by which these gene families and their products interact to initiate NCC formation. Each is a two-step model, involving specification of NC by:

- sequential activity of ectoderm-derived *Bmp* and *Wnt* at the border of the neural tube, with Bmp conferring competence on the neural plate to respond to Wnts (Fig. 2.9);
- (2) combined activity by which Notch signaling in the dorsal neural tube modulates the activity of Bmp and so induces NC (Fig. 2.9); and/or
- (3) interaction between different signaling pathways such as Fgf —> homeobox, (msh-like 1 gene) Msx1 and Wnt —> Pax3 to induce NC.

All three modes of specification, or combinations of two or more modes, may operate in a single species. The pathway utilized may vary from species to species, or one or more pathways may act as a backup for a third and most usually used pathway. Although termed 'modes' and 'pathways', there may well be considerable conservation in signaling across the vertebrates. At least one Bmp and one Wnt gene signal high up in the cascade. Differences between species may be as 'simple' as which



**Fig. 2.9** A comparison of the signaling molecules involved in induction of NC at the neural ectoderm (*gray*)–epidermal ectoderm border of avian, zebrafish, and frog embryos as seen in a crosssection with the neural plate (*gray*) and lateral epidermal ectoderm *above* and the notochord and somitic mesoderm *below*. For the genes activated by these signaling molecules, see the text. In all three taxa, *sonic hedgehog* (*Shh*) from the notochord activates *Bmp4* and *Bmp7* in the neural plate via the Delta–Notch pathway to establish the neural–epidermal ectoderm border. Additional signals from the epidermal ectoderm differ by taxa; *Wnt6* in chicken embryos, *Wnt8* in zebrafish, and *Wnts1*, *3a* and 7 in frog embryos (shown on the *left* and *right*, respectively). Additional signals required to generate the border in *Xenopus* include *Wnt8*, *Fgf2*, and retinoic acid (RA) from the somitic mesoderm, and *Fgf8*; whether the *Fgf8* is somitic or epidermal ectodermal in origin is uncertain (shown as *Fgf8?*). Arrows show signaling to the border, except for *Shh*, which signals to the neural plate. Data from various sources. Presentation adapted from Jones and Trainor (2005)

Bmp or which Wnt paralogs is used or precisely when in the cascade the Bmp or Wnt is activated. Nonetheless, because differences occur, the evidence for *Xenopus*, chicken, and mouse embryos are discussed separately.

## Xenopus

Once neural ectoderm is induced by notochord (see above), induction of NC occurs at the epidermal/neural ectodermal border. Depending on the species, this may complete the induction, or epithelial ectodermal signaling may continue to be required for the induction/specification of particular types of NCCs. Lateral mesoderm (Fig. 2.10) is involved in NC induction in *Xenopus*, acting in concert with axial mesoderm. Lateral (paraxial) mesoderm evokes at least four NC markers from *Xenopus* neural ectoderm—*Snail2*, *FoxD3*, *Zic3*, and *Sox9*—axial mesoderm evokes only a subset, while in the absence of mesoderm, *Fgf8* upregulates all but *Snail2* in the list above. The implication is that *Fgf8* from lateral mesoderm is a necessary and may be a sufficient signal to evoke NC in *Xenopus*.<sup>20</sup>

Although the role of mesoderm was thought not to extend beyond the initial induction of neural ectoderm by the notochord in *Xenopus* (Fig. 2.10), paraxial



Fig. 2.10 The sequence of the major steps in the induction of the neural crest, as seen in early amphibian embryos. (A) Step 1: the mesodermal (M) induces neural ectoderm (Ne). (B) Step 2: neural and epidermal ectodermal induce neural crest (NC) at the neural–epidermal boundary. Step 3: ventral mesoderm (VM) may play a role in epidermal ectodermal induction of neural crest

mesoderm may participate in inducing the NC by regulating a gradient of Bmp associated with induction of NC and inhibition of epidermal differentiation; future epidermal ectoderm dorsalized by Noggin produces melanophores, even though no notochord is present; chicken neural ectoderm associated with mesoderm forms melanocytes but not neurons. In tissue recombination experiments, and on the basis of the induction of high levels of expression of *Snail2* and the differentiation of melanophores, lateral mesoderm was found to be a more potent inducer of NC than is notochord. Studies with whole embryos support this conclusion: removing lateral mesoderm reduces markers of NC induction/differentiation; removing notochord has no effect.<sup>21</sup>

One of the Wnt genes, *Xwnt7b*, expressed in future epidermal ectoderm, plays an active role in the induction of *Xenopus* NCCs (Fig. 2.9). The evidence is based on (i) the finding that *Xwnt7b* induces the NC markers *Xsnail2* and *Xtwist* (which encodes a bHLH transcription factor) in epidermal ectoderm cotreated with Noggin and in neuralized ectoderm *in vitro* and (ii) that exogenous *Xwnt7b* enhances the expression of *Xtwist in vivo*.

A role for *Wnt8* in NC induction in *Xenopus* (and in zebrafish) has also been demonstrated through inhibition of Bmp4 at gastrulation, a stage of development when Noggin does not inhibit Bmp4 (Baker *et al.*, 1999). *Wnt8* from somitic mesoderm is involved is establishing the border at which NC arises in *Xenopus* and in zebrafish (Fig. 2.9). *AmphiWnt8* is also expressed in the paraxial mesoderm in amphioxus, and although functional studies have not been performed, the patterns of expression are consistent with the possibility that *AmphiWnt8* may play a role in neural induction, upregulating *Pax3/7* and *Msx* at the border between epidermal and neural ectoderm (see Chapter 4).

Fgf2 from somitic mesoderm and Fgf8 from mesoderm or epidermal ectoderm (Fig. 2.9) are involved in NC induction in *Xenopus*; neural differentiation declines and melanophore differentiation increases if gastrula ectoderm from increasingly older embryos is exposed to Fgf2, a finding that is consistent with altered competence $^{\oplus}$  of the ectoderm with age and with a progressive shift from neural to NC induction.

#### Chicken Embryos

Fgf2 appears to play a role in neural and NC induction in avian embryos. Overexpressing Fgf2—achieved by Rodríguez-Gallardo *et al.* (1997) by placing Fgfsoaked beads within the primitive streak—induces ectopic neural cells from epidermal ectoderm; whether NC also arose in these ectopic neural cells was not reported.

Juxtaposing neural and nonneural ectoderm from H.H. stage 4–10 avian embryos elicits NC; juxtaposing the same tissues from older (H.H. stage 8–10) embryos dorsalizes the ectoderm, conclusions based on upregulation of such dorsal and NC markers as *Wnt1*, *Wnt3a*, and *Snail2*. This result is consistent with a two-step model for induction of the NC in chicken embryos, involving *Wnt6* and *Fgf* as major upstream regulators (Fig. 2.9). *Snail2*, which by activating Rhob can enhance NCCs production, may be part of the second step (del Barrio and Nieto, 2002).

Mouse Embryos: In mice, Snail2 is not required for NC or mesoderm formation.

*Snail2* is not expressed in murine premigratory NCCs but is expressed in migrating NCCs. *Snail2* alone does not provide a sufficient signal to induce NC in *Xenopus*, indicating modulation of NCC inducing pathways between different vertebrates. As neither *Snail1* nor *Snail2* is involved in NC induction or NCC delamination, a two-step model involving *Snail* genes cannot apply to mice, although *Snail1* does play a role in establishing the left–right symmetry of murine embryos. Nevertheless, murine and chicken *Snail2* are functionally equivalent in that *Snail2* from either species can function in chicken hindbrain.<sup>22</sup>

#### A Role for Notch in NCC Induction

Members of the Notch family of transmembrane domain proteins—Notch1–Notch4 in mammals—are receptors for two families of transmembrane ligands, Jagged (Jagged1, Jagged2) and delta-like (Delta-like1, 3, and 4). Alagille syndrome, which includes heart and facial defects, results either from *absence of the gene JAG1* (5–7% of individuals) or spontaneous mutation(s) in *JAG1* (perhaps as many as 50% of cases).

<sup>&</sup>lt;sup> $\oplus$ </sup> Competence is the term used in developmental biology for the ability of a group of embryonic cells or an embryonic region to respond to inductive signals. Competence is gained and lost progressively during development (see this chapter and Box 2.2). Loss of competence is the proximate explantation for the loss of the lateral line and cement glands in the direct-developing Puerto Rican frog, *Eleutherodactylus coqui* (see this chapter and Box 2.3), for the inability of *premature death* (*p*) mutant Mexican axolotls to form NC-derived cartilages (see Chapter 7), for the loss of teeth in birds (see Box 3.5), and for the variability in regulative ability in subpopulations of cells along the body axis or in different species (see Chapter 10).

Receptor–ligand interaction enables signal transduction between adjacent cells and regulation of gene expression by activating bHLH repressors of transcription. After receptor binding, the Notch intracellular domain is cleaved off and transported to the nucleus, where it binds to a C-repeat binding factor (CBF) in nonmammalian vertebrates and to the conserved DNA-binding protein RBP-Jk in mammals.

Notch signaling plays a critical role in NC specification by facilitating **lateral induction** (Box 2.2) in gastrulae, determining the ectodermal domain from which NC will arise. In chicken and amphibian embryos, Notch is involved in determination of the ectodermal NC domain for CNC, reflecting a conserved role for Notch in generating boundaries—for example, at wing margins in *Drosophila*, and at limb bud margins in vertebrates—and in refining boundaries, for example, somite boundaries.

# **Box 2.2 Lateral induction**

Lateral (homoiogenetic, horizontal, planar) induction is the spreading of an induced state by cells that were induced following interaction with an inductor derived from an adjacent cell layer, the latter sometimes known as vertical induction (Figs. 2.6 and 2.11). Neural induction proceeds laterally in *Xenopus*; that is, additional neural tissue is induced from already induced neural tissues through a signal traveling along the ectoderm, rather than from continuous vertical induction from the notochord below (Fig. 2.11).

Lateral induction has been demonstrated by the neuralization of ectoderm transplanted adjacent to the neural tube or placed in culture with neural tube, or by replacing early future neural plate ectoderm of Mexican axolotls or alpine newts with uninduced gastrula ectoderm (Servetnick and Grainger, 1991). There is loss of ectodermal competence, lateral spread of neural induction along the ectoderm, and placode formation in association with weak competence at the boundary. In chicken embryos, trunk but not cranial neural ectoderm is induced laterally, cranial neural tube requiring contact with the invaginating Hensen's node (Box 9.1). Hindbrain from zebrafish can induce ventral epidermis to become NC, a finding that is inconsistent with lateral induction in this species.

Some studies raise the issue of whether induction of NC always follows the induction of neural ectoderm. Although Mitani and Okamoto (1991) claimed evidence for separate inductions of neural tube and NC in a microculture assay of *Xenopus* early gastrula cells, close range and/or lateral inductions cannot be ruled out in such an experimental approach. Mitano and Okamoto used antibody markers for neurons, melanophores, and epidermal cells, but not NC markers. Using the genes *Snail1, Snail2, and Noggin* as NC markers, Mayor and colleagues (1995) claimed that NC was induced independently of the neural plate. *Noggin* is an important inducer of rostral neural tissues and associated structures such as the

cement gland (Box 2.3), but does not induce hindbrain or spinal cord; induction of postotic and TNC is controlled by genes other than *Noggin*.<sup>a</sup>

<sup>a</sup> See Lamb *et al.* (1993) for *Noggin* as an inducer of rostral neural structures and Holtfreter (1968) and Nieuwkoop *et al.* (1985) for older studies on lateral induction affecting the NC.

## **Box 2.3 Cement glands**

**Cement glands** located on the ventral surface of the head of anuran tadpoles (see panel J in Fig. 2.6) are used for attachment during feeding.<sup>a</sup> In *Xenopus*, the cement glands arise following a series of interactions initiated during the induction of rostral neural and epidermal ectoderm. Disrupting any of these interactions blocks cement gland formation. Induction is evidenced by differential expression of epidermal and nonepidermal keratins and by the expression of an antibody against tyrosine hydroxylase associated with the glands (Drysdale and Elinson, 1993).

A gene involved in cement gland induction, *XOtx2*, the *Xenopu* s ortholog of the *Drosophila* gap gene *Orthodenticle*, is expressed in rostral neurectoderm during gastrulation. Ectopic expression of *XOtx2* is a sufficient signal to induce an extra cement gland.

*Dlx* is another gene expressed in *Xenopus* cement glands. In Puerto Rican coqui, which lack cement glands, *Dlx* is expressed in a region of ectoderm that corresponds to the ectodermal region from which cement glands arise in *Xenopus*. Fang and Elinson (1996) used cross-species transplantation and tissue recombinations to investigate the potential developmental mechanisms responsible for the loss of the cement glands in coqui. They found that coqui cranial tissues can induce cement glands from *Xenopus* ectoderm, but that coqui ectoderm cannot respond to inductive signals from *Xenopus*; competence of coqui ectoderm to respond to induction is modified without modifying the inductive signal. Therefore, loss of competence, not loss of induction, leads to loss of cement glands in coqui.

Loss of ectodermal competence is also responsible for the loss of balancers in some amphibians, for loss of limbs in avian mutants such as *limbless*, and for loss of teeth in birds.<sup>b</sup>

An important series of messages lies in these examples of the ways in which cell and tissue interactions are modified in association with the loss of structures during evolution:

(1) An organ may be lost without the loss of the entire developmental system that produces that organ.

- (2) Loss of organs is often mediated through modification (not loss) of inductive interactions.
- (3) Modification of competence is the usual means by which inductive interactions are altered.
- (4) Inductive signaling can persist even if competence to respond is lost.
- (5) Provided that competence can be restored, the potential exists for the organ to reappear.

<sup>a</sup> In their description of larval cement glands in 20 species of frogs, Nokhbatolfoghahai and Downie (2005) documented five patterns—not necessarily restricted to families—and three species that lacked cement glands, two of which bore traces of the glands as evaginations. <sup>b</sup> See Maclean and Hall (1987) and Hall (1987, 1999a\*, 2005b\*) for examples of loss of ectodermal competence.

After NC is specified Notch plays a further critical role, specifying the fates of NCCs through **lateral inhibition**; Notch limits the number of cells that adopt a primary fate, holding them in reserve for a second fate. The cells held in reserve express a high level of the Notch ligand Delta1. Adoption of the second fate requires activation of Notch signaling, which, depending on species, may or may not involve maintaining cells in a proliferative state, and may or may not always act by inhibiting the expression of genes required for cells to adopt a neuronal fate.

In chicken embryos *Notch* signaling is modulated by *Lunatic fringe* (*Lfng*), which encodes for a glycosyltransferases that modifies *Notch* and its ligands. *Lfng* is expressed in the neural tube except along the dorsal midline. The border between expression and nonexpression therefore marks the site of future NC formation. In the presence of excess *Lfng*, Nellemans and colleagues (2001) found a 68% increase in CNCCs as a result of enhanced proliferation of existing NCCs; *Lfng* upregulated *Delta1* leading to the redistribution of *Notch1* and enhanced development of CNCCs.

In *Xenopus*, Notch signaling is required for the expression of *Xsnail2*, which is induced by *Xmsx1*, which in turn is induced by *Bmp4* under Notch control

Notch -> Bmp4 -> Xmsx1 -> Xsnail2

Similarly, in chicken embryos, *Bmp4* (and therefore Notch) is required for expression of *Snail2* as CNCCs are specified; epidermal expression of the Notch ligand, Delta1 is required to upregulate *Bmp4* and to induce NC in chicken embryos (Endo *et al.*, 2002).

Notch signaling appears to play a minor role in induction of the NC in fish and mammals. Knocking out *Delta1* in mice or *DeltaA* or *Notch1a* in zebrafish does not eliminate NC. It may reduce the numbers of NCCs that form, although redundancy with other pathways may obscure the primary effects in these knockouts.

## A Role for Bmps in NCC Induction and Beyond

As just discussed, as members of the TGF- $\beta$  family of secreted factors Bmps are regulated by Notch signaling. Bmps also are regulated in the extracellular environment by binding proteins, Noggin and Chordin being the two most well studied. At the transcriptional level, *Bmps* are regulated by **Smad proteins**<sup> $\otimes$ </sup> translocated to the nucleus.

In *Xenopus*, Smad7 inhibits Bmp4-mediated induction of mesoderm, thereby activating a default neural induction pathway. Smad4 is not required for NCC migration in mouse embryos but is required for the correct patterning of the epithelium of the first pharyngeal arch, which, in turn, patterns the craniofacial skeletal elements (see Chapter 7). Smad4 is required for the development of tooth buds beyond the dental lamina stage, and for development of the NCC contribution to the cardiac outflow tract (see Chapter 8). In all three situations, Smad4 mediates epithelial–mesenchymal interactions (see Chapters 7 and 8).<sup>24</sup>

Bmps play multiple roles in development, so it is perhaps not surprising that they play multiple roles in NCCs in chicken, *Xenopus*, and zebrafish embryos, including:

- induction of the neural crest;
- epithelial → mesenchymal transformation and migration of NCCs (*Noggin* is downregulated in gradient fashion within the ectoderm, setting the earliest time for NCC delamination from the neural tube);
- specification of some types of NCCs, especially cells of the autonomic nervous system; and
- regulation of those mesenchymal NCCs that form craniofacial skeletal and heart structures via *Smad4*.<sup>25</sup>

After NC induction and specification, NCCs reuse Bmps at different times, in different places, and in different ways. Bmp2 is expressed in distinct fields in facial epithelia and in NC-derived mesenchyme but not in somatic or prechordal mesoderm.

As discussed earlier, acquiring or maintaining a dorsal fate involves interaction between neural ectoderm and signals from the epidermal ectoderm. Genes preferentially expressed in the dorsal neural tube (from which NCCs arise) initially have a uniform distribution throughout the neural tube but are inhibited ventrally by genes, such as *Shh*, although cells of the ventral neural tube can be switched to NCCs if they are grafted into the migration pathway taken by NCCs (see Box 6.3).

 $<sup>^{\</sup>otimes}$  *Smads* (*Small Mothers Against Decapentaplegic*) are three classes of transcription factors. Their most common role is to modulate the action of ligands of members of the Tgf $\beta$  family of growth factors, with which they complex before entering the nucleus to transcribe gene activity. *Smad1*, *Smad4*, and *Smad7*, each play roles in the NC or in NCCs.

As discussed when evaluating neural induction, a gradient of Bmp expression along the neural tube, at its highest rostrally and lowest caudally, is suggested from analyses of mutant zebrafish that lack Bmp2b, and in which the NC fails to form. The role of such a gradient, whether it is counteracted by an inverse gradient of the Bmp inhibitor, Noggin, and whether it is required to induce NC in all taxa remain active areas of research, as evidenced by studies in which Bmp2 has been shown to be required for NCC migration but not for NC induction in mouse embryos, even though Bmp2 is required for induction in other vertebrates; blocking Bmp2 or Bmp4 in murine embryos with Noggin depletes CNCCs, resulting in small pharyngeal arches and inhibition of chondrogenesis (because Bmp2 is required for NCC migration) but does not block NC induction.<sup>26</sup>

Bmp4 is expressed initially in the lateral neural plate and subsequently in the dorsal neural tube and midline ectoderm. It now appears that *Shh* mediates regionalization of the medial portion of the neural plate—the region from which neurons and NC arise—while Bmp from the adjacent epidermal ectoderm regionalizes the lateral neural plate, from which placodes arise (Figs. 2.11 and 6.4; and see Box 10.1). *Bmp4* and *Bmp7* are expressed in epidermal ectoderm adjacent to the border with neural tube (Fig. 2.12); either one can substitute for ectoderm to promote NCC delamination and activate NC markers such as *Snail1*. The Bmp4 expressed at the edges of the neural plate and in the dorsal neural tube also signals to paraxial mesoderm; grafting Bmp4-producing cells into paraxial mesoderm induces *Msx1* and *Msx2* expression (see the following section) and is associated with ectopic cartilage formation. Similarly, suppressing Bmp inhibits its ventralizing action in dorsal locations.<sup>27</sup>

## Zic3 and Zic5

The pair-rule family of homeobox genes in *Drosophila* is responsible for the subdivision of the embryonic body into regions. *Zic3*, an ortholog of the *Drosophila* pair-rule gene *odd-paired*, encodes a zinc-finger transcription factor that promotes NC over neural differentiation. *Zic3*, which is blocked by Bmp4, is expressed in



**Fig. 2.11** The role of sonic hedgehog (Shh) and Bmp4 and Bmp7 in the induction of the neural crest. Shh in the notochord (N) induces neural ectoderm from the neural plate (NP). Bmp in the neural plate and epidermal ectoderm induces neural crest (NC) at the neural–epidermal ectoderm boundary. Epidermal ectodermal BmpP diffuses laterally to induce placodal ectoderm (P). Neural plate Bmp diffuses to the mesoderm (M) to induce somitic mesoderm



**Fig. 2.12** Expression of Bmp4 and Bmp7 as seen in cross-sections through the developing neural folds, dorsal neural tube, and epidermal ectoderm in a chicken embryo of H.H. stage 10. At the level of the open neural folds (**A**, **B**, **C**, and **D**), Bmp4 is expressed in the neural folds and in the epidermal ectoderm flanking the neural folds (**A** and **C**), while Bmp7 is only expressed in epidermal ectoderm (**B** and **D**). At the level of the closed neural tube (**E**, **F**, **G**, and **H**), Bmp4 is concentrated in the dorsal midline of the neural tube (**E** and **G**), while Bmp7 is concentrated in the epidermal ectoderm, especially in the region of the future forebrain (**H**). Bar = 80  $\mu$ m (**A**, **B**, **C**, and **D**); 100  $\mu$ m (**E**, **F**, **G**, and **H**). Reproduced from Liem *et al.* (1995) from a figure kindly provided by Karel Liem and with the permission of the publisher, Copyright © Cell Press

neural ectoderm and NC, appearing first in the neural plate at gastrulation (Fig. 2.13). *Zic3* is one of the earliest genes so far identified as involved in neural ectoderm, induction, and/or proliferation of NC. Overexpressing *Zic3* induces NCC markers in animal cap explants and expands the population of NCCs (Fig. 2.13), both of which can be induced by Bmp4 or Bmp7.<sup>28</sup>

cont./Xtwi Zic3 V/Xtwi A

**Fig. 2.13** Expression of *Zic3* in the South African clawed-toed frog *Xenopus laevis*. (A) Expression in a stage-16 neurula (anterior to the *left*) is in the lateral edges of the neural plate (*white arrowheads*) and in the neural crest (*black arrowheads*). (B) A control embryo (cont.) and an embryo injected with *Zic3* mRNA at the eight-cell stage (*Zic3*). *Xtwist* (*Xtwi*) is used as a marker for neural crest cells; in the cephalochordate *Branchiostoma belcheri*, *twist* is expressed in mesoderm and pharyngeal endoderm. In control *Xenopus* embryos, *Xtwist* visualizes an expanded cephalic neural crest (*black arrowheads*, *arrow*) and ectopic clusters of pigment cells (*white arrowheads*). Reproduced from Nakata *et al.* (1997) from a figure kindly supplied by Jun Aruga. Copyright © (1997) National Academy of Sciences, U.S.A.

A second pair-rule gene, Zic5, also is expressed in Xenopus NC. Overexpression enhances NC markers (with corresponding loss of epidermal markers) and induces NC in animal cap ectoderm. A dominant-negative construct blocks NC formation *in vivo*. While Zic3 primarily functions rostrally, Zic5 evokes more caudal NC, converting cells from epidermal to NC (Nakata *et al.*, 2000).

# Msx Genes and Specification of NCCs

Once NC is induced by Notch and Bmp4, Msx genes are required to upregulate *Snail2* to specify populations of cells at the border as NCCs:

Grafting Bmp4 into paraxial mesoderm induces *Msx1* and *Msx2* expression and associated formation of ectopic (presumably NC) cartilage.

Msx genes are homeobox-containing genes. A code of 13 homeobox-containing (*Hox*) genes patterns cranial and pharyngeal regions of vertebrate embryos (see Box 1.2). A 14th Hox gene, *Hox14*, has been identified in some vertebrates (note b in Box 1.2). Links between Bmp and *Hox* genes in the induction of NC are being uncovered; in *Xenopus, Msx1* mediates the role of Bmp4 in inhibiting epidermal and neural ectodermal induction. In concert with *Msx* genes, Bmp2 and Bmp4 regulate apoptosis of NCCs, with Bmp4 eliciting apoptosis, a topic discussed in

Chapter 10. Because Msx1 induces apoptosis, while *Snail2* inhibits apoptosis; the balance between the two genes—coupled with the regulation of transcription caspase enzymes and other genes—generates discrete boundaries with NC-forming territories, and therefore facilitates formation of the NC.<sup>29</sup>

Three Msx genes have been characterized from mouse embryos (Fig. 2.14).<sup> $\oplus$ </sup> Msx1 and 2 have similar patterns of expression in early mouse embryos, initially in the dorsal neural tube and in migrating NCCs, then in pharyngeal arches, facial processes, tooth germs, hair buds, and limb buds. Msx3 is confined to the dorsal neural tube. In embryos with 5–8 pairs of somites, Msx3 is expressed segmentally in the hindbrain in all rhombomeres except r3 and r5, from which lower numbers of NCCs emerge (Fig. 2.15). By the 18-somite stage, expression is no longer segmental but is uniform within dorsal hindbrain and dorsal rostral spinal cord (Figs. 2.14 and 2.15 [Color Plate 3]). As for the other Msx genes,



**Fig. 2.14** Expression of Msx3 in 8–9-day-old mouse embryos. (**A**) Msx3 is expressed strongly in rhombomeres 1, 2, and 4 and in the spinal cord, and weakly in r3 in this 7-somite embryo seen in lateral view with anterior to the *left* and r3 and r5 identified. (**B**) This 10-somite embryo, seen in dorsal view with anterior to the *right*, shows weak expression of Msx3 in r3 and lack of expression in r5. (**C**) There is uniform expression of Msx3 throughout the hindbrain and spinal cord in this 18-somite embryo seen in dorsal view (anterior to the *right*). (**D**) The gap in expression in r5 seen in the normal embryos (**B**) is not seen in this 10-somite embryo carrying the *Kreisler* (*Krmlkr*) mutation. Kreisler codes for a transcription factor that regulates rhombomere segment identity through *Hox* genes. Indeed, r5 may not have developed in this embryo. Reprinted from a figure kindly provided by Paul Sharpe from *Mechanisms of Develop*, Volume 55, Shimeld *et al.* (1996). Copyright (**C**) (1996) with permission from Elsevier Science (see Color Plate 3)

<sup>&</sup>lt;sup> $\oplus$ </sup> Zebrafish have at least five *Msx* genes (*MsxA–MsxE*), although these are not orthologous to *Msx1* and 2 of amphibians, birds, and mammals, a finding that is consistent with separate gene duplications in fish and with potentially different functions of *Msx* genes in fish and tetrapods.



**Fig. 2.15** Expression of Msx3 in 9.5–11.5-day-old mouse embryos seen in dorsal view with anterior to the *right* (**A**, **B**, and **C**) and in histological cross-section (**D**). (**A**) Expression is strong in hindbrain and spinal cord at 9.5 days. The arrow marks the hindbrain–midbrain boundary, expression being negative in the midbrain. (**B**) Expression is similar at 10.5 days of gestation. 2, 3, 4, and 5, rhombomeres 2, 3, 4 and 5; OV, the otic vesicle, which displays nonspecific trapping of the antibody. (**C**) At 11.5 days of gestation, expression is restricted dorsally and is absent from rhombomeres 3–5. (**D**) A transverse section of the neural tube of an embryo of 9.5 days of gestation shows Msx3 expression in the dorsal neural tube and in NCCs adjacent to the neural tube. Reprinted from a figure kindly provided by Paul Sharpe from *Mech Develop*, Volume 55, Shimeld *et al.* (1996). Copyright (**C**) (1996) with permission from Elsevier Science (see Color Plate 3)

*Msx3* can be upregulated by Bmp4 and expression (which is normally restricted dorsally) extended ectopically into the ventral neural tube.<sup>30</sup>

# **Establishing Cranial and Trunk Neural Crest**

Hensen's node is the site of future notochord in chicken embryos; the role of Kupffer's vesicle, the teleost homolog of Hensen's node, is discussed in Box 9.1. As discussed earlier, neural ectoderm is induced by notochord during primary neurulation as notochordal cells invaginate beneath the ectoderm and extend caudally, visible externally as the primitive streak. During this caudal extension, Hensen's node imposes rostrocaudal patterning onto the future NC, in all likelihood with the same signals (FGFs, Wnt, and retinoic acid) that posteriorize the neural tube as a whole. A consequence of rostrocaudal patterning is the regionalization of the NC into cranial and trunk, which can broadly be equated with NCCs arising from the brain and from the spinal cord, respectively.

Evidence is accumulating to indicate that CNC and TNC may be able to produce similar cell types, provided they are exposed to the appropriate signals, which they can be when maintained *in vitro*, but which they are not *in vivo* or *in ovo*. Nevertheless, differentiation of NC-derived cell types is regionalized *in vivo*, and it is important to know how that restriction in potential occurs, because the mechanisms that pattern the NC into distinct regions along the rostrocaudal axis appear to differ between chicken and mouse embryos are discussed separately.

# Chicken Embryos

In the section on establishing the epidermal-neural border earlier in this chapter, we saw that an ectopic ectodermal-neural border can be established and NC induced at that border when Hensen's node from a chicken embryo is grafted into the lateral (normally ectodermal) epiblast of a Japanese quail embryo. Notochord induces ectoderm to become neural plate (and suppresses epidermal ectodermal fate). Through subsequent signaling (Fig. 2.11) NC forms at the neural-epidermal border.

Fate mapping and lineage analysis of Hensen's node in H.H. stage 4 chicken embryos demonstrate that the node consists of presumptive notochord, endoderm, and somitic mesoderm, and that the progeny of individual cells within the node can contribute to all three regions. Fate is restricted by Hensen's node during a narrow window between H.H. stages 4 and 6 (18–25 h of incubation). This conclusion comes from co-culturing NCCs with Hensen's node and observing modification of NCC fate, a determining factor being the age of the embryo from which Hensen's node is derived:

- Nodes from young (H.H. stage 4) embryos respecify TNCCs as cranial; cranial markers, such as fibronectin and actin, are upregulated, and the trunk marker, melanin, is downregulated.
- This ability is lost from Hensen's node by H.H. stage 6, which corresponds with the timing of neural induction and regionalization by Hensen's node *in ovo*.
- Nodes from H.H. stages 2–4 induce rostral and caudal nervous system, while nodes from H.H. stages 5 and 6 induce only caudal nervous system. In part, this reflects declining competence of the epiblast at H.H. stage 4.<sup>31</sup>

One of the molecules involved in fate determination is Bmp6, a protein localized in the posterior marginal zone of the epiblast. Additional primitive streaks can be induced in ectopic locations in the epiblast following localized injection of Bmp6, which changes the fate of epiblast cells from epidermal to neural. Once ectodermal is fated to be neural, regression of Hensen's node and the accompanying induction of notochord and neural ectoderm impart rostral–caudal patterning onto the NC.<sup>32</sup>

Tgf $\beta$  has been identified as a candidate molecule establishing rostral identity through a mechanism that may involve regulating NCC–substrate adhesion. Although cranial and trunk crest have similar amounts of Tgf $\beta$  messenger RNA (mRNA), cranial crest is more sensitive to exogenous Tgf $\beta$ . Immortalized Hensen's node cells secrete a Tgf $\beta$ -dependent factor that enhances cranial but suppresses TNC. Furthermore, treating TNC with Tgf $\beta$  enhances CNC markers: 400 picomolar Tgf $\beta$  decreases the number of melanocytes (TNCCs) that form, while increasing the number of fibronectin-positive (cranial) cells; blocking Tgf $\beta$  downregulates cranial and upregulates trunk markers in CNCCs. Such a mechanism, tied to NC induction as outlined above, would impose rostral–caudal patterning onto the NC during primary neurulation.

## Mouse Embryos

Quinlan and colleagues (1995) mapped the neurectodermal fate of epiblast cells at the egg-cylinder stage of mouse development and demonstrated that neural primordia exhibit cranio-caudal patterning before neurulation.

An early regionalization of future cranial and TNC in murine embryos is suggested by a <sup>3</sup>H-thymidine-labeling study, in which rostral or caudal ectoderm from embryos labeled immediately before the onset of NCC migration (late primitive streak stage, 8 days of gestation; Table 2.1) was inserted into the equivalent position in unlabeled embryos, which were then maintained in whole embryo culture for 3 days. Rostral ectoderm formed cranial neuroepithelium, while caudal ectoderm formed trunk neuroepithelium, indicating that segregation into future reanial and neural tissue (and NC?) occurs before the incorporation of future neural ectoderm into the neural tube (assuming that NC is patterned at the same time as the neural ectoderm, as suggested by the 1995 study). Also recall that the most caudal NCCs in mice consist of two cell populations, one derived from neurectoderm (primary neural crest) and the other from the tail bud (secondary neural crest).<sup>33</sup> This fundamental, but little appreciated process of secondary neurulation, and the knowledge that the tail region does not develop directly from primary germ layers, were discussed in Chapter 1.

# **Ectoderm from the Most Rostral Neural Tube**

NCCs and cells of the central nervous system are closely related, indeed so closely related that they share a common lineage: central neurons and NC derivatives can arise from the same cloned cells. Nevertheless, not all the cells in the neural folds form neurons or even NC.<sup>34</sup>

Intracelomic grafting of future rostral neural ectoderm from H.H. stage 4–5 chicken embryos demonstrates that much of the neural tube arises from the medial and not the lateral region of the neural folds. (The origin of placodes from lateral neural folds is discussed in Chapter 6.) However, NC does not arise from neural folds in the region of the future forebrain. Surprisingly, this most rostral 'neural' ectoderm forms facial ectoderm (Fig. 2.16). Quail/chicken chimeras demonstrate that the facial ectoderm of chicken embryos arises from the neural folds of the forebrain and is patterned into regions or **ectomeres**, the epidermal ectodermal equivalent of the neuromeres in the hindbrain, discussed in the following section. The superficial ectoderm of the roof of the mouth, of the olfactory cavities, and of the head and face in avian embryos, all arise from the neural folds and neural plate of the prosencephalon; that is, from cells immediately rostral to the most rostral NC (Fig. 2.16). While the fate of prosencephalic neural crest is committed early (certainly by H.H. stages 10–14), the fate of the more caudal mesencephalic-metencephalic neural crest is not set until later.<sup>35</sup>

In this connection, the two studies by Couly and Le Douarin (1985, 1987) on the existence of ectomeres may be especially important. Neural ectoderm, NC, and



**Fig. 2.16** Diagrammatic representations of the origin of the most rostral placodes and of the craniofacial ectoderm in embryonic chickens as seen from the dorsal surface. Placodes such as the olfactory may arise from the prosencephalic neural folds (*open squares*), from ectoderm adjacent to the neural folds (lens), or from neural folds and adjacent ectoderm (trigeminal). Ectoderm from the prosencephalic neural folds gives rise to the ectoderm of the frontonasal processes but not to neural crest or neural ectoderm. Ectoderm from the rostral mesencephalon and adjacent ectoderm (*black circles*) gives rise to ectoderm of the maxillary and mandibular processes and to the trigeminal placode. The mesencephalic contribution to the trigeminal placode includes neural crest. Based on data from Couly and Le Douarin (1985, 1987, 1990) and Dupin *et al.* (1993)

facial ectoderm are set aside during the primary embryonic induction by a code of *Hox* genes. Alternatively, although not necessarily to the exclusion of early determination of some cell lineages, restriction may occur during NCC migration; facial ectoderm and pharyngeal endoderm play important roles in eliciting differentiation from lineages of NCCs at different stages of migration (see Box 3.4).

# **Rostrocaudal Patterning of CNC**

The pharyngeal region of vertebrate embryos forms by coordinated interactions between NCCs, pharyngeal arches, and the developing brain under the direction of a code based on overlapping expression boundaries of *Hox* genes (see Box 1.2). Alternate (odd-numbered) rhombomeres (neuromeres) in mice have characteristic boundaries of *Hox* gene expression. Other gene products are also expressed segmentally; odd-numbered rhombomeres of the hindbrain of chicken embryos bind to HNK-1 and express *Msx2* and Bmp (see Chapter 10).

# Hox Genes

Using knowledge of expression boundaries in the rhombomeres of embryonic chicken hindbrains, Paul Hunt and colleagues demonstrated that the rostral limits of expression of *Hoxb1–Hoxb4* coincide with particular rhombomere boundaries. As

with cranial-nerve patterning, these expression patterns are intrinsic to each rhombomere, provided that the rhombomeres develop in their normal position. Consequently, they are maintained if rhombomeres are transplanted to another site along the neural axis, being driven by the *Hox* code they carry with them (Figs. 2.17 and 2.18 [Color Plate 4]).<sup>36</sup> For example:

- *Hoxb1* is expressed only in r4, even if r4 is allowed to form more rostrally within the neural tube (Fig. 2.17).
- *Hox3a* has its rostral boundary of expression at the border between r4 and r5, an autonomous expression boundary that is reflected in neural tube and NC and retained if r4 and r5 are transplanted.<sup>37</sup>

Vielle-Grosjean et al. (1997) described a *Hox* code with a high degree of conservatism of Hox1–Hox4 in the hindbrain and pharyngeal arches of human embryos. Differential downregulation of individual *Hox* genes in different human tissues occurs later in development.

NCCs migrating from chicken hindbrains express a combination of *Hox* genes appropriate to their rhombomere of origin and carry this combination to the pharyngeal arches. Pharyngeal-arch ectoderm expresses the same combination of *Hox* genes as does NC-derived mesenchyme of that arch; similar expression boundaries are detected in early mouse embryos in surface ectoderm, cranial ganglia, migrating NCCs, and in the mesenchyme of the pharyngeal arches. Consequently, a **fundamental unity of mechanism patterning this region** of the embryo is the common pattern of *Hox* gene expression in the hindbrain, NC, and the pharyngeal arches into which NCCs migrate.

A similar pattern is seen in zebrafish, in which the transcription factor *Krox20* is expressed in r3, r5, and in migrating NCCs. This is a relic of an ancient segmentation; *AmphiKrox* is expressed with a one-somite periodicity in the neural tube of the cephalochordate, amphioxus (Jackman and Kimmel, 2002). Interestingly, expression of *Krox20* in the pharyngeal NC in chicken embryos does not equate

**Fig. 2.17** Expression of *Hoxb1* (visualized with β-galactosidase) is restricted to r4 in 9.5-day-old mouse embryos. The *arrow* marks the position of the otic vesicle. Reproduced in *black* and *white* from the colored original in Guthrie *et al.* (1992) from a figure kindly supplied by Andrew Lumsden. Reprinted with permission from *Nature* 356:157–159. Copyright © (1992), Macmillan Magazines Limited





**Fig. 2.18** *Hox*-gene expression in the rhombomeres of the hindbrain (r1–r7) and in the pharyngeal arches (p1–p4) is shown in this reconstruction of a mouse embryo of 9.5 days of gestation. *Colored bars* in the neural tube and *colored arrows* in migrating neural crest cells represent expression domains of *HoxA–HoxD*, which are also shown in the panel at the *bottom*. Some genes, such as *Hoxa2*, are expressed in the hindbrain but not in migrating neural crest cells. Reproduced from Manley and Capecchi (1995), with the permission of Company of Biologists Ltd. (see Color Plate 4)

with segmentation of the hindbrain, highlighting the existence of different postmigration patterning mechanisms in different species. A *cis*-acting enhancer element 26 kB upstream of *Krox20* in conserved among chickens, mice, and humans and can rescue the pattern of *Krox20* expression in transgenic mice. Localization of *Krox20* is to r5 in these embryos because of two conserved *Krox20* binding sites in the enhancer and a conserved binding site for Sox10, which, in concert, direct expression to the r5 NC.<sup>38</sup>

Hox group 3 paralogous genes (*Hoxa3*, *Hoxb3* and *Hoxd3*) act in a combinatorial fashion in patterning neurectoderm, and in patterning NC and mesoderm-derived mesenchyme, although it appears from studies by Manley and Capecchi (1997) that the identity of specific *Hox* genes may be less critical than the number of genes functioning in a region or developmental field (see Box 10.1).

## A Role for Mesoderm

As we have seen, neural ectoderm, NC, and pharyngeal-arch endoderm share boundaries of expression of the same *Hox* genes. Initially thought to reflect transfer of the *Hox* code from

neural ectoderm —> neural crest —> pharyngeal-arch endoderm

The similar expression boundaries may not reflect a straightforward *Hox* code transfer; for example, separate enhancer elements are present in *Hox* gene clusters in the neural tube and in NCCs.

A study by Frohman and colleagues (1990) based on the expression pattern of the murine homeobox-containing gene *Hoxb1* suggests that the primary pattern lies with head mesoderm, not within rhombomeres of the hindbrain. According to this scenario, the *Hox* code arises in mesoderm, is transferred to rhombomeres of the hindbrain, then to the pharyngeal arch mesenchyme (via migrating NCCs), and finally to pharyngeal endoderm and superficial ectoderm:

```
mesoderm —> hindbrain —> arch mesenchyme —> arch
endoderm and superficial ectoderm
```

This study raises an important point. Is the primary rostrocaudal regionalization of neural ectoderm and NC derived from neural induction, or is it secondarily imposed upon the neural tube from mesoderm?

The cranial nerves of avian embryos are patterned by dorsoventral signals that are both cranial mesodermal and rhombomeric in origin. A role for paraxial mesoderm is supported by studies in which rhombomeres were transplanted more rostrally or more caudally along the neural axis than their normal locations, and the resulting altered patterns of *Hox* gene expression analyzed. The boundary of *Hox* gene expression is controlled, in part, by paraxial mesoderm, in part by signals from the neural epithelium itself, with the constraint that posterior properties and posterior *Hox* genes overrule anterior properties and anterior genes. Transplanting rhombomeres from caudal —> rostral alters neither the pattern of *Hox* genes expressed nor the fate of the cells. Transplantation from rostral —> caudal modifies *Hox* code and cell fate to that appropriate to the new location.<sup>39</sup>

#### The Midbrain–Hindbrain Boundary

The midbrain-hindbrain boundary (the isthmus), a major organizing center in vertebrate embryos, is regulated by members of at least five gene families: Otx2, Wnt1, Fgf8, the *Engrailed* genes *En2*, *En5*, and En8, and three Pax genes, *Pax2*, *Pax5*, and *Pax8*. Figure 4.8 shows the relationships between these *Pax* genes in vertebrates. Mutations in these patterning genes can delete mid- and hindbrain.

Through secretion of Fgf8, the isthmus functions as a developmental organizer and patterns the midbrain; Fgf8 grafted into the caudal diencephalon can induce an ectopic midbrain. Studies in mouse embryos show that Fgf8 expressed in the isthmus plays a regulatory role in the specification of first- and second-arch NCCs; second-arch craniofacial structures form if NCCs express *Hoxa2* (see Boxes 7.1 and 10.3) and Fgf8 downregulates *Hoxa2* in first arch crest (Trainor et al., 2002).

 $En \ 2$  is upregulated during neural induction in a region-specific manner. XEn2 is expressed at the boundary between mid- and hindbrain and in the mandibular arches, optic tectum, and anterior pituitary. Although En2 is normally restricted to

the boundary between mid- and hindbrain in avian embryos it can be induced ectopically in association with repatterning neural ectoderm. Injecting antibodies against *Pax2* into zebrafish embryos leads to malformations of the midbrain–hindbrain boundary, downregulation of *Pax2* transcripts in the caudal midbrain, and alterations of *Wnt1* and *En2*, two genes regulated by *Pax2*.

Of evolutionary interest is the expression of En2 at the midbrain-hindbrain boundary in Japanese lamprey embryos. In the lamprey, however, *Engrailed* is expressed not in the NC but in one muscle of the mandibular arch, the velothyroideus (see Chapter 4).<sup>40</sup>

## Dlx Genes and Dorsoventral Patterning of CNC

Another genetic cascade governing pharyngeal-arch development and specification is the differential dorsoventral expression of *Distal-less* (*Dlx*) genes<sup>®</sup> in mouse pharyngeal endoderm, expression domains that pattern the endoderm dorsoventrally. Expression boundaries of *Hox* and *Dlx* pattern the pharyngeal arches in orthogonal rostrocaudal and dorsoventral<sup> $\diamond$ </sup></sup> directions. *Dlx* is a marker for the forebrain and patterns the forebrain and the rostral craniofacial skeleton, which develops from the first and second pharyngeal arches; specific arch deficiencies occur in embryos carrying *Dlx* mutations. Additional roles for *Dlx* genes are discussed in Chapters 4 and 7.<sup>41</sup>

#### Notes

- See Hörstadius (1950, pp. 4–6) for early studies on the origin of the NC, mostly based on analysis of amphibian embryos. Garcia-Martinez *et al.* (1993) and Basch *et al.* (2006) mapped the future NC in blastula-stage chicken embryos. Schoenwolf and Alvarez (1991) used quail/chicken chimeras to establish the timing of determination of neural–epidermal cell fate. See Colas and Schoenwolf (2001) for an overview of the cellular and molecular bases of neurulation.
- 2. See Quinlan *et al.* (1995), Tam and Quinlan (1996) and Tam and Selwood (1996) for these studies.
- 3. Hill and Watson (1958) studied the northern quoll ('native cat'), *Dasyurus hallucatus*, bandicoots (*Perameles* spp.), kangaroos and rock wallabies (*Macropus* spp., *Petrogale* spp.), and American opossums (*Didelphis* spp.).
- 4. For induction of NC in birds and for the combined use of quail/chicken chimeras and HNK-1 or *Snail2*, see Dickinson *et al.* (1995), Bronner-Fraser (1995), and Selleck and Bronner-Fraser

 $<sup>^{\</sup>otimes}$  *Distal-less* (*Dlx*) genes are a family of seven homeodomain transcription factors, the vertebrate ortholog of the gene *distal-less* (*Dll*) in *Drosophila*. With respect to the NC and NCCs, *Dlx* genes play important roles in forebrain and craniofacial development. The roles of five *Dlx* genes (*Dlx1-4* and *Dlx7*) are discussed in the text. Amphioxus expresses the invertebrate ortholog, *Dll*, in the neural tube (see Chapter 4).

 $<sup>^{\</sup>diamond}$  The terms dorsoventral, mediolateral, and proximodistal can be used interchangeably for this second axis that extends from the dorsal midline.

(1995\*). For overviews and recent studies on NC induction see Baker and Bronner-Fraser (1997a), Barembaum and Bronner-Fraser (2005), Basch *et al.* (2006), Correia *et al.* (2007) and Schmidt *et al.* (2007).

- 5. See Meulemans and Bronner-Fraser (2004) and Sauka-Spengler *et al.* (2007) for the first and latest of these studies, and see Davidson and Erwin (2006) for gene regulatory networks and the evolution of major organismal features. Marianne Bronner-Fraser has generously provided access to a manuscript which was in press at the time of this being written (April, 2008).
- 6. See Le Douarin and Kalcheim (1999\*) and Tucker (2004\*) for summaries of the characterization of the HNK-1 antigen. See Kuratani (1991) for HNK-1 expression in alternate rhombomeres, and Bronner-Fraser (1987) for perturbation studies with HNK-1 antibody. Information on HNK-1 labeling in different groups may be found in the relevant sections of Chapters 5, 6, 7, and 8.
- 7. See Luider *et al.* (1992) for HNK-1-positive cells in the embryonic gut, and Basch *et al.* (2006) and Lacosta *et al.* (2007) for *Pax7*.
- 8. Molecular markers often have to be used in conjunction with cellular markers to avoid ambiguity or false-positive results. Because *Xsnail2* is expressed in NC-derived and in mesoderm-derived mesenchyme, patterns of expression must be interpreted with caution.
- 9. Nieto *et al.* (1994) and Duband *et al.* (1995) demonstrated the involvement of *Snail2* at the onset of NCC migration. Sechrist *et al.* (1995) used *Snail2* to monitor regulation of the NC; Mancilla and Mayor (1996), Mayor *et al.* (1997), La Bonne and Bronner-Fraser (2000) and Aybar *et al.* (2003) analyzed the role of *XSnail2*; and Nagatomo and Hashimoto, 2007) studied *Xhairy2*.
- 10. For a comprehensive overview of the Tgf $\beta$  superfamily and their receptors in the context of mouse craniofacial development, including tables of family members and their receptors, see Dudas and Kaartinen (2005). See Taneyhill *et al.* (2007) and Coles *et al.* (2007) for Cad6B as a *Snail2* target, and for overviews.
- 11. See Hong and Saint-Jeannet (2005\*), Kelsh (2006\*), and Saint-Jeannet (2006\*) for analyses of and access to the primary literature on *Sox10*.
- 12. See Morales et al. (2007) for Sox5, and del Barrio and Nieto (2002) for Snail2 and RhoB.
- 13. See Logan and Nusse (2004\*) and Hayward *et al.* (2008\*) for *Wnt* signaling in development, Raible and Ragland (2005\*) for *Wnt* signaling in NCCs, C. Tan *et al.* (2001) for *Kermit*, Baranski *et al.* (2000) for *Frizzled* upregulation in avian NCCs, and Deardorff *et al.* (2001) for *Xfz3*.
- 14. See Gessert *et al.* (2007) for Pescadillo and Schmidt *et al.* (2007) for canonical and non-canonical signaling pathways operating in tandem.
- 15. See Northcutt (1995) and Smith Fernandez et al. (1998) for discussions of whether the vertebrate forebrain is segmented.
- 16. See McMahon and Bradley (1990) and Ikeya *et al.* (1997) and H. Y. Lee *et al.* (2004) for the studies on *Wnt* genes, and Urbánek *et al.* (1997\*) for gene families that establish the midbrain–hindbrain boundary.
- 17. See Dunn *et al.* (2000) for Wnts and melanocytes, and Luo *et al.* (2003) for Hoxa2 and Ap $2\alpha$ .
- For cascades of signals in embryonic induction, see Nieuwkoop *et al.* (1985), Gurdon (1992), Hall (2005b\*) and Gilbert (2006). See Hemmati-Brivanlou and Melton (1997), Sasai and de Robertis (1997), Weinstein and Hemmati-Brivanlou (1997), Marchant *et al.* (1998) and Hurtado and de Robertis (2007) for the role of Bmps in neural induction.
- 19. For development of the NC at the border between neural and epidermal ectoderm, see Bronner-Fraser (1995), Selleck and Bronner-Fraser (1995\*) and Graveson *et al.* (1997). See Nieuwkoop and Albers (1990) for altered ectodermal responsiveness, Xu *et al.* (1995) for the Bmp4 receptor, and Wilson and Hemmati-Brivanlou (1995) for activin.
- 20. See Selleck and Bronner-Fraser (1995) for melanocyte induction, Marchant *et al.* (1998) for the Bmp gradient, Barembaum and Bronner-Fraser (2005\*) for an overview of early steps in NC induction, Sasai *et al.* (2001) for *FoxD3*, and Monsoro-Burq *et al.* (2003) for *Fgf*8 and lateral mesoderm.

- La Bonne and Bronner-Fraser (1998) found that although *Snail2* is a marker for NC, *XSnail2* alone is not a sufficient signal to induce NC in *Xenopus*, although downregulation of *Xsnail1* and *Xsnail2* inhibits NCC migration, resulting in deficiencies, especially in the rostral craniofacial skeleton (Carl *et al.*, 1999).
- 22. See Dickinson *et al.* (1995) and del Barrio and Nieto (2002) for studies with chicken embryos, La Bonne and Bronner-Fraser (1998) for those with *Xenopus*, Jiang *et al.* (1998) for mice, Murray and Gridley (2006) for *Snail* genes in mice.
- 23. See Cornell and Eisen (2005\*) and Jones and Trainor (2005\*) for reviews of Notch–Delta signaling.
- 24. See Bhushan et al. (1998) for Smad7, and Ko et al. (2007) and Nie et al. (2008) for Smad4.
- 25. See Raible and Ragland (2005\*) and Nie *et al.* (2006\*) for overviews of the roles of Bmp in NCC and craniofacial development, and Nie *et al.* (2008) for *Smad4* signaling.
- 26. See Marchant *et al.* (1998) and Barth *et al.* (1999) for initial studies, Kanzler *et al.* (2000) and Correia *et al.* (2007) for *Bmp2* and *Bmp4* in murine NCC migration but not induction, and see Jones and Trainor (2005) and Trainor (2005) for recent summaries. At the gastrula stage in *Xenopus*, Noggin does not inhibit *Bmp4*, although *Wnt8* does (J. C. Baker *et al.*, 1999). A gradient of *Noggin* in the neuroepithelium of chicken embryos inactivates *Bmp4* in response to signals from the paraxial mesoderm, and so coordinates the timing of NCC delamination (Sela-Donenfeld and Kalcheim, 2000).
- 27. For involvement of Bmp4 and Bmp7 in neural or NC induction in *Xenopus*, chicken, mouse, and zebrafish, see Dickinson *et al.* (1995), Liem *et al.* (1995), Selleck and Bronner-Fraser (1995\*), and the literature discussed by Hall (1999a\*), Le Douarin and Kalcheim (1999\*), Nie *et al.* (2006), and Saint-Jeannet (2006). For overviews of molecular control of neural induction, see Sasai and de Robertis (1997) and Hurtado and de Robertis (2007). See Watanabe and Le Douarin (1996) for Bmp4 and chondrification of paraxial mesoderm, and Korade and Frank (1996) for NC from ventral neural tube cells.
- 28. See Nakata *et al.* (1997) and Suzuki *et al.* (1997) for *Zic3* and *Msx1* and their relationship to Bmps and NC induction, and Woo and Fraser (1998) for the zebrafish study.
- See Bennett *et al.* (1995) for the distribution of Bmp in murine orofacial tissues, Tribulo *et al.* (2004) for *Msx1*, *Snail2*, and apoptosis, and Hall and Ekanayake (1991) and Chapter 7 for other growth factors that regulate differentiation of NCCs.
- 30. See Ekker et al. (1997) and Shimeld et al. (1996) for Msx genes in zebrafish and mice.
- 31. Similarly, the ability of specific rostrocaudal regions of the neural ectoderm to induce lens formation and the ability of specific regions of the epidermal ectoderm to respond to those inductions are determined during neural induction and primary axis formation.
- 32. See Selleck and Stern (1991) for the fate map and lineage analysis of Hensen's node, and Shah *et al.* (1997) for the studies with chicken Bmp6 (formerly known as Vg1). In a different context, prostate cancers secrete Bmp6, which, in turn, induces further Bmp6, which plays a role in the invasiveness and metastasis of the cancer (Dai *et al.*, 2005).
- 33. See Beddington and Robertson (1998) for the <sup>3</sup>H-thymidine study. Thomas and Beddington (1996) determined that anterior primitive endoderm may be responsible for initial patterning of the rostral neural plate (especially the prosencephalon) in murine embryos. See Schoenwolf and Nichols (1984) and Schoenwolf *et al.* (1985) for caudal NC and tail bud formation in mice and avian embryos. See Chapter 1 and Hall (1997, 1999a\*) for secondary neurulation.
- 34. See Bronner-Fraser and Fraser (1997\*), Fraser and Bronner-Fraser (1991), Selleck and Bronner-Fraser (1995\*) and Mujtaba *et al.* (1998) for evidence for the common lineage of central neurons and NCCs.
- 35. See Couly and Le Douarin (1990) for these studies on patterning of neural fold ectoderm, Fernández-Garre *et al.* (2002) for fate mapping of the neural plate at H. H. stage 4, and Alvarado-Mallart (1993) for the timing of commitment of the mesencephalic–metencephalic neuroepithelium, a commitment that requires at least two steps.
- 36. See Kuratani (1991) for HNK-1 expression in alternate rhombomeres, and Hunt and Krumlauf (1992\*), Kessel (1992\*), and Krumlauf (1993\*) for the *Hox* code.

- 37. See Saldivar *et al.* (1996) for cell autonomous rather than environmentally regulated expression of *Hox3a*, and Guthrie *et al.* (1992), Kuratani and Eichele (1993) and Wilkinson (1995) for intrinsic patterning of cranial nerves and rhombomere *Hox* gene expression patterns.
- 38. See Nieto *et al.* (1995) for *Krox20* expression in zebrafish, and Ghislain *et al.* (2003) for the enhancer elements that restrict expression to r5 in tetrapods.
- 39. See Kuratani and Aizawa (1995) for the transplantation studies, Kuratani and Aizawa (1995) for cranial nerve patterning, and Carstens (2004) for importance of understanding neuromeric organization for pediatric surgery.
- 40. See Hemmati-Brivanlou *et al.* (1990) for expression of *Engrailed*, Simeone *et al.* (1992) for nested expression domains of Otx1 and Otx2 in the murine rostral hindbrain, and Rhinn *et al.* (1998) for Otx2 in murine pharyngeal endoderm and its role in specification of fore- and midbrain territories. For gene families that establish the midbrain–hindbrain boundary, see Urbánek *et al.* (1997\*).
- See Qiu *et al.* (1997\*) for the dorsoventral *Dlx* code. An issue (# 5) of Volume 235 of the *J Dev Dyn* (2006) is devoted to craniofacial development and patterning.