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Neural Crest and Cranial Ectodermal Placodes

Clare Baker

GENERAL OVERVIEW AND CHAPTER LAYOUT

The entire peripheral nervous system (PNS) of vertebrates is derived from two transient embryonic cell populations: the neural crest (Hall, 1999; Le Douarin and Kalcheim, 1999) and cranial ectodermal placodes (Webb and Noden, 1993; Baker and Bronner-Fraser, 2001; Begbie and Graham, 2001a). Both originate from ectoderm at the border between the prospective neural plate and epidermis. Neural crest cells delaminate in a rostrocaudal wave and migrate through the embryo along specific migration pathways. They give rise to all peripheral glia, all peripheral autonomic neurons (postganglionic sympathetic and parasympathetic neurons; enteric neurons), all sensory neurons in the trunk, and some cranial sensory neurons, together with many non-neural derivatives such as pigment cells, endocrine cells, facial cartilage and bone, teeth, and smooth muscle. Cranial ectodermal placodes are paired, discrete regions of thickened cranial ectoderm that give rise to the paired peripheral sense organs (olfactory epithelium, inner ear, anamniote lateral line system plus the lens of the eye), most cranial sensory neurons, and the adenohypophysis (anterior pituitary gland). Neural crest, cranial ectodermal placodes, and their derivatives comprise many of the key defining characteristics of the craniates (vertebrates plus hagfish) within the chordate phylum (Gans and Northcutt, 1983; Northcutt and Gans, 1983; Maisey, 1986; Baker and Bronner-Fraser, 1997).

The neural crest and cranial ectodermal placodes share many similarities. Both arise from ectoderm at the neural plate border. Both give rise to multiple neuronal and non-neuronal cell types, including some overlapping derivatives, such as cutaneous sensory neurons in the trigeminal ganglion. Like cells in the central nervous system (CNS) (see Chapter 9), both placode-derived and neural crest cells have considerable migratory ability, although unlike CNS cells, they migrate in the periphery. There are also important differences between the neural crest and cranial ectodermal placodes. Neural crest cells form along the entire length of the neuraxis, except the rostral forebrain, while placode formation is restricted to the head. Neural crest cells give rise to various derivatives not formed by placodes, such as autonomic neurons, melanocytes, cartilage, and smooth muscle. Conversely, unlike neural crest cells, placodes form sensory ciliary receptor cells (sensory cells with a single modified cilium, e.g., olfactory receptor neurons, inner ear hair cells).

The neural crest and cranial ectodermal placodes were discovered independently toward the end of the 19th century; neural crest cells in chick embryos (His, 1868) and placodes in shark embryos (van Wijhe, 1883). They have been studied continuously ever since. What mechanisms and molecules control their formation in the embryo, their adoption of specific migration pathways, and their diversification into so many different cell types? This chapter summarizes our current understanding of these processes in both the neural crest and placodes.

After a brief description of the derivatives of the neural crest (section Neural Crest Derivatives), the chapter follows the order of neural crest cell development *in vivo*. The embryonic origin of neural crest cells at the border between the neural plate and epidermis is described, together with our current knowledge of the molecular nature of neural crest induction (sections Embryonic Origin of the Neural Crest; Neural Crest Induction). Neural crest cell migration pathways through the embryo are then outlined, including developments in our understanding of the molecular cues that guide migrating neural crest cells (section Neural Crest Migration). Finally, an overview is given of current hypotheses on how the diversity of neural crest cell derivatives is achieved (section Neural Crest Lineage Diversification), with particular emphasis on the formation of different cell types in the PNS (section Control of Neural Crest Cell Differentiation in the PNS).

The chapter then introduces the cranial ectodermal placodes (section Overview of Cranial Ectodermal Placodes). The evidence for a common "preplacodal field" at the anterior neural plate border is described (section A Preplacodal Field at the Anterior Neural Plate Border). Our current knowledge of the mechanisms of induction and neurogenesis within each individual placode is then discussed (sections Sense Organ Placodes; Trigeminal and Epibranchial Placodes). For the purposes of this part of the chapter, the placodes are divided into two groups: those that contribute to the paired sense organs (olfactory, lateral line, otic and lens placodes) (section Sense Organ Placodes), and those that only (or mainly) form sensory neurons (trigeminal and epibranchial placodes) (section Trigeminal and Epibranchial Placodes). The hypophyseal placode, which forms the endocrine

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cells of the adenohypophysis, falls outside the scope of this chapter and is not discussed.

NEURAL CREST DERIVATIVES

Neural crest cells form a startling array of different cell types, including cartilage and bone in the head, teeth, endocrine cells, peripheral sensory neurons, all peripheral autonomic neurons (enteric, postganglionic sympathetic, and parasympathetic neurons), all peripheral glia, and all epidermal pigment cells (Fig. 1). The neural crest origin of these cells has been determined by a variety of ablation and cell-labeling experiments, some of which are described in detail in the section on Experimental Approaches. Neural crest cells emigrating at different rostrocaudal levels along the neuraxis give rise to different but overlapping sets of derivatives (see Table 1). There are traditionally four rostrocaudal divisions of the neural crest along the neuraxis based on these differences: cranial (posterior diencephalon to rhombomere 6); vagal (axial level of somites 1–7); trunk (axial level of somites 8–28); and lumbosacral (axial level posterior to somite 28).

Cranial neural crest cells form a large amount of "mesectoderm," that is, ectodermal derivatives that are mesodermal in character, such as cartilage, bone, teeth, smooth muscle, and other connective tissues. Most of the vertebrate skull is derived from cranial neural crest cells (Fig. 1B). Cranial neural crest cells also form melanocytes (Fig. 1A), Schwann cells, all the satellite glia of the cranial ganglia, parasympathetic neurons, sensory neurons in some cranial sensory ganglia (see Fig. 11), and endocrine cells. Vagal and lumbosacral neural crest cells together provide all the neurons and glia of the enteric nervous system, plus sensory ganglia, parasympathetic ganglia, melanocytes, and endocrine cells (see Table 1). Trunk neural crest cells form the neurons and satellite glia of the sympathetic and dorsal root ganglia, together with Schwann cells, melanocytes, and endocrine cells in the adrenal medulla (Table 1; Figs. 1C and 5).

Most of the vagal neural crest is technically a subdivision of the cranial neural crest, since the boundary between the hindbrain and spinal cord falls at the level of somite 5 (Lumsden, 1990; Cambronero and Puelles, 2000). Vagal neural crest clearly also forms mesectoderm, including musculoconnective elements of the major arteries (Le Lièvre and Le Douarin, 1975; Etchevers *et al*., 2001) and the aorticopulmonary septum of the heart (Kirby *et al*., 1983). Although in birds, mesectoderm is only formed down to the level of the fifth somite (Le Lièvre and Le Douarin, 1975), corresponding precisely to the caudal boundary of the hindbrain, mesectoderm production cannot be used as a dividing line between cranial and trunk neural crest cells in all vertebrates. Trunk neural crest cells give rise to dorsal fin mesenchyme in fish and amphibians (Raven, 1931; DuShane, 1935; Collazo *et al*., 1993; Smith *et al*., 1994). They may contribute dermal bone to the fin rays of bony fish during normal development, although fish neural crest cells have not yet been followed late enough in development to prove this (Smith *et al*., 1994). When experimentally challenged with inducing tissues in culture, trunk neural crest cells from the level of the thoracic somites can form

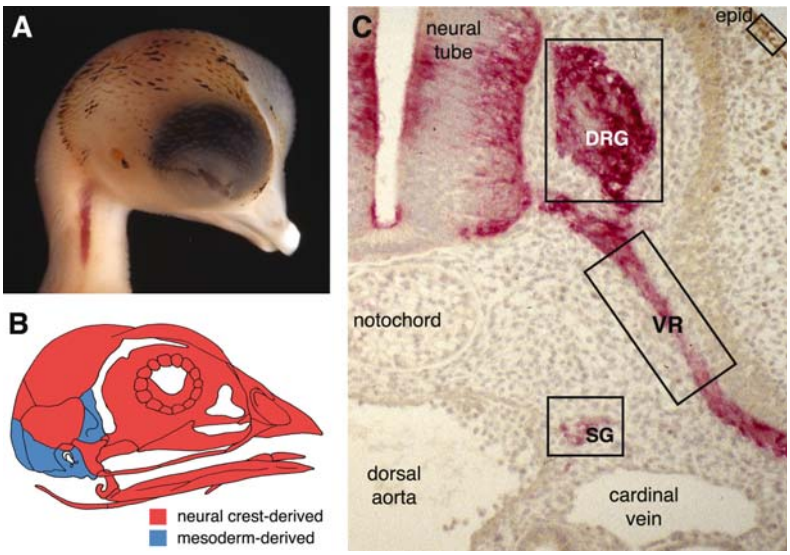

FIGURE 1. Diversity of neural crest derivatives. (A) Melanocytes, seen here as darkly pigmented feathers on the head of a quail–chick chimera. This 11 day-old chick embryo received a unilateral isotopic graft of migrating quail mesencephalic neural crest cells at the 10-somite stage. (B) Schematic to show that most of the vertebrate cranium derives from the neural crest. Redrawn from Couly *et al*. (1993). (C) Transverse section through the trunk of a 4 day-old chick embryo, stained with an anti-neurofilament antibody (dark staining), to show the location of trunk neural crest derivatives (boxes). These include all neurons and satellite cells of the dorsal root ganglia (DRG) and sympathetic ganglia (SG), Schwann cells along the ventral root (VR), and melanocytes in the epidermis (epid).

TABLE 1. Derivatives of the Neural Crest at Different Axial Levels

Axial level	Cell type	Tissues
Cranial (caudal diencephalon to rhombomere 6)	Mesectoderm	Most bones and cartilages of the neurocranium (brain capsule) and splanchnocranium (facial and pharyngeal)
		Tooth papilla; odontoblasts; dentine matrix
		Meninges of the brain
		Corneal "endothelium"
		Dermis of head and neck
		Tendons
		Non-endothelial components (pericytes, connective, and smooth muscle) of aortic arch-derived arteries
		Smooth muscle (feather arrector muscles in birds; in head blood vessels and aortic arch arteries)
		Connective component of striated muscles (facial and ocular)
		Subcutaneous adipose tissue
		Mesenchymal component of pituitary, salivary, thyroid and parathyroid glands, and the thymus
	Melanocytes	Epidermal pigment cells
	Neurons	
	Sensory	Proximal region of trigeminal (V) ganglion
		Proximal ganglia of cranial nerves VII, IX, and X
		Mesencephalic nucleus of the trigeminal nerve (inside brain)
	Parasympathetic	Postganglionic neurons in ciliary, ethmoidal (dorsal pterygopalatine), sphenopalatine (ventral pterygopalatine), submandibular, otic ganglia
	Glia	Schwann cells
		Satellite cells in cranial ganglia
	Endocrine	Calcitonin-producing cells of the ultimobranchial body (in mammals, parafollicular cells in the thyroid gland)
		Carotid body
Vagal (post-otic hindbrain: somite levels 1–7)	Mesectoderm	Aorticopulmonary septum of the heart
		Non-endothelial components (pericytes, connective, and smooth muscle) of aortic arch-derived arteries
	Melanocytes	Epidermal pigment cells
	Neurons	
	Sensory	Proximal ganglia of cranial nerves IX and X
		Dorsal root ganglia (somite levels 6–7 only)
	Parasympathetic	Postganglionic neurons of parasympathetic nerves innervating thoracic and abdominal visceral organs, including cardiac ganglia
	Sympathetic	Postganglionic neurons in superior cervical ganglion (somite levels 1–4 in the mouse)
	Enteric (sensory, motor, and interneurons)	Enteric ganglia
	Glia	Schwann cells
		Satellite cells in peripheral ganglia (including enteric)
	Endocrine	Calcitonin-producing cells of the ultimobranchial body (in mammals, parafollicular cells in the thyroid gland)
		Carotid body and groups of carotid cells in walls of large arteries arising from heart
Trunk (somite levels 8–28)	Mesectoderm	Fin mesenchyme in fish and amphibians
	Melanocytes	Epidermal pigment cells
	Neurons	
	Sensory	Dorsal root ganglia
	Sympathetic	Postganglionic neurons in sympathetic ganglia
	Glia	Schwann cells
		Satellite cells in peripheral ganglia
	Endocrine	Adrenal chromaffin cells (somite levels 18–24)
(caudal to somite 28)	Melanocytes	Epidermal pigment cells
	Neurons	
	Sensory	Dorsal root ganglia
	Parasympathetic	Remak's ganglion (birds); postganglionic neurons of pelvic splanchnic nerves
	Sympathetic	Postganglionic neurons in sympathetic ganglia
	Enteric (sensory, motor, and interneurons)	Enteric ganglic in post-umbilical gut

TABLE 1. (*Continued*)

Axial level	Cell type	Tissues
Lumbosacral (cont'd)	Glia	Schwann cells Satellite cells in peripheral ganglia (including post-umbilical enteric ganglia)
Pygostyle (birds only: somite levels 47–53)	Melanocytes Glia	Epidermal pigment cells Schwann cells

Source: Le Douarin and Kalcheim (1999); Etchevers *et al.* (2001); Durbec *et al.* (2001); Durbec *et al.* (1996); Smith *et al.* (1994); Collazo *et al.* (1993); Lim *et al.* (1987); Catala *et al.* (2000).

teeth and bone (Lumsden, 1988; Graveson *et al*., 1997). Trunk neural crest cells can also form smooth muscle *in vitro* (e.g., Shah *et al*., 1996). Results from both amphibian and chick embryos suggest that under the right circumstances, trunk neural crest cells can even form cartilage (Epperlein *et al*., 2000; McGonnell and Graham, 2002; Abzhanov *et al*., 2003). These experiments are examples of many such showing that restrictions in the fate of neural crest cell populations, at a given axial level (i.e., what they form during normal development), do not seem to result from restrictions in potential (the range of possible derivatives), at least at the population level. This will be discussed more fully in the section on Axial Fate-Restriction.

One proposed derivative of the neural crest has aroused controversy: The large sensory neurons that make up the mesencephalic nucleus of the trigeminal nerve (mesV) within the midbrain. These neurons were fate-mapped in the chick to mesencephalic neural crest cells that reenter into the brain immediately after delamination (Narayanan and Narayanan, 1978). Certainly, mesV precursors are not present in the migrating mesencephalic neural crest cell population that has moved away from the brain beneath the adjacent surface ectoderm (Baker *et al*., 1997). The neural crest origin of mesV neurons has been challenged by a study of molecular marker expression (Hunter *et al*., 2001), but the question will only be settled by combining a fate-mapping study with molecular markers. Similar large sensory neurons (Rohon-Beard neurons) in the dorsal neural tube in the trunk of fish and amphibian embryos were originally proposed to be a neural crest derivative (Du Shane, 1938; Chibon, 1966). Studies of different zebrafish mutants have shown that Rohon-Beard neurons share a lineage with neural crest cells (Artinger *et al*., 1999; Cornell and Eisen, 2000, 2002). However, if neural crest cells are defined as cells that have delaminated from the neuroepithelium (section Neural Crest Induction), then Rohon-Beard neurons cannot be described as derivatives of the neural crest.

EMBRYONIC ORIGIN OF THE NEURAL CREST

Neural crest cells were first recognized in the neurula-stage chick embryo as a strip of cells lying between the presumptive epidermis and the neural tube (His, 1868). This area is already distinct at the open neural plate stage in amphibians (Brachet, 1907; Raven, 1931; Knouff, 1935; Baker and Graves, 1939) (see Fig. 13A). The prospective neural crest of urodele amphibians was fate-mapped in early gastrula stages, using vital dyes, to a narrow band of ectoderm between the presumptive neural plate and epidermis (Vogt, 1929). The prospective neural crest was also fate-mapped in the chick gastrula to a region between the prospective neural plate and epidermis, using isotopic grafts of tritiated-thymidine labeled epiblast tissue (Rosenquist, 1981). During neurulation, the neural plate border region forms the neural folds, which rise up and move together until they fuse to form the neural tube (Fig. 2). The prospective neural crest is thus brought from the lateral edges of the open neural plate to the dorsal midline, that is, the "crest" of the neural tube (although cranial neural crest cells are not always incorporated into the neural tube; see section Epithelial–Mesenchymal Transition). In fish, and in the tail region of tetrapods, the neural tube forms by secondary neurulation, in which the ectoderm thickens ventrally and the lumen of the neural tube forms by cavitation. However, the morphogenetic movements of secondary neurulation also involve infolding of the neural plate (Schmitz *et al*., 1993; Papan and Campos-Ortega, 1994; Catala *et al*., 1996). In the zebrafish, two bilaterally symmetrical thickenings form on either side of a medial thickening: These fuse to form the neural keel (Schmitz *et al*., 1993). Prospective neural crest cells (as well as prospective neural and epidermal cells) are contained within the lateral thickenings; they subsequently converge toward the dorsal midline (Schmitz *et al*., 1993; Thisse *et al*., 1995). Neural crest cells, therefore, originate from the border between the neural plate and epidermis in all vertebrates.

Presumptive neural crest cells do not form a segregated population in the neural plate border region. When single cells in this region of open neural plate stage chick embryos were labeled and their progeny examined, it was found that individual cells within this field could form epidermis, neural crest and neural tube derivatives in the trunk (Selleck and Bronner-Fraser, 1995). Similarly, when small groups of cells were labeled at the cranial neural plate border, neural crest precursors were found to be intermingled with epidermal, placodal, and neural tube precursors (Streit, 2002). The epidermal lineage only segregates from the CNS and neural crest cell lineages when the neural tube closes (Selleck and Bronner-Fraser, 1995). Neural crest and CNS cell lineages do not seem to segregate at any stage within the neural tube: Single cells within the dorsal neural tube can form both neural tube and neural crest cell derivatives (Bronner-Fraser and Fraser, 1989). Dorsal root ganglion neurons and glia, and

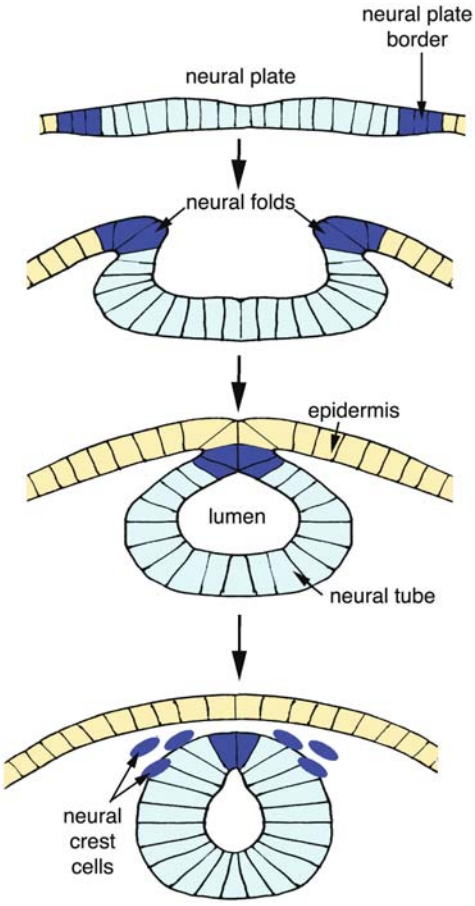

FIGURE 2. Schematic of neurulation in the trunk region of the vertebrate embryo, showing the location of prospective neural crest cells at the lateral borders of the neural plate. As the neural folds rise up and approximate to form the neural tube, prospective neural crest cells are brought dorsally to the "crest" of the neural tube. Cranial neural crest cells, however, are not always incorporated into the neural tube (section Embryonic Origin of the Neural Crest).

melanocytes, are generated by the dorsal neural tube as late as embryonic day 5 (E5) in the chick, several days after "classical" neural crest cell emigration has ceased (Sharma *et al*., 1995). Furthermore, ventral neural tube cells grafted into neural crest cell migration pathways are able to form neural crest cell derivatives, although they eventually lose the potential to form neurons (Korade and Frank, 1996). Hence, neural crest cells do not constitute a separate population from the CNS until they delaminate from the neuroepithelium. Delamination, therefore, is a crucial defining characteristic of neural crest cells (section Neural Crest Induction).

As will be seen in the section on Evidence for Non-Neural Ecoderm Involvement, neural crest cells can be generated experimentally not only from the neural plate, but also from non-neural ectoderm (prospective epidermis), when these tissues are exposed to appropriate signals. Therefore, all ectodermal cells have the potential to form neural crest cells, at least during early stages of development. However, during normal development, neural crest cells only arise at the border between neural plate and epidermis, which is underlain by nonaxial mesoderm. What mechanisms and molecules underlie the induction of neural crest cells in this region?

NEURAL CREST INDUCTION

Neural crest cells form at the border between prospective neural plate and prospective epidermis, above nonaxial (paraxial and lateral plate) mesoderm. The neural plate border itself is a recognizable domain, characterized by expression of various genes, including those encoding transcription factors such as *Pax3, Zic*, and *Snail* family members. Many of these genes are maintained in neural crest cells (see Table 1; LaBonne and Bronner-Fraser, 1999; Gammill and Bronner-Fraser, 2003). However, induction of the neural plate border is not equivalent to induction of the neural crest. The most rostral part of the neural plate border (prospective rostral forebrain) fails to produce neural crest (Adelmann, 1925; Knouff, 1935; Jacobson, 1959; Chibon, 1967a; Couly and Le Douarin, 1985; Sadaghiani and Thiébaud, 1987), except possibly for a few in the mouse (Nichols, 1981; Osumi-Yamashita *et al*., 1994). In the head, the neural plate border also gives rise to cranial ectodermal placodes (section A Preplacodal Field at the Anterior Neural Plate Border). Furthermore, neural plate border markers and morphology can be induced experimentally without inducing neural crest cells (McLarren *et al*., 2003).

The available evidence (reviewed in Kalcheim, 2000; Mayor and Aybar, 2001; Knecht and Bronner-Fraser, 2002; Gammill and Bronner-Fraser, 2003) suggests that neural crest induction can be divided into three main steps: (1) establishment of the neural plate border, which is initially anterior in character, via intermediate levels of bone morphogenetic protein (BMP) activity and Dlx transcription factor activity; (2) posteriorization of the neural plate border, and induction of neural crest cell precursors within it, by Wnt and/or FGF signaling; (3) epithelial–mesenchymal transition. Until a cell delaminates from the neuroepithelium into the periphery, it is not a *bona fide* neural crest cell. Indeed, failure to emigrate can lead to neural differentiation of neural crest precursors within the neuroepithelium (Borchers *et al*., 2001). Hence, induction of delamination can be considered as the final step in neural crest induction.

Selected molecular markers of neural crest cells, many of which are used in assays for neural crest cell induction, are listed in Table 2 (also see Gammill and Bronner-Fraser, 2003). In *Xenopus*, induction of the genes encoding the zinc finger transcription factors, Slug and Twist (section Snail/Slug and FoxD3 Are Required for Neural Crest Precursor Formation), is commonly used as a proxy for neural crest cell induction. The HNK-1 epitope, a carbohydrate expressed on migrating neural crest cells, among other cell types, is frequently used in the chick to identify neural crest cells (see Table 2). The winged-helix transcription factor FoxD3 (sections Snail/Slug and FoxD3 Are Required for Neural Crest Precursor Formation; FoxD3 Promotes Neural Crest Cell Delimitation at All Axial Levels) and the HMG-box transcription factors Sox9 and Sox10 (section Sox10

TABLE 2. Some Genes Expressed in Premigratory and Migrating Neural Crest Cells

Molecule	Type	NC precursors	Migrating NC cells	Role in:	Selected references
Frizzled3	Wnt receptor	+	−	NC cell induction	Deardorff *et al*. (2001)
Pax3	Paired-domain transcr. factor	+	+ (early); reexpressed later	Postmigratory	Mansouri *et al*. (2001)
Zic family	Zinc finger transcr. factors	+	+	NC cell induction	Nakata *et al*. (2000)
AP-2α	Transcr. factor	+	+	NC precursor cell formation	Luo *et al*. (2003)
Sox9	HMG-domain transcr. factor	+	+	NC precursor cell formation and postmigratory	Spokony *et al*. (2002); Cheung and Briscoe (2003)
Sox10	HMG-domain transcr. factor	+	+	NC precursor cell formation and postmigratory	Britsch *et al*. (2001); Dutton *et al*. (2001); Honoré *et al*. (2003)
FoxD3 (=forkhead6)	Winged helix transcr. factor	+	+	NC cell induction	Dottori *et al*. (2001); Sasai *et al*. (2001)
Slug/Snail family	Zinc finger transcr. factors	+	+ (early)	NC cell induction, migration	LaBonne and Bronner-Fraser (2000); del Barrio and Nieto (2002)
Twist	bHLH transcr. factor	+ (cranial)	+ (cranial)	Unknown	Gitelman (1997)
Endothelin receptor B	Endothelin-3 receptor	+	+	Postmigratory	Nataf *et al*. (1996)
RhoB	GTP-binding protein	+	+ (early)	Emigration	Liu and Jessell (1998)
ADAM13	Metallo-protease	−	+	Emigration/ migration	Alfandari *et al*. (2001)
Cadherin7	Cell–cell adhesion	−	+	Migration	Nakagawa and Takeichi (1998)
$p75^{NTR}$	Low-affinity neurotrophin receptor	−	+	Unknown	Stemple and Anderson (1992)
HNK1 epitope	Glucuronic acid-containing carbohydrate	−	+	Unknown	Le Douarin and Kalcheim (1999)

Note: References are selected to enable further reading: they are not comprehensive. bHLH, basic helix-loop-helix; HMG, high mobility group; NC, neural crest; transcr., transcription.

Is Essential for Formation of the Glial Lineage), which are expressed in neural crest precursors and migrating neural crest cells, are more recently identified neural crest cell markers.

Step 1: Establishment of the Neural Plate Border

Molecular signals involved in neural plate induction are discussed at length in Chapter 1 and will not be reviewed here. The classical "default" model for neural plate induction, whereby high levels of bone morphogenetic proteins (BMPs) specify epidermis, and low levels specify neural plate (see Chapter 1), led to the suggestion that intermediate levels of BMP activity specify the border between the two tissues (reviewed in Mayor and Aybar, 2001). Indeed, intermediate BMP activity levels are sufficient to induce some anterior neural plate border genes in *Xenopus* ectoderm *in vitro* (Wilson and Hemmati-Brivanlou, 1995; Knecht and Harland, 1997; Villanueva *et al*., 2002). Importantly, however, no concentration of BMP antagonist is sufficient to induce neural crest cells alone, that is, in the absence of neural and epidermal markers (Wilson *et al*., 1997; LaBonne and Bronner-Fraser, 1998). This is consistent with the fact that the anterior neural plate border does not produce neural crest cells, and with the hypothesis that additional signals are required to induce neural crest cell precursors within the neural plate border region (see next section).

In *Xenopus*, overexpression of BMP antagonists *in vivo* leads to lateral expansion of neural crest markers, contiguous with their normal domain, at the expense of epidermal ectoderm (Mayor *et al*., 1995; LaBonne and Bronner-Fraser, 1998). Conversely, overexpression of BMP4 has little effect on neural crest markers, but shifts the border medially at the expense of the neural plate (LaBonne and Bronner-Fraser, 1998). Zebrafish embryos carrying mutations in the BMP signaling pathway also show reduced or expanded domains of neural crest cell precursors, depending on the effect of the mutation on BMP activity levels (Nguyen *et al*., 1998). In the chick, the balance between

BMP4 and its antagonists is important for establishing and/or maintaining the prospective neural plate border: This region, which itself expresses BMP4, is the only region responsive to changes in the level of BMP signaling at neural plate stages (Streit and Stern, 1999).

These results suggest that BMP signaling is required for neural plate border formation and maintenance, and that changes in BMP activity levels can affect neural crest cell formation, although they are not sufficient to induce neural crest cells.

Members of the Dlx family of transcription factors play an important role in positioning the neural plate border during gastrulation (McLarren *et al*., 2003; Woda *et al*., 2003). In the chick, gain-of-function experiments have shown that Dlx5, itself a marker of the neural plate border, represses neural fates without inducing epidermis (McLarren *et al*., 2003). Furthermore, Dlx5 acts non-cell autonomously (presumably by activating downstream signaling pathways) to promote the expression of other neural plate border markers in adjacent cells, such as the transcription factor Msx1, and BMP4 itself (McLarren *et al*., 2003). However, Dlx5 activity is not sufficient to induce either neural crest cells or placodes (McLarren *et al*., 2003). In *Xenopus*, gain-of-function and loss-of-function experiments have shown that Dlx3 and Dlx5 activity positions the neural plate border, and that Dlx protein function in non-neural ectoderm is required for the subsequent induction of both neural crest and placodes (Woda *et al*., 2003).

In summary, the activity of BMP signaling molecules and Dlx transcription factors appears to specify the neural plate border region. However, the activity of these molecules is insufficient to specify neural crest cells (or placode cells). Intermediate BMP activity levels induce neural plate border that is anterior in character. Hence, additional signals are required to posteriorize the neural plate border and induce neural crest precursor cells within it.

Step 2: Induction of Neural Crest Precursors

It is becoming increasingly evident that Wnt and/or FGF family members are involved both in posteriorizing the neural plate border and inducing neural crest precursor cells within it. These do seem to be separable processes, however, as neural crest induction can be experimentally uncoupled from the anterior–posterior patterning of the neural plate (e.g., Chang and Hemmati-Brivanlou, 1998; Monsoro-Burq *et al*., 2003).

Posteriorizing Signals (Wnts and FGFs)

A posteriorizing signal derived from the paraxial mesoderm enables rostral neural plate tissue to form neural crest cells in the chick (Muhr *et al*., 1997) and establishes *Pax3* expression at the neural plate border in both chick and *Xenopus* embryos (Bang *et al*., 1997, 1999). In the chick, this posteriorizing activity is mediated by Wnt family members, in particular Wnt8c and Wnt11, in conjunction with permissive FGF signaling (Nordström *et al*., 2002). Paraxial mesoderm produces several other factors, including FGFs and retinoic acid, that are able to posteriorize the neural plate to induce posterior cell fates. In the chick, though, FGFs and retinoic acid are insufficient to induce caudal character in neural cells *in vitro*: This requires Wnt activity from the caudal paraxial mesoderm (Muhr *et al*., 1997, 1999; Nordström *et al*., 2002).

Induction of Neural Crest Precursors (Wnts and FGFs)

In both *Xenopus* and chick embryos, Wnt family members are both sufficient to induce neural crest cells from neuralized ectoderm *in vitro*, and necessary for neural crest induction *in vivo* (reviewed in Wu *et al*., 2003). Wnts can induce neural crest markers in conjunction with BMP inhibitors in ectodermal explants *in vitro* (Saint-Jeannet *et al*., 1997; Chang and Hemmati-Brivanlou, 1998; LaBonne and Bronner-Fraser, 1998). Conversely, inhibiting Wnt function *in vivo* by overexpressing a dominant negative Wnt ligand prevents early neural crest cell marker expression (LaBonne and Bronner-Fraser, 1998). Morpholino oligonucleotide-mediated blockage of the translation of the Wnt receptor Frizzled3, or its proposed adaptor protein Kermit, both reduce *Slug* expression in *Xenopus* (Deardorff *et al*., 2001; Tan *et al*., 2001), again showing a requirement for Wnt signaling in neural crest cell formation. Furthermore, the *Xenopus Slug* promoter contains a functional binding site for a downstream effector of Wnt signaling (LEF/β-catenin) that is required to drive its expression in neural crest precursors, showing that the requirement for Wnt is direct (Vallin *et al*., 2001).

Wnt activity is also necessary and sufficient for neural crest cell induction in the chick (García-Castro *et al*., 2002). Overexpression of a dominant negative Wnt ligand inhibits *Slug* expression *in vivo*: This can be rescued by application of exogenous Wnt (García-Castro *et al*., 2002). Conversely, *Drosophila* Wingless (a Wnt1 homologue that triggers the Wnt signaling pathway in vertebrates) can induce neural crest cells from neural plate in a chemically defined medium that lacks any other growth factors and hormones (García-Castro *et al*., 2002). Importantly, BMP4, which was previously shown to induce neural crest cells from neural plate *in vitro*, in the presence of various additives (Liem *et al*., 1995), is unable to induce neural crest cells from the neural plate in their absence (García-Castro *et al*., 2002). Synergism with other factors present in the medium may also underlie the induction of neural crest cells by BMP2/4 from dissociated rat neural tube cells (Lo *et al*., 2002) or neuroepithelial stem cells (Mujtaba *et al*., 1998).

Wnt signaling seems to control the domain of expression of Iro1 and Iro7, homeodomain transcription factors homologous to the Iroquois family of factors that, in *Drosophila*, regulate the expression of proneural genes (section Proneural Genes: An Introduction) (Itoh *et al*., 2002). Functional knockdown of both Iro1 and Iro7 using morpholino antisense oligonucleotides leads to loss of *FoxD3* expression (Itoh *et al*., 2002). This not only suggests that these transcription factors are upstream of *FoxD3*, but also provides indirect evidence that Wnt signaling regulates neural crest induction (Itoh *et al*., 2002). Furthermore, Wnt signaling is required for the induction of c-Myc, a basic helix-loop-helix zipper transcription factor whose expression is

required for *Slug* and *FoxD3* expression and neural crest cell formation in *Xenopus* (Bellmeyer *et al*., 2003).

The above results clearly show that Wnts are both necessary and sufficient to mediate neural crest cell induction from neuralized ectoderm. Several different models of neural crest induction have been proposed over the years, variously stressing the importance of nonaxial mesoderm and neural plate–epidermal interactions. (Some of the data supporting a role for both paraxial mesoderm and neural plate–epidermal interactions in neural crest induction are described in the following sections.) However, since both paraxial mesoderm and epidermis express Wnt family members, it is likely that both tissues are involved *in vivo*. Wnt8 is expressed in the paraxial mesoderm, and Wnt6 and Wnt7b are expressed in non-neural ectoderm (Chang and Hemmati-Brivanlou, 1998; García-Castro *et al*., 2002).

Nonetheless, Wnts may not be the whole story. Work in *Xenopus* has suggested that not only Wnt8, but also retinoic acid and FGFs, are able to induce *Slug* expression, both in the anterior neural plate border, and in tissue transformed into anterior neural plate border by intermediate levels of BMP activity (Villanueva *et al*., 2002; Monsoro-Burq *et al*., 2003). Furthermore, FGF signaling is required for induction of neural crest markers by paraxial mesoderm in *Xenopus* (Monsoro-Burq *et al*., 2003). Hence, although most of the evidence so far favors Wnts as the primary signals that induce neural crest cell precursors within the neural plate border (see Wu *et al*., 2003), FGF involvement cannot be ruled out.

Evidence for Paraxial Mesoderm Involvement in Neural Crest Induction

Several lines of evidence have suggested a role for nonaxial mesoderm in neural crest cell induction. In 1945, Raven and Kloos showed in an amphibian model that fragments of lateral archenteron roof (prospective paraxial and lateral plate mesoderm) can induce neural crest cells from overlying ectoderm, in the absence of neural tissue, when grafted into the blastocoel (Raven and Kloos, 1945). Over fifty years later, prospective paraxial mesoderm was shown to induce neural crest marker expression and melanocytes from competent ectoderm in *Xenopus* explant cocultures (Bonstein *et al*., 1998; Marchant *et al*., 1998; Monsoro-Burq *et al*., 2003). In the chick, paraxial mesoderm can induce neural plate explants to form melanocytes (though not neurons) (Selleck and Bronner-Fraser, 1995). Hence, paraxial mesoderm is sufficient to induce at least some neural crest cell markers and derivatives *in vitro*, both from non-neural ectoderm and neural plate. Importantly, removing prospective paraxial mesoderm at the start of gastrulation in *Xenopus* leads to a reduction in *Slug* expression and melanocyte formation *in vivo* (Bonstein *et al*., 1998; Marchant *et al*., 1998). This suggests that paraxial mesoderm is not only sufficient to induce neural crest cells *in vitro*, but also necessary for neural crest cell induction *in vivo*.

The molecular model of neural crest induction described thus far (i.e., intermediate BMP activity plus Wnt/FGF signaling) can explain the induction of neural crest cells by paraxial mesoderm. Paraxial mesoderm expresses both BMP inhibitors, such as Noggin and Follistatin (e.g., Hirsinger *et al*., 1997; Marcelle *et al*., 1997; Liem *et al*., 2000), and Wnt and FGF family members (see previous section). The BMP inhibitors may induce intermediate levels of BMP activity in non-neural ectoderm, while the Wnt/FGF signals may subsequently induce neural crest cells from this neuralized ectoderm. However, this model has not been tested directly.

Evidence for Non-Neural Ectoderm Involvement in Neural Crest Induction

A role for non-neural ectoderm in neural crest cell induction was first proposed in the late 1970s and early 1980s. Rollhäuser-ter Horst used interspecific grafts between different species of urodele amphibians to follow the fate of gastrula ectoderm juxtaposed to different tissues (Rollhäuser-ter Horst, 1979, 1980). The ectoderm failed to form neural crest cells *in vitro* either when cultured alone, or when cocultured with neural-inducing tissue, but did form neural crest cells when both tissues were grafted to the belly of host embryos (Rollhäuser-ter Horst, 1979). This suggested a requirement for the host epidermis as well as neural-inducing tissue. When the gastrula ectoderm was grafted in place of the host neural folds, it also formed neural crest cells (Rollhäuser-ter Horst, 1980), again suggesting a role for interactions between neural and non-neural ectoderm in neural crest induction.

Moury and Jacobson similarly used pigmented and albino axolotl embryos as donors and hosts, respectively, to show that both neural folds and neural crest cells form at any newly created boundary between neural plate and epidermis (Moury and Jacobson, 1989, 1990). Under these circumstances, both epidermis and neural plate form neural crest cells. Interestingly, the neural plate forms melanocytes while the epidermis forms sensory neurons (Moury and Jacobson, 1990). In *Xenopus*, labeled neural plate grafted into epidermis *in vivo* leads to *Slug* upregulation in both donor and host tissues, at the interface between them (Mancilla and Mayor, 1996). Likewise, when quail neural plate is grafted into chick epidermis *in vivo*, both quail and chick tissue generate migratory HNK-1-positive cells (Selleck and Bronner-Fraser, 1995). *Slug* is also induced after similar experiments using unlabeled chick tissue (although in which tissues is unclear) (Dickinson *et al*., 1995).

Although these *in vivo* experiments suggested a role for interactions between neural plate and epidermis in neural crest cell induction, all the grafted tissues were also exposed to signals from the underlying mesoderm. However, *in vitro* cocultures of neural plate and epidermis, in the absence of mesoderm, are sufficient to induce *Slug* expression in *Xenopus* (Mancilla and Mayor, 1996) and neural crest cells in the chick (*Slug* expression; formation of melanocytes and catecholaminergic neurons) (Dickinson *et al*., 1995; Selleck and Bronner-Fraser, 1995). Hence, a local interaction between neural and non-neural ectoderm is sufficient to induce neural crest cells *in vitro*. This finding has been exploited in a subtractive hybridization screen of a macroarrayed chick cDNA library, in order to provide the

first gene expression profile of newly induced neural crest cells (Gammill and Bronner-Fraser, 2002).

The interaction between neural and non-neural ectoderm seems to recapitulate all of the steps of neural crest induction seen *in vivo*, including induction of the neural plate border, since neural folds form at all experimentally generated neural/epidermal interfaces (Moury and Jacobson, 1989). Both epidermal and neural plate cells may contribute to the new neural plate border region, perhaps explaining why both tissues form neural crest cells after such interactions.

In summary, there is substantial evidence to implicate both paraxial mesoderm and non-neural epidermis in neural crest cell induction *in vivo*. Their involvement is probably due to their expression of Wnt (and/or FGF) family members, which can induce neural crest cell precursors within the neural plate border region.

AP2α and SoxE Transcription Factors Are Involved in the Earliest Steps of Neural Crest Precursor Formation

The transcription factor AP2α is expressed during early stages of neural crest development in all vertebrates, as well as in other tissues, such as the epidermis (see Luo *et al*., 2003). In *Xenopus*, AP2α expression, which covers a broader territory than other early neural crest precursor markers such as *Sox9* (see next paragraph) and *Slug*, is upregulated by BMP and Wnt signaling (Luo *et al*., 2003). Morpholino-mediated functional knockdown of AP2α results in failure of neural fold formation and the loss of *Sox9* and *Slug* expression (Luo *et al*., 2003). These results suggest an important role for AP2α in the earliest stages of neural crest precursor formation. However, the broad expression pattern of AP2α, in particular in epidermis, implies that other factors must be involved in restricting neural crest precursor formation to the correct region.

Sox9 and Sox10 are members of the E subgroup of high-mobility-group (HMG) domain Sox transcription factors. Sox9 is one of the earliest markers of premigratory neural crest cell precursors within the neural plate border; its expression is maintained during early stages of neural crest migration (Spokony *et al*., 2002; Cheung and Briscoe, 2003). Morpholino-mediated functional knockdown of either Sox9 or Sox10 in *Xenopus* blocks neural fold formation, as well as blocking expression of neural plate border markers and neural crest precursor markers, including *Slug* (Spokony *et al*., 2002; Honoré *et al*., 2003). Unlike Dlx activity (see section Establishment of the Neural Plate Border), Sox9 activity is sufficient to induce neural crest precursor markers, including *Slug* and *FoxD3*, in both dorsal and ventral regions of the chick neural tube (Cheung and Briscoe, 2003). However, Sox9-induced ectopic neural crest precursors rarely delaminate except in the most dorsal regions of the neural tube (Cheung and Briscoe, 2003). This suggests that additional signals are required for neural crest cell delamination, and that these signals are only present dorsally (see section Epithelial–Mesenchymal Transition).

Importantly, Sox9-mediated induction of neural crest precursor markers in the chick does not induce BMP or Wnt family members, nor require BMP activity, suggesting that, like AP2α, Sox9 lies downstream of these signaling pathways (Cheung and Briscoe, 2003). Blocking either FGF signaling or Wnt signaling in *Xenopus* also blocks Sox10 expression at the neural plate border (Honoré *et al*., 2003), again suggesting that the SoxE transcription factors lie downstream of identifed neural crest precursor inducing signals.

Although morpholino-mediated functional knockdown of *Sox9a* in zebrafish does not affect neural crest precursors, it is possible that *Sox9b* may instead play this role in zebrafish (Yan *et al*., 2002). Neural crest-specific knockout of *Sox9* in mice does not cause neural crest precursor defects (Mori-Akayama *et al*., 2003), but it is possible that overlapping expression of the other SoxE subgroup members, Sox8 and Sox10, may compensate for the loss of Sox9.

In summary, it seems likely that AP2α, Sox9, and Sox10 may be crucial downstream target of BMP and Wnt/FGF signals in the formation of neural crest precursors. AP2α seems to lie upstream of Sox9, whose activity in turn induces the expression of multiple other markers of neural crest cell precursors, including *Slug* and *FoxD3* (see next section). However, delamination from the neuroepithelium (i.e., neural crest cell formation) requires additional signals that, at least in the chick, may only be present in the dorsal neural tube.

Snail/Slug and FoxD3 Are Required for Neural Crest Precursor Formation

The Snail superfamily of zinc finger transcriptional repressors contains two major families: Snail and Scratch (Nieto, 2002). In vertebrates, the Snail family is further subdivided into Snail and Slug subfamilies, both of which are essential during two stages of neural crest formation: (1) The formation of neural crest cell precursors within the neuroepithelium, and (2) delamination of cranial neural crest cells (section Snail Family Members Promote Cranial Neural Crest Cell Delamination). In *Xenopus, Slug* is first expressed at late gastrula stages, long before neural crest delamination occurs (Mayor *et al*., 1995). Slug acts as a transcriptional repressor (LaBonne and Bronner-Fraser, 2000; Mayor *et al*., 2000). *Slug* overexpression in *Xenopus* leads to an expansion of the neural crest domain at the expense of epidermis, and to overproduction of at least some neural crest derivatives (LaBonne and Bronner-Fraser, 1998). Conversely, other early neural crest precursor markers are lost after expression of a dominant negative *Slug* construct or antisense *Slug* RNA, showing that Slug function is necessary for the formation of neural crest precursors (Carl *et al*., 1999; LaBonne and Bronner-Fraser, 2000). However, not all *Slug*-expressing cells delaminate to form neural crest cells (Linker *et al*., 2000).

The winged-helix transcription factor FoxD3 (Forkhead6) is also important in early stages of neural crest cell formation (Dottori *et al*., 2001; Kos *et al*., 2001; Pohl and Knöchel, 2001; Sasai *et al*., 2001). Like Slug, FoxD3 is a transcriptional repressor (Pohl and Knöchel, 2001; Sasai *et al*., 2001) and is expressed both in premigratory neural crest cell precursors and migrating neural crest cells. In *Xenopus*, inhibiting FoxD3 function *in vivo*

using a dominant negative *FoxD3* construct represses the expression of early neural crest precursor markers, including *Slug*, and leads to a corresponding expansion of the neural plate (Sasai *et al*., 2001). Hence, like *Slug*, FoxD3 function is required for the formation of neural crest precursors. However, overexpression of *FoxD3* in the chick neural tube does not upregulate *Slug*, suggesting that *Slug* is not an obligate downstream target of FoxD3 (Dottori *et al*., 2001). Instead, the two genes seem to act in concert, in partially overlapping pathways, to promote neural crest cell formation (Sasai *et al*., 2001).

In addition to their importance for the formation of neural crest cell precursors, both FoxD3 and Slug can promote neural crest cell delamination (sections FoxD3 Promotes Neural Crest Cell Delamination at All Axial Levels; Snail Family Members Promote Cranial Neural Crest Cell Delamination).

Step 3: Epithelial–Mesenchymal Transition

The final step in neural crest induction is the activation of the epithelial–mesenchymal transition that leads to delamination from the neuroepithelium into the periphery. As described at the beginning of the section on Neural Crest Induction, a cell cannot be described as a *bona fide* neural crest cell until it emigrates from the neuroepithelium. Hence, induction of delamination is the final step in the induction of the neural crest.

In all vertebrates, neural crest cell precursors delaminate in a rostrocaudal wave along the neuraxis. Whether or not neural crest cell precursors are initially incorporated into the neural tube depends on the timing of neural crest cell delamination relative to the timing of fusion of the neural folds. This varies from species to species and on the axial level within the embryo. Cranial neural crest cells, in particular, which are the first to delaminate, may not be incorporated into the neural tube. In the mouse, cranial neural crest delamination begins in the midbrain/rostral hindbrain well before neural tube closure, when the neural folds are approaching one another in the cervical region (Nichols, 1981). In frogs, cranial neural crest cells form large masses that segregate from the neural tube prior to its closure; these masses do not take part in the morphogenetic movements of neurulation (Schroeder, 1970; Olsson and Hanken, 1996). In the chick, however, cranial neural crest cells delaminate as the neural folds meet or during early apposition, beginning at midbrain levels (Tosney, 1982). Trunk neural crest cells in the chick only emigrate after the epidermis and neural tube have separated (Tosney, 1978).

In the chick, the first sign of imminent neural crest cell delamination at cranial levels is that the neural crest cell precursor cell population becomes less tightly packed, and the cells extend long cellular processes into the intercellular spaces within the population (Tosney, 1982). As emigration starts, the basal lamina over the neural crest cells becomes fragmented, and the cells extend long processes into the adjacent cell-free space (Tosney, 1982). Clearly, major changes in cytoskeletal architecture, cell–cell and cell–matrix interactions occur during this epithelial–mesenchymal transition. Recent molecular evidence has given us a more detailed insight into the genes and signaling pathways controlling these processes.

The Basal Lamina Must Be Degraded before Delamination Can Occur

Neural crest cells do not seem to be able to penetrate an intact basal lamina (Erickson, 1987). The basal lamina clearly breaks down over neural crest cell precursors before they delaminate from the neuroepithelium (e.g., Tosney, 1982; Raible *et al*., 1992) and this may be due to neural crest secretion of proteases, although it remains to be demonstrated. Neural crest cell precursors produce various proteolytic enzymes, including the serine protease plasminogen activator (Valinsky and Le Douarin, 1985; Agrawal and Brauer, 1996), BMP1/Tolloid metalloproteases (Martí, 2000), and members of the metalloprotease/disintegrin family (Alfandari *et al*., 1997; Cai *et al*., 1998). Some of these proteases are only found in cranial neural crest cell precursors and migrating cranial neural crest cells, for example, the metalloprotease/disintegrin ADAM13 in *Xenopus* (Alfandari *et al*., 1997, 2001). However, a role for these proteases in neural crest cell delamination has not yet been shown.

Inhibiting Protein Kinase C Signaling Promotes Delamination

If avian neural tube explants are treated with protein kinase C inhibitors, cells immediately, and precociously, delaminate and migrate away from the neural tube (Newgreen and Minichiello, 1995, 1996). This occurs on both dorsal and ventral sides of the neural tube (although ventral cells are less sensitive than dorsal cells) (Newgreen and Minichiello, 1995, 1996). This stimulatory effect of protein kinase C inhibitors does not require protein synthesis (Newgreen and Minichiello, 1995). Similarly, protein kinase C inhibition triggers delamination, migration, and expression of the neural crest marker *Sox10*, in neuroectoderm cells produced from mouse embryonic stem cells in culture (Rathjen *et al*., 2002). These results suggest that delamination can be induced by signals that modulate protein kinase C activity.

Delamination is Associated with Downregulation of Cadherin6B

Calcium-dependent cell–cell adhesions are required to prevent precocious emigration of neural crest cells (Newgreen and Gooday, 1985). In the chick, most neural tube cells express the calcium-dependent cell–cell adhesion molecule N-cadherin, while epidermal cells express E-cadherin; however, the dorsal neural tube, which contains neural crest cell precursors, expresses neither N-cadherin nor E-cadherin (Akitaya and Bronner-Fraser, 1992). In accordance with this, N-cadherin itself does not seem to be required for neural crest cell formation or migration, as pigmentation and cranial cartilages are normal in *N-cadherin* mutant zebrafish (Lele *et al*., 2002). Instead, neural crest cell precursors within the neuroepithelium express *cadherin6B*; this expression is lost in emigrating neural crest cells (Nakagawa and Takeichi, 1995, 1998). Type II (atypical) cadherins are then upregulated in subpopulations of migrating neural crest cells, for example *cadherin7* and *cadherin 11*; these

may be involved in controlling the rate of neural crest cell migration and/or in some aspects of fate specification (Nakagawa and Takeichi, 1995; Borchers *et al*., 2001).

FoxD3 Promotes Neural Crest Cell Delamination at All Axial Levels

FoxD3 is essential for the formation of neural crest cell precursors (section Snail/Slug and FoxD3 Are Required for Neural Crest Precursor Formation), and it may also play a role in neural crest cell delamination. Ectopic expression of FoxD3 in the chick neural tube promotes neural crest cell delamination at all axial levels (Dottori *et al*., 2001). This is achieved without upregulating *Slug* or, apparently, *RhoB* (section BMP4 Induces RhoB, Which Is Essential for Neutral Crest Cell Delamination), suggesting that FoxD3 and Slug function independently in regulating neural crest cell delamination (Dottori *et al*., 2001). The precise mechanism of action of FoxD3 in promoting delamination remains unclear.

Snail Family Members Promote Cranial Neural Crest Cell Delamination

Snail family transcription factors are required for the formation of neural crest cell precursors (section Snail/Slug and FoxD3 Are Required for Neural Crest Precursor Formation). Several different lines of evidence also support a role for *Snail* family genes in epithelial–mesenchymal transitions. Overexpression of mouse *Slug* in bladder carcinoma cells leads to desmosome dissociation at sites of cell–cell contact, a necessary prerequisite for epithelial–mesenchymal transition (Savagner *et al*., 1997). Overexpression of mouse *Snail* in epithelial cells represses transcription of the cell–cell adhesion molecule *E-cadherin*, and leads to epithelial–mesenchymal transition and migratory and invasive cell behaviors (Batlle *et al*., 2000; Cano *et al*., 2000). Since *Snail* and/or *Slug* genes are expressed in premigratory neural crest cell precursors in all vertebrates, a role in neural crest cell delamination from the neuroepithelium seems likely.

Early antisense experiments in chick embryos suggested a role for Slug in cranial neural crest cell migration (Nieto *et al*., 1994). Cranial neural crest cell migration is inhibited in *Xenopus* in the presence of antisense *Slug* RNA or a dominant negative *Slug* construct (Carl *et al*., 1999; LaBonne and Bronner-Fraser, 2000). Overexpression of *Slug* in the chick neural tube leads to an increase in the number of migrating cranial neural crest cells, although not of trunk neural crest cells (del Barrio and Nieto, 2002). Other experiments have also shown that, unlike FoxD3, increased Slug activity alone does not cause trunk neural crest cell delamination in the trunk (Sela-Donenfeld and Kalcheim, 1999). The basis of this difference between head and trunk is unknown.

BMP Signaling is Required for Delamination

In the trunk of the chick embryo, neural crest cells only begin to delaminate in areas adjacent to the epithelial somites: They do not emigrate at the level of the segmental plate mesoderm (Teillet *et al*., 1987). The timing of neural crest cell emigration in the trunk can be correlated with expression of the BMP2/4 antagonist Noggin (Sela-Donenfeld and Kalcheim, 1999). *Noggin* is strongly expressed in the dorsal neural tube opposite the segmental plate mesoderm, more weakly expressed opposite newly epithelial somites, and absent opposite fully dissociated somites, while BMP4 is expressed in the dorsal neural tube at all levels (Sela-Donenfeld and Kalcheim, 1999). Noggin overexpression (i.e., inhibition of BMP activity) inhibits neural crest cell delamination both *in vivo* and *in vitro*, and this can be rescued *in vitro* by BMP4 (Sela-Donenfeld and Kalcheim, 1999). This suggests that a balance between BMP4 and its antagonists plays a role in the onset of neural crest cell delamination in the trunk (Sela-Donenfeld and Kalcheim, 1999). This balance is now known to be controlled by the paraxial mesoderm itself: The dorsomedial region of developing somites produces a signal that downregulates *noggin* transcription in the dorsal neural tube (Sela-Donenfeld and Kalcheim, 2000). This enables the coordination of neural crest cell emigration with the formation of a suitable mesodermal substrate for migration (section Migration Pathways of Trunk Neural Crest Cells) (Sela-Donenfeld and Kalcheim, 2000).

BMP signaling is also essential for cranial neural crest cell migration in the mouse (Kanzler *et al*., 2000). When *noggin* is expressed in transgenic embryos under the control of a *Hox2a* enhancer, leading to *noggin* overexpression in the hindbrain, hindbrain-level neural crest cells fail to emigrate (Kanzler *et al*., 2000). Although *Bmp4* is not expressed in the dorsal hindbrain in the mouse, *Bmp2* is expressed there, and hindbrain neural crest cells fail to migrate in *Bmp2* mutant embryos. Hence, it seems that BMP2 activity is necessary for cranial neural crest cell emigration in the mouse (Kanzler *et al*., 2000).

These results show that BMP signaling is essential not just to establish the neural plate border, but also at a later stage, to promote neural crest cell delamination.

BMP4 Induces RhoB, Which Is Essential for Neural Crest Cell Delamination

The small GTP-binding protein RhoB is expressed in neural crest precursors within the neuroepithelium and is downregulated shortly after delamination (Liu and Jessell, 1998). Rho proteins have been implicated in the assembly of the actin cytoskeleton required for motility (see Frame and Brunton, 2002). Treatment of chick neural tube explants with a Rho-specific inhibitor has shown that Rho function is essential for neural crest cell delamination, and that the actin cytoskeleton in neural crest cell precursors is perturbed (Liu and Jessell, 1998). *RhoB* also seems to be a downstream target of Slug activity, though whether direct or indirect is unknown (del Barrio and Nieto, 2002). It is not, however, detectably induced by FoxD3 (Dottori *et al*., 2001). Nor, interestingly, is RhoB detectably induced by Sox9, which induces neural crest precursor formation but is not sufficient to promote efficient delamination, except at the dorsalmost region of the neural tube (Cheung and Briscoe, 2003; section AP2α and SoxE Transcription Factors Are Involved in the Earliest Steps of Neural Crest Precursor Formation). However, RhoB is induced by BMP4: Indeed, it was originally identified in a PCR-based screen for genes induced by BMP4 in

neural plate cells (Liu and Jessell, 1998). Since BMP4 is essential for delamination of neural crest cell precursors and induces RhoB, it seems that BMP4 activity is the most likely candidate for the dorsally located signal that induces neural crest cell formation from premigratory neural crest cell precursors. It will be important to establish whether all RhoB-expressing neural crest cell precursor cells do, in fact, emigrate from the neural tube.

Transition from G1 to S Phase of the Cell Cycle Is Required for Neural Crest Cell Delamination

In the chick, most trunk neural crest cells emigrate from the neural tube in the S phase of the cell cycle, when their nuclei are located at or near the basal margin of the neuroepithelium (Burstyn-Cohen and Kalcheim, 2002). Blocking the cell cycle transition from G1 to S phase blocks neural crest delamination, both *in vivo* and in explants (Burstyn-Cohen and Kalcheim, 2002). Thus, the cell cycle status of neural crest cell precursors is an essential prerequisite for the epithelial–mesenchymal transition that forms neural crest cells. It is possible that BMP signaling in the dorsal neural tube induces a cascade of signals that influence G1/S transition, perhaps by upregulating cyclin D1. Alternatively, independent pathways downstream of BMP signaling and the cell cycle may converge on common downstream targets to initiate delamination.

Summary of Neural Crest Induction

Neural crest induction is a multistep, multisignal process that can be divided into three distinct phases. Firstly, the neural plate border is induced during gastrulation, probably by intermediate levels of BMP activity, and with the involvement of Dlx transcription factors. Secondly, Wnt and/or FGF signals from surrounding tissues (paraxial mesoderm and non-neural ectoderm) posteriorize the neural plate border and induce neural crest cell precursors within it. Finally, BMP activity in the dorsal neural tube induces RhoB in a subset of neural crest cell precursors. After G1/S transition, these cells undergo an epithelial–mesenchymal transition, delaminate from the neuroepithelium as neural crest cells, and migrate into the periphery.

As neural crest cells delaminate from the neuroepithelium, they are faced with very different mesodermal environments depending on their axial level. In the head, they encounter the apparently disorganized cranial paraxial mesenchyme, while in the trunk, the paraxial mesoderm is segmented into repeating blocks, the somites. In both head and trunk, however, neural crest cells follow ordered pathways to their target sites, where they differentiate into an impressive array of different derivatives. The mechanisms underlying this migration are discussed in the following section.

NEURAL CREST MIGRATION

Experimental Approaches

Two main experimental approaches have been used to map the migration pathways and, concurrently, define the derivatives of the neural crest. First, ablation studies have been performed, to determine what cell types and tissues are lacking as a result. Although such experiments yielded a wealth of information, particularly from fish and amphibians, drawbacks included the possibility of regulation to restore the missing cells, and indirect effects on other tissues. The second approach has been to label the neural folds, including premigratory neural crest cell precursors: Labeled neural crest cells delaminating into the periphery can be distinguished from surrounding unlabeled cells. Early studies in amphibian embryos employed vital dyes to label donor embryos, from which neural folds were explanted and grafted into unlabeled host embryos (e.g., Detwiler, 1937). Heterospecific grafts were also used extensively in amphibians as differences in pigmentation and/or cell size enabled donor and host tissues to be distinguished. Such grafts have also been combined with staining techniques that reveal differences in nuclear morphology (e.g., Sadaghiani and Thiébaud, 1987; Krotoski *et al*., 1988).

Tritiated thymidine labeling of the nuclei of donor embryos, followed by grafting of labeled neural folds into unlabeled hosts, was introduced in the 1960s for the chick (Weston, 1963) and immediately applied in amphibians (Chibon, 1964). This method was used in avian embryos for about 12 years (e.g., Johnston, 1966; Noden, 1975). It was superseded, however, by Le Douarin's discovery that the quail nucleolus is associated with a large mass of heterochromatin, enabling it to be distinguished clearly from chick nuclei after appropriate staining (Le Douarin, 1969, 1973). Hence, quail neural folds could be grafted into chick hosts, and the fate of the donor quail cells followed throughout development, up to and including hatching (although graft rejection occurs eventually). This technique was used in a series of elegant fate-mapping studies to define all the derivatives of the neural crest in the avian embryo along the length of the neuraxis (e.g., Le Douarin and Teillet, 1973, 1974; Teillet, 1978; Noden, 1978a, b) (reviewed in Le Douarin and Kalcheim, 1999). Today, a quail-specific antibody enables easier identification of grafted quail cells within the chick host, and the quail–chick chimera technique is still commonly used to study neural crest cell fate, migration, and potential (e.g., Baker *et al*., 1997; Catala *et al*., 2000; Etchevers *et al*., 2001).

Migrating neural crest cells have also been followed using monoclonal antibodies, such as the HNK1 antibody in chick and rat embryos (e.g., Rickmann *et al*., 1985; Bronner-Fraser, 1986; Erickson *et al*., 1989). Modern, nontoxic vital dyes have been extensively used to map neural crest cell migration pathways and derivatives *in situ*, avoiding any risk of artifacts introduced by invasive surgery or differences in behavior between donor and host cells. The lipophilic dye DiI can be injected into the lumen of the neural tube to label all neural tube cells, including premigratory neural crest cells, which can subsequently be followed as they migrate through the periphery (e.g., Serbedzija *et al*., 1989, 1990; Collazo *et al*., 1993). Time-lapse *in ovo* confocal microscopy, combined with DiI labeling, has also enabled migrating hindbrain neural crest cells to be followed *in vivo* at high resolution (e.g., Kulesa and Fraser, 2000). Membrane-impermeant dyes, such as lysinated rhodamine dextran, can be

injected into individual neural crest cell precursors and migrating neural crest cells *in vivo*, allowing the progeny of single cells to be followed during development (Bronner-Fraser and Fraser, 1988, 1989; Fraser and Bronner-Fraser, 1991). Retroviral-mediated gene transfer has also enabled the clonal analysis of the progeny of single neural crest cells *in vivo* (Frank and Sanes, 1991). In mice, the fate of migrating cranial neural crest cells has been followed by using Cre–Lox transgenic technology to activate constitutive β-galactosidase expression under the control of the *Wnt1* promoter (Chai *et al*., 2000).

Together, these different cell-labeling approaches have enabled a detailed picture to be drawn of the migration pathways followed by neural crest cells through the periphery.

Migration Pathways of Cranial Neural Crest Cells

Cranial neural crest cells migrate beneath the surface ectoderm, above the paraxial cephalic mesoderm (see Figs. 3 and 4B), although a few cells penetrate the paraxial mesoderm.

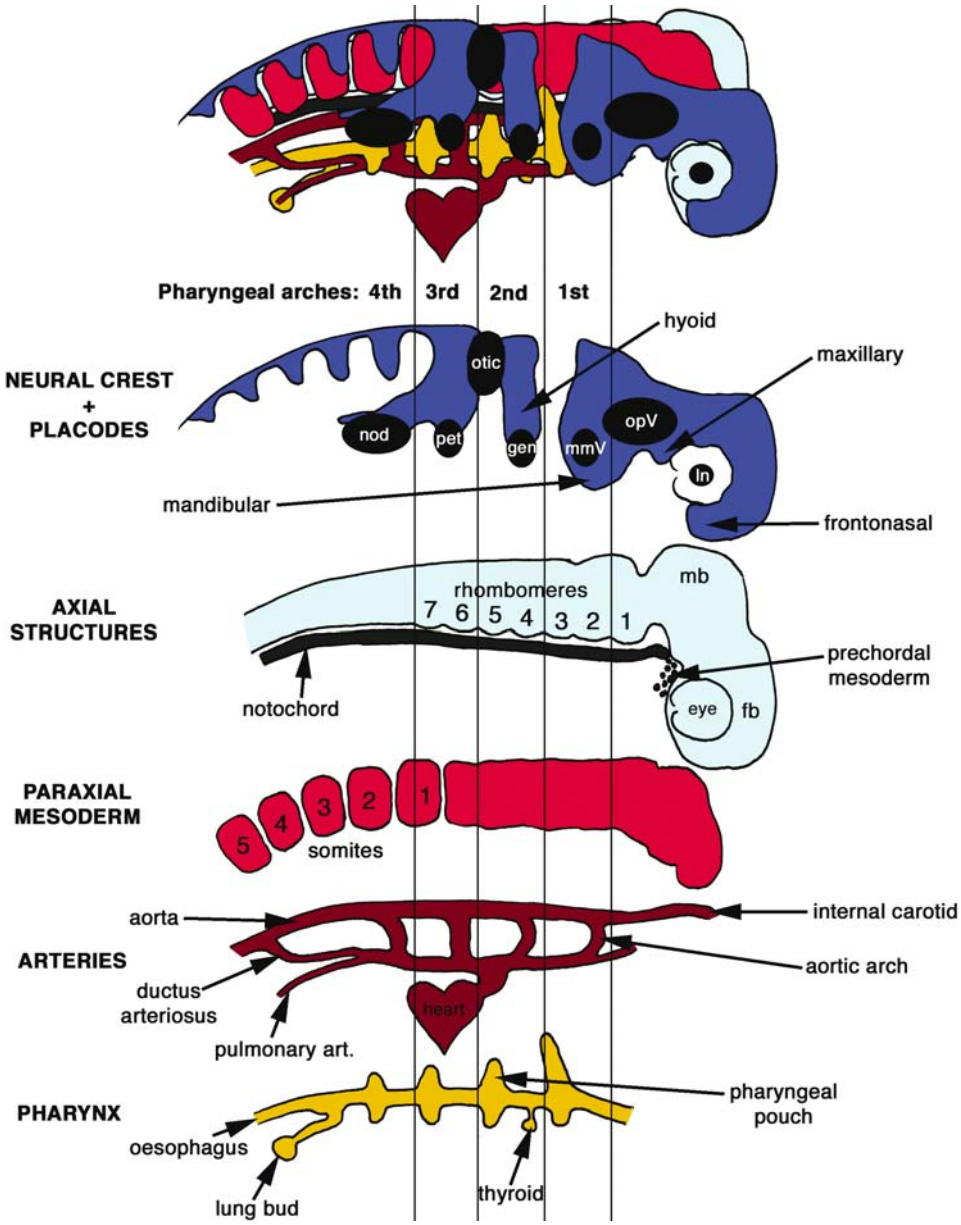

FIGURE 3. Schematic lateral views of a generalized 20–30 somite-stage amniote embryo with the surface ectoderm removed (except to show the positions of the cranial ectodermal placodes). Each tissue type from the embryo at the top is shown separately below, illustrating the relative positions of the migrating neural crest, placodes (filled black circles), axial structures, paraxial mesoderm, arteries, and pharyngeal endoderm. The olfactory placodes cannot be seen in this view. The vertical lines indicate which regions are in register with each pharyngeal arch. Redrawn from Noden (1991). art., artery; fb, forebrain; gen, geniculate; ln, lens; mb, midbrain; mmV, maxillomandibular trigeminal; nod, nodose; opV, ophthalmic trigeminal; pet, petrosal.

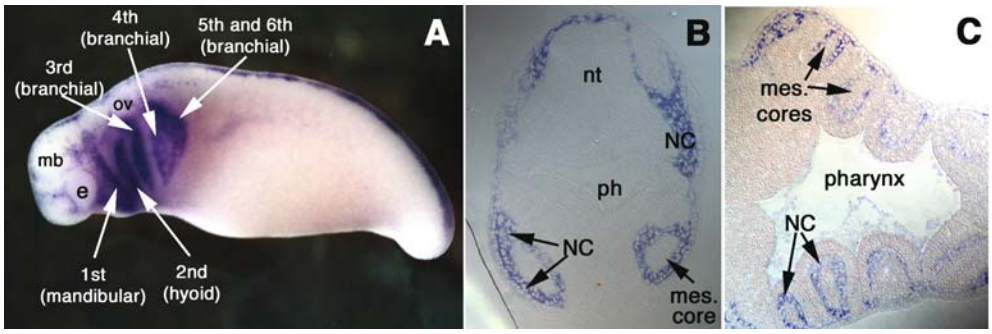

FIGURE 4. Cranial neural crest migration streams in the axolotl visualized by *in situ* hybridization for the *AP-2* gene. (A) Stage 29 (16-somite stage) axolotl embryo showing six *AP-2*$^{+}$ neural crest migration streams in the head (mandibular, hyoid, and four branchial streams). Premigratory trunk neural crest cell precursors can be seen as a dark line at the dorsal midline of the embryo. (B) Transverse section through a stage 26 (10–11 somite stage) axolotl embryo showing *AP-2*$^{+}$ neural crest cells (NC) moving out from the neural tube (nt) and down to surround the mesodermal core of the mandibular arch. (C) Horizontal section through the pharynx of a stage 34 (24–25 somite stage) axolotl embryo showing *AP-2*$^{+}$ neural crest cells (NC) around the mesodermal cores of each pharyngeal arch. e, eye; mb, midbrain; mes., mesodermal; NC, neural crest; nt, neural tube; ov, otic vesicle; ph, pharynx. Staging follows Bordzilovskaya *et al*. (1989). All photographs courtesy of Daniel Meulemans, California Institute of Technology, United States of America.

They migrate as coherent populations; indeed, at the hindbrain level, migrating neural crest cells are connected in chains by filopodia (Kulesa and Fraser, 1998, 2000). They populate the entire embryonic head and form much of the neurocranium (brain capsule) and all of the splanchnocranium (viscerocranium or visceral skeleton), that is, the skeleton of the face and pharyngeal arches. They also form neurons and satellite glia in cranial sensory and parasympathetic ganglia, Schwann cells, endocrine cells, and epidermal pigment cells (see Table 1).

Pharyngeal Arches and Neural Crest Streams

The patterning of cranial neural crest cell migration is intimately bound up with the segmental nature of both the hindbrain (rhombomeres; see Chapter 3) and the periphery (pharyngeal arches). Pharyngeal arches are also known as branchial arches, from the Latin *branchia* ("gill"), because in aquatic vertebrates the more caudal arches are associated with gills. However, "pharyngeal" is the more appropriate term, because all arches form in the pharynx, but not all arches support gills. Pharyngeal arches form between the pharyngeal pouches, which are outpocketings of the pharyngeal (fore-gut) endoderm that fuse with the overlying ectoderm to form slits in the embryo (see Fig. 3). The pharyngeal slits form the gill slits in aquatic vertebrates; the first pharyngeal slit in tetrapods forms the middle ear cavity. Paraxial mesoderm in the core of the pharyngeal arches (Figs. 4B, C) gives rise to striated muscles. Cranial neural crest cells migrate subectodermally to populate the space around the mesodermal core (Figs. 4B, C), where they give rise to all skeletal elements of the arches, and the connective component of the striated muscles.

The first pharyngeal arch is the mandibular, which forms the mandible (lower jaw). The second arch is the hyoid, which forms jaw suspension elements in fish but middle ear bones in tetrapods, together with parts of the hyoid apparatus/bone (supporting elements for the tongue and roof of the mouth). Varying numbers of arches follow more caudally. The third and fourth arches also contribute to the hyoid apparatus and to laryngeal cartilages in tetrapods; in mammals, the fourth arch forms thyroid cartilages. More caudal arches in fish and aquatic amphibians support gills and form laryngeal cartilages in tetrapods. Importantly, pharyngeal arch formation *per se*, and the regionalization of gene expression patterns within them (excluding those of neural crest-derived structures) are both independent of neural crest cell migration (Veitch *et al*., 1999; Gavalas *et al*., 2001).

Cranial neural crest cells migrate in characteristic streams associated with the pharyngeal arches (Figs. 3 and 4A). There are three or more major migration streams in all vertebrates. The first stream, from the midbrain and rhombomeres 1 and 2 (r1,2), populates the first (mandibular) arch; the second stream, from r3–5, populates the second (hyoid) arch, and the third, from r5–7, populates the third arch (Fig. 4). In fish and amphibians, additional caudal streams populate the remaining arches: The axolotl, for example, has four branchial (gill) arches caudal to the mandibular and hyoid arches (Fig. 4A). How is the migrating neural crest cell population sculpted to achieve these different streams?

Separation of the First, Second, and Third Neural Crest Streams (Amniotes)

In chick and mouse embryos, there are neural crest cell-free zones adjacent to r3 and r5 (Fig. 3). It was suggested that neural crest cells at r3 and r5 die by apoptosis to generate adjacent neural crest-free zones (Graham *et al*., 1993). However, both r3 and r5 give rise to neural crest cells during normal development in both chick and mouse, though r3 generates fewer neural crest cells than other rhombomeres (Sechrist *et al*., 1993; Köntges and Lumsden, 1996; Kulesa and Fraser, 1998; Trainor *et al*., 2002b). Neural crest cells from r3 and r5 migrate rostrally and caudally along the neural tube to join the adjacent neural crest streams; that is, r3-derived neural crest joins the r1,2 (first arch) and r4 (second arch) streams, while r5-derived neural crest joins the r4 (second arch) and r6,7 (third arch) streams (Sechrist

et al., 1993; Köntges and Lumsden, 1996; Kulesa and Fraser, 1998; Trainor *et al*., 2002b). This deviation of the r3 and r5 neural crest generates the neural crest-free zones adjacent to r3 and r5, forming the three characteristic streams in birds and mice (Fig. 3). Hence, the first arch is populated by neural crest cells from the midbrain and r1–3, the second arch by neural crest cells from r3–5, and the third arch by neural crest cells from r5–7.

Neural crest cells leaving r5 are confronted by the otic vesicle (Fig. 3), which provides an obvious mechanical obstacle to migration. No such obstacle exists at r3; instead, paraxial mesoderm at the r3 level is inhibitory for neural crest cell migration, at least in amniotes (Farlie *et al*., 1999). This inhibition is lost in mice lacking ErbB4, a high-affinity receptor for the growth factor Neuregulin1 (NRG1) (Golding *et al*., 1999, 2000). ErbB4 is expressed in the r3 neuroepithelium, while NRG1 is expressed in r2; ErbB4 activation by NRG1 may somehow signal the production of inhibitory molecules in r3-level paraxial mesoderm (Golding *et al*., 2000). A few hours after removing either r3 itself, or the surface ectoderm at the r3 level, r4 neural crest cells move aberrantly into the mesenchyme adjacent to r3, suggesting that both r3 itself and r3-level surface ectoderm are necessary to inhibit neural crest cell migration (Trainor *et al*., 2002b).

Separation of the Third and Fourth Streams (Anamniotes)

Fish and amphibians also have additional cranial neural crest streams that populate the more caudal pharyngeal arches. In amphibians, at least, neural crest cells destined for different arches do not separate into different streams adjacent to the neural tube; instead, separation occurs at or just before entry into the arches (Robinson *et al*., 1997). Another difference in *Xenopus*, in which the otic vesicle is adjacent to r4 rather than r5, is that all r5-derived neural crest cells seem to migrate into the third arch (Robinson *et al*., 1997).

In *Xenopus*, migrating neural crest cells in the third and fourth cranial neural crest streams are separated by repulsive migration cues. These are mediated by the ephrin family of ligands, acting on their cognate Eph-receptor tyrosine kinases (Smith *et al*., 1997; Helbling *et al*., 1998; reviewed in Robinson *et al*., 1997; for a general review of ephrins and Eph family members, see Kullander and Klein, 2002). The transmembrane ligand ephrinB2 is expressed in second arch neural crest cells and mesoderm. One ephrinB2 receptor, EphA4, is expressed in third arch neural crest cells and mesoderm, while a second ephrinB2 receptor, EphB1, is expressed in both third and fourth arch neural crest cells and mesoderm (Smith *et al*., 1997). Inhibition of EphA4/EphB1 function using truncated receptors results in the aberrant migration of third arch neural crest cells into the second and fourth arches. Conversely, ectopic activation of EphA4/EphB1 (by overexpressing ephrinB2) results in the scattering of third arch neural crest cells into adjacent territories (Smith *et al*., 1997). Hence, the complementary expression of ephrinB2 and its receptors in the second and third arches, respectively, is required to prevent mingling of second and third arch neural crest cells before they enter the arches. Since ephrinB2 is also expressed in second arch mesoderm, it is also required to target third arch neural crest cells correctly away from the second arch and into the third arch. *EphrinB2*-null mice also show defects in cranial neural crest cell migration, particularly of second arch neural crest cells, which scatter and do not invade the second arch (Adams *et al*., 2001).

Migrating *Xenopus* cranial neural crest cells also express EphA2; overexpression of a dominant negative (kinase-deficient) EphA2 receptor similarly leads to the failure of the third and fourth neural crest streams to separate, as neural crest cells from the third stream migrate posteriorly (Helbling *et al*., 1998).

Neural Crest Streams and Cranial Skeleto-Muscular Patterning

Cranial neural crest cells form not only many of the skeletal elements of the head, but also the connective component of the striatal muscles that are attached to them (see Table 1). When the long-term fate of neural crest cells arising from the midbrain and each rhombomere was mapped using quail-chick chimeras, it was found that each rhombomeric population forms the connective components of specific muscles, together with their respective attachment sites on the neurocranium and splanchnocranium (Köntges and Lumsden, 1996). Cranial muscle connective tissues arising from a given rhombomere attach to skeletal elements arising from the same initial neural crest population, explaining how evolutionary changes in craniofacial skeletal morphology can be accommodated by the attached muscles (Köntges and Lumsden, 1996). Similar results have also been obtained in frog embryos, where connective tissue components of individual muscles of either of the first two arches originate from the neural crest migratory stream associated with that arch (Olsson *et al*., 2001). Hence, the streaming of cranial neural crest cells into the different pharyngeal arches is important for patterning not only skeletal elements, but also their associated musculature.

Migration Pathways of Trunk Neural Crest Cells

The migration pathways of trunk neural crest cells have been most extensively studied in avian embryos (e.g., Weston, 1963; Rickmann *et al*., 1985; Bronner-Fraser, 1986; Teillet *et al*., 1987). As described in this section, neural crest cells only leave the neural tube opposite newly epithelial somites (Fig. 5A) (for reviews of somite formation and maturation, see Stockdale *et al*., 2000; Pourquié, 2001). Here, they enter a cell-free space that is rich in extracellular matrix. They only migrate into the somites at a level approximately 5–9 somites rostral to the last-formed somite, where the somites first become subdivided into different dorsoventral compartments, the sclerotome and dermomyotome (Fig. 5B) (Guillory and Bronner-Fraser, 1986). The sclerotome is formed when the ventral portion of the epithelial somite undergoes an epithelial–mesenchymal transition to form loose mesenchyme. This mesenchyme will eventually form the cartilage and bone of the ribs and axial skeleton. The dorsal somitic compartment, the dermomyotome, remains epithelial, and will eventually form dermis, skeletal muscle, and vascular derivatives.

There are two main neural crest cell migration pathways in the avian trunk (Fig. 5C): (1) a ventral pathway between the neural tube and somites, followed by neural crest cells that eventually give rise to dorsal root ganglia, Schwann cells, sympathetic ganglia, and (at somite levels 18–24 in birds) adrenal chromaffin cells, and (2) a dorsolateral pathway between the somite and the overlying ectoderm, followed by neural crest cells that eventually form melanocytes.

Ventral Migration Pathway

In the chick, neural crest cells that delaminate opposite epithelial somites initially migrate ventrally between the somites. Once the sclerotome forms, they migrate exclusively through the rostral half of each sclerotome, leading to a segmental pattern of migration (Rickmann *et al*., 1985; Bronner-Fraser, 1986). This pathway is almost identical to that followed by motor axons as they grow out from the neural tube, shortly after neural crest cells begin their migration (Rickmann *et al*., 1985). Mouse neural crest cells are similarly restricted to the rostral sclerotome (Serbedzija *et al*., 1990).

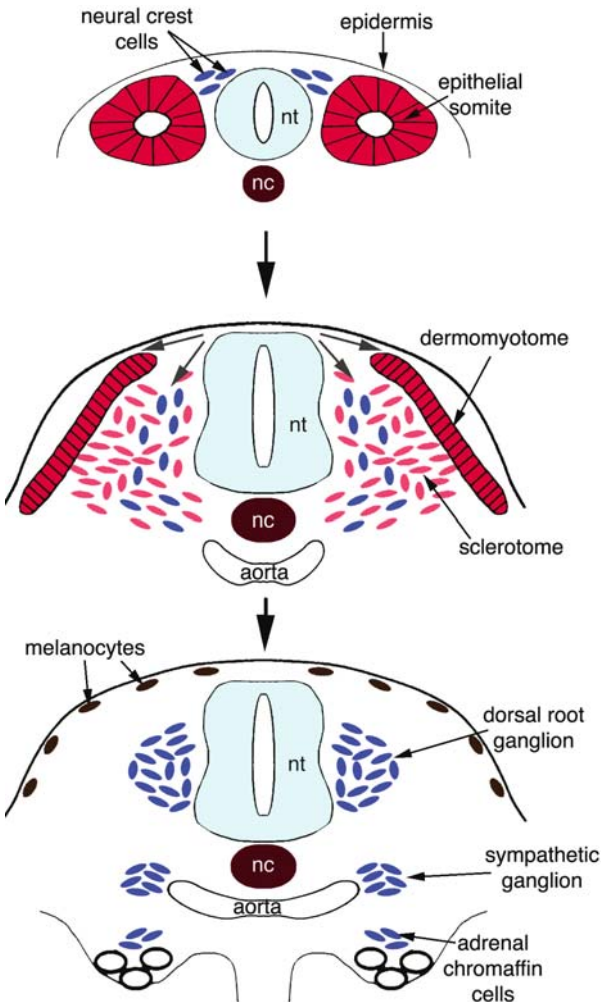

FIGURE 5. Schematic showing trunk neural crest cell migration pathways and derivatives (also see Fig. 1C). Neural crest cells migrate ventrally through the sclerotome to form neurons and satellite glia in the dorsal root ganglia and sympathetic ganglia, chromaffin cells in the adrenal gland (and Schwann cells on the ventral root; not shown). Neural crest cells also migrate dorsolaterally beneath the epidermis to form melanocytes. nc, notochord; nt, neural tube.

Neural crest cells that remain within the rostral sclerotome aggregate to form the dorsal root ganglia (primary sensory neurons and satellite glial cells), while those that move further ventrally form postganglionic sympathetic neurons (Fig. 8; section The Autonomic Nervous System: An Introduction) and adrenal chromaffin cells (Fig. 5C). The restriction of neural crest cells to the rostral half of each somite therefore leads to the segmental distribution of dorsal root ganglia; as will be seen in the section on Molecular Guidance Cues for Trunk Neural Crest Cell Migration, it results from the presence of repulsive migration cues in the caudal sclerotome.

Neural crest cells that delaminate opposite the caudal half of a somite migrate longitudinally along the neural tube in both directions. Once they reach the rostral half either of their own somite, or of the adjacent (immediately caudal) somite, they enter the sclerotome (Teillet *et al*., 1987). Hence, each dorsal root ganglion is derived from neural crest cells emigrating at the same somite level and from one somite anterior to that level. In contrast, each sympathetic ganglion is derived from neural crest cells originating from up to six somite-levels of the neuraxis: This is approximately equal to the numbers of spinal cord segments contributing to the preganglionic sympathetic neurons that innervate each ganglion (see Fig. 8) (Yip, 1986).

There are some differences in the ventral neural crest migration pathway between different vertebrates. In fish and amphibians, the somites are mostly myotome, with very little sclerotome. In these animals, the ventral migration pathway is essentially a medial migration pathway, between the somites and the neural tube/notochord. In *Xenopus*, neural crest cells following this pathway give rise to dorsal root ganglia, sympathetic ganglia, adrenomedullary cells, and also pigment cells (Krotoski *et al*., 1988; Collazo *et al*., 1993). This is also a segmental migration, but in this case, the neural crest cells migrate between the neural tube and the caudal half of each somite (Krotoski *et al*., 1988; Collazo *et al*., 1993). The ventral pathway is the main pathway followed by pigment cell precursors in *Xenopus*; only a few pigment cells follow the dorsolateral pathway beneath the ectoderm (Krotoski *et al*., 1988; Collazo *et al*., 1993). In zebrafish, neural crest cells enter the medial pathway at any rostrocaudal location; however, they subsequently converge toward the middle of the somite so that their ventral migration is restricted to the region halfway between adjacent somite boundaries (Raible *et al*., 1992). Rostral sclerotome precursors and motor axons also follow this pathway toward the center of the somite. However, rostral sclerotome cells are not required for this convergence of neural crest cells and motor axons, suggesting that unlike the situation in avian embryos (section Molecular Guidance Cues for Trunk Neural Crest Cell Migration), neural crest and motor axon guidance cues are not derived from the sclerotome (Morin-Kensicki and Eisen, 1997).

Dorsolateral Migration Pathway

Neural crest cells that migrate along the dorsolateral pathway, between the somites and the ectoderm, give rise to epidermal melanocytes in all vertebrates. In chick embryos, melanocytes only differentiate after they have invaded the ectoderm, while in amphibians, melanocytes often differentiate during migration (see, e.g., Keller and Spieth, 1984). In *Xenopus*, the subectodermal pathway is only a minor pathway for pigment cells, as most pigment cell precursors follow the ventral pathway (Krotoski *et al*., 1988; Collazo *et al*., 1993). However, in most amphibians, such as the axolotl, the dorsolateral pathway is a major pathway for pigment cell precursors (see, e.g., Keller and Spieth, 1984).

By injecting DiI into the lumen of the neural tube at progressively later stages, the fate of later-migrating neural crest cells can be specifically examined (Serbedzija *et al*., 1989, 1990). The earliest injection labels all neural crest cells, while subsequent injections label neural crest cells leaving the neural tube at progressively later times. These experiments showed that neural crest cell derivatives are "filled" in a ventral–dorsal order, since the label is progressively lost first from sympathetic ganglia, and then from dorsal root ganglia, in both mouse and chick embryos (Serbedzija *et al*., 1989, 1990). The last cells to leave the neural tube exclusively migrate along the dorsolateral pathway. (The same ventral–dorsal filling of derivatives is also seen in the head, where early-migrating mesencephalic neural crest cells form both dorsal and ventral derivatives, while late-migrating cells exclusively form dorsal derivatives; Baker *et al*., 1997.)

Entry onto the dorsolateral pathway is delayed relative to entry onto the ventral pathway in the chick and zebrafish. In the chick, trunk neural crest cells only begin migrating dorsolaterally 24 hr after migration has begun on the ventral pathway (Erickson *et al*., 1992; Kitamura *et al*., 1992). This is concomitant with the dissociation of the epithelial dermomyotome to form a mesenchymal dermis. (In the vagal region of chick embryos, however, neural crest cells immediately follow the dorsolateral pathway, via which they reach the pharyngeal arches; Tucker *et al*., 1986; Kuratani and Kirby, 1991; Reedy *et al*., 1998.) In the zebrafish, there is also a delay of several hours before neural crest cells follow the dorsolateral pathway (Raible *et al*., 1992; Jesuthasan, 1996). In contrast, neural crest cells follow both dorsolateral and ventral pathways simultaneously in the mouse (Serbedzija *et al*., 1990), while in the axolotl, the dorsolateral pathway is followed before the ventral pathway (Löfberg *et al*., 1980).

In the zebrafish, the lateral somite surface triggers collapse and retraction of neural crest cell protrusions but not Rohon-Beard growth cones, suggesting that the delay in entry onto the dorsolateral pathway is mediated by a repulsive cue on the dermomyotome that acts specifically on neural crest cells (Jesuthasan, 1996). In the chick trunk, inhibitory glycoconjugates, including peanut agglutinin-binding molecules and chondroitin-6-sulfate proteoglycans, are expressed on the dorsolateral pathway during the period of exclusion of neural crest cells; their expression decreases concomitant with neural crest cell entry (Oakley *et al*., 1994). Dermomyotome ablation abolishes expression of these molecules and accelerates neural crest cell entry onto the dorsolateral pathway (Oakley *et al*., 1994). Chondroitin-sulfate proteoglycans and the hyaluronan-binding proteoglycan aggrecan are also found in the perinotochordal space, which similarly excludes neural crest cells (see, e.g., Bronner-Fraser, 1986; Pettway *et al*., 1996; Perissinotto *et al*., 2000). It has also been suggested that, at least in the chick, only melanocyte precursors are able to enter the dorsolateral pathway (Erickson and Goins, 1995). However, this cannot be an absolute restriction, since multipotent neural crest cells (able to form not only melanocytes, but also sensory and autonomic neurons) have been isolated from the trunk epidermis of quail embryos (Richardson and Sieber-Blum, 1993).

Other Migration Pathways in the Trunk

In amphibians, neural crest cells also migrate dorsally to populate the dorsal fin (Löfberg *et al*., 1980; Krotoski *et al*., 1988; Collazo *et al*., 1993). In *Xenopus*, DiI-labeling showed the existence of two migration pathways toward the ventral fin (Collazo *et al*., 1993). One pathway leads along the neural tube and through the dorsal fin around the tip of the tail, while the other leads ventrally toward the anus and directly down the presumptive enteric region to the ventral fin (Collazo *et al*., 1993).

Molecular Guidance Cues for Trunk Neural Crest Cell Migration

Various extracellular matrix molecules that are permissive for neural crest migration are prominent along neural crest migration pathways, including fibronectin, laminin, and collagen types I, IV, and VI (reviewed in Perris, 1997; Perris and Perissinotto, 2000). Function-blocking antibodies and antisense oligonucleotide experiments targeted against the integrin receptors for these molecules perturb neural crest cell migration (reviewed in Perris and Perissinotto, 2000). PG-M/versicans (major hyaluronan-binding proteoglycans) are expressed by tissues lining neural crest cell migration pathways and may be conducive to neural crest cell migration (Perissinotto *et al*., 2000).

The most important guidance cues for neural crest cells seem to be repulsive. As discussed in the section on Dorsolateral Migration Pathway inhibitory extracellular matrix molecules such as chondroitin-sulfate proteoglycans and aggrecan are expressed in regions that do not permit neural crest cell entry, such as the perinotochordal space. Most molecular information is available about guidance cues that act to restrict neural crest cell migration to the rostral sclerotome in chick and mouse embryos (reviewed in Kalcheim, 2000; Krull, 2001). Microsurgical rotation of the neural tube or segmental plate mesoderm showed that the guidance cues responsible for the rostral restriction of neural crest cell migration, and also sensory and motor axon growth, reside in the mesoderm, not in the neural tube (Keynes and Stern, 1984; Bronner-Fraser and Stern, 1991). Similarly, when compound somites made up only of rostral somite-halves are surgically created, giant fused dorsal root ganglia form, while very small, irregular dorsal root ganglia form when only caudal halves

are used (Kalcheim and Teillet, 1989). This also demonstrates the importance of the mesoderm in segmenting trunk neural crest cell migration. The presence of alternating rostral–caudal somite halves is also important for the correct formation of the sympathetic ganglionic chains (Goldstein and Kalcheim, 1991).

Many different molecules that are localized to the caudal sclerotome have been proposed as candidate repulsive cues for neural crest cells (see Krull, 2001). It is probable that multiple cues are present and act redundantly. Peanut agglutinin-binding molecules seem to be important, since application of peanut agglutinin leads to chick neural crest cell migration through both rostral and caudal half-sclerotomes; however, their identity is unknown (Krull *et al*., 1995). F-spondin, an extracellular matrix molecule originally isolated in the floor-plate, is also involved: Overexpression of F-spondin in the chick inhibits neural crest cell migration into the somite, while anti-F-spondin antibody treatment enables neural crest cell migration into previously inhibitory domains, including the caudal sclerotome (Debby-Brafman *et al*., 1999). Semaphorin 3A (Sema3A; collapsin1), a secreted member of the semaphorin family of proteins that act as (primarily) repulsive guidance cues for axon growth cones (reviewed in Yu and Bargmann, 2001), is also expressed in the caudal sclerotome (Eickholt *et al*., 1999). Migrating neural crest cells express the Sema3A receptor, Neuropilin1, and selectively avoid Sema3A-coated substrates *in vitro* (Eickholt *et al*., 1999). Mice mutant for either *sema3A* or *neuropilin1* show normal neural crest migration through the caudal sclerotome (Kawasaki *et al*., 2002), but it is possible that other related molecules compensate for their loss.

Finally, as in the cranial neural crest (section Migration Pathways of Cranial Neural Crest Cells), ephrin–Eph interactions are also important (reviewed in Robinson *et al*., 1997; Krull, 2001). In the chick, trunk neural crest cells express the receptor EphB3, while its transmembrane ligand, ephrinB1, is localized to the caudal sclerotome (Krull *et al*., 1997). Neural crest cells enter both rostral and caudal sclerotomes in explants treated with soluble ephrinB1 (Krull *et al*., 1997). Similar ephrin–Eph interactions are also important in restricting rat neural crest cells to the rostral somite: Both ephrinB1 and ephrinB2 are expressed in the caudal somite, while neural crest cells express the receptor EphB2 and are repelled by both ligands (Wang and Anderson, 1997). Ephrin B ligands are also expressed in the dermomyotome in the chick: these seem to repel EphB-expressing neural crest cells from the dorsolateral pathway at early stages of migration, but promote entry onto the dorsolateral pathway at later stages, particularly of melanoblasts (Santiago and Erickson, 2002).

Importantly, ephrins do not simply block migration, but act as a directional cue. Eph^+ neural crest cells will migrate over a uniform $ephrin^+$ substrate, but when given a choice between $ephrin^+$ and ephrin-negative substrates, they preferentially migrate on the latter (Krull *et al*., 1997; Wang and Anderson, 1997).

Migration Arrest at Target Sites

Surprisingly little is known about the signals that control the arrest of neural crest cells at specific target sites.

FGF2 and FGF8 have been shown to promote chemotaxis of mesencephalic neural crest cells *in vitro*; both of these molecules are expressed in tissues in the pharyngeal arches, although an *in vivo* role has not been demonstrated (Kubota and Ito, 2000). Sonic hedgehog (Shh) in the ventral midline seems to act as a migration arrest signal for mesencephalic neural crest-derived trigeminal ganglion cells (Fedtsova *et al*., 2003). A local source of Shh blocks migration of these cells in chick embryos, while in Shh knockout mice, trigeminal precursors migrate toward the midline and condense to form a single fused ganglion (Fedtsova *et al*., 2003). Shh has also been shown to inhibit dispersal of avian trunk neural crest cells *in vitro* (Testaz *et al*., 2001), so it is possible that Shh may be a general migration arrest signal for neural crest cells.

Glial cell line-derived neurotrophic factor (GDNF), a ligand for the receptor tyrosine kinase Ret, has chemoattractive activity for Ret-expressing enteric neural crest cell precursors in the gut (Young *et al*., 2001). GDNF is expressed throughout the gut mesenchyme; it may promote neural crest cell migration through the gut and prevent neural crest cells leaving the gut to colonize other tissues, although this has not been proven (Young *et al*., 2001).

Sema3A, described in the last section as a potential repulsive guidance cue for neural crest cells migrating through the sclerotome (Eickholt *et al*., 1999), is required for the accumulation of sympathetic neuron precursors around the dorsal aorta (Kawasaki *et al*., 2002). In mice mutant either for *sema3A* or the gene encoding its receptor, *neuropilin1*, neural crest cells migrate normally through the caudal sclerotome, but sympathetic neuron precursors are widely dispersed, for example in the forelimb, where *sema3A* is normally expressed (Kawasaki *et al*., 2002). Sema3A also promotes the aggregation of sympathetic neurons in culture, suggesting a potential role for Sema3A in clustering sympathetic neuron precursors at the aorta (Kawasaki *et al*., 2002). Since *sema3A* is expressed in the somites (in the dermomyotome as well as in the caudal sclerotome) and in the forelimb, it is possible that secreted Sema3A forms a dorsoventral gradient, trapping sympathetic neuron precursors by the aorta, at the ventral point of the gradient (Kawasaki *et al*., 2002).

Summary of Neural Crest Migration

Neural crest cell migration pathways in the head and trunk are generally conserved across all vertebrates. Distinct streams of migrating cranial neural crest cells populate different pharyngeal arches. These streams are formed at least partly via the action of repulsive guidance cues from the mesoderm, including an unidentified ErbB4-regulated inhibitory cue in r3-level mesoderm in amniotes, and repulsive ephrin–Eph interactions between neural crest cells and pharyngeal arch mesoderm in amphibians. In the amniote trunk, the restriction of neural crest cell migration to the rostral sclerotome is mediated by multiple repulsive cues from the caudal sclerotome, including ephrins. This restriction is essential for the segmentation of the PNS in the trunk. Although relatively little is known about how migration arrest is controlled, a few potential molecular cues have been identified. These include Sema3A, which is required for the accumulation of sympathetic neuron precursors at the dorsal aorta.

NEURAL CREST LINEAGE DIVERSIFICATION

The astonishing diversity of neural crest cell derivatives has always been a source of fascination, and much effort has been devoted to understanding how neural crest lineage diversification is achieved (reviewed in Le Douarin and Kalcheim, 1999; Anderson, 2000; Sieber-Blum, 2000; Dorsky *et al*., 2000a; Sommer, 2001). The formation of different cell types in different locations within the embryo raises two distinct developmental questions (Anderson, 2000). First, how are different neural crest cell derivatives generated at distinct rostrocaudal axial levels? During normal development, for example, only cranial neural crest cells give rise to cartilage, bone, and teeth; only vagal and lumbosacral neural crest cells form enteric ganglia; and only a subset of trunk neural crest cells form adrenal chromaffin cells (see Table 1). Are these axial differences in neural crest cell fate determined by environmental differences or by intrinsic differences in the neural crest cells generated at different axial levels? Second, how are multiple different neural crest cell derivatives generated at the same axial level? For example, vagal neural crest cells form mesectodermal derivatives, melanocytes, endocrine cells, sensory neurons, and all three autonomic neuron subtypes (parasympathetic, sympathetic, and enteric). How is this lineage diversification achieved? These two questions will be examined in turn.

Axial Fate-Restriction Does Not Generally Reflect Restrictions in Potential

The restricted fate of different neural crest cell precursor populations along the neuraxis (see Table 1) has been extensively tested in avian embryos using the quail-chick chimera technique. Neural fold fragments from one axial level of quail donor embryos were grafted into different axial levels of chick host embryos (reviewed in Le Douarin and Kalcheim, 1999). These experiments revealed that, in general, neural crest cell precursors from all axial levels are plastic, as a population; that is, a premigratory population from one axial level can form the neural crest cell derivatives characteristic of any other axial level. For example, caudal diencephalic neural crest precursors, which do not normally form neurons or glia, will contribute appropriately to the parasympathetic ciliary ganglion and proximal cranial sensory ganglia after grafts to the mesencephalon or hindbrain (Noden, 1975, 1978b). Trunk neural crest precursors, which do not normally form enteric neurons, will colonize the gut and form enteric neurons, expressing appropriate neurotransmitters, when they are grafted into the vagal region (Le Douarin and Teillet, 1974; Le Douarin *et al*., 1975; Fontaine-Pérus *et al*., 1982; Rothman *et al*., 1986). Cranial and vagal neural crest cells, which do not normally form catecholaminergic derivatives, can form adrenergic cells both in sympathetic ganglia and the adrenal glands, when grafted to the "adrenomedullary level" (somites 18–24) of the trunk (Le Douarin and Teillet, 1974). These results suggest that axial differences in neural crest fate reflect axial differences in the environment, not intrinsic differences in the neural crest cells themselves, at least at the population level.

There are some exceptions to this general rule, however. For example, the most caudal neural crest cells in the chick embryo (those derived from the level of somites 47–53), only form melanocytes and Schwann cells during normal development (Catala *et al*., 2000). Furthermore, when tested both by *in vitro* culture and heterotopic grafting, they seem to lack the potential to form neurons (Catala *et al*., 2000).

Until very recently, it was accepted that trunk neural crest cells are intrinsically different from cranial neural crest cells in that they lack the potential to form cartilage. Trunk neural crest cells do not form cartilage when trunk neural folds are grafted in place of cranial neural folds in either amphibian or avian embryos (Raven, 1931, 1936; Chibon, 1967b; Nakamura and Ayer-Le Lièvre, 1982). One study suggested that trunk neural crest cells do not migrate into the pharyngeal arches after such grafts in the axolotl (Graveson *et al*., 1995) and hence are not exposed to cartilage-inducing signals from the pharyngeal endoderm. Even when trunk neural crest cells are cocultured *in vitro* with pharyngeal endoderm, however, under the same conditions that elicit cartilage from cranial neural crest cells, they do not form cartilage (Graveson and Armstrong, 1987; Graveson *et al*., 1995). Nonetheless, a study in the axolotl using DiI-labeled trunk neural folds found some aberrant migration by trunk neural crest cells in the head, and incorporation of a few trunk neural crest cells into cartilaginous skeletal elements (Epperlein *et al*., 2000).

Cervical and thoracic trunk neural crest cells isolated from avian embryos will eventually form both bone and cartilage when cultured for many days in a medium commonly used for growing these tissues (McGonnell and Graham, 2002; Abzhanov *et al.*, 2003). Interestingly, this late differentiation *in vitro* correlates temporally with a downregulation of *Hox* gene expression in a subset of trunk neural crest cells in long-term culture (Abzhanov *et al*., 2003). This alteration in *Hox* expression may enable trunk neural crest cells to respond to chondrogenic signals (section Cranial Neural Crest Cells Are Not Prepatterned). Furthermore, when implanted as loosely packed aggregates directly into the mandibular and maxillary primordia, trunk neural crest cells were found scattered in multiple cartilaginous elements, including Meckel's cartilage and the sclera of the eyes (McGonnell and Graham, 2002). Hence, it appears that trunk neural crest cells do have the potential to form cartilage, although this is only expressed under particular experimental conditions. Notably, the formation of cartilage *in vivo* is only observed when the cells are scattered among host neural crest cells, rather than when they are present as a coherent mass (McGonnell and Graham, 2002). It is possible that these scattered cells alter their *Hox* gene expression pattern to accord with the surrounding host neural crest cells, enabling them to respond to chondrogenic signals (section Cranial Neural Crest Cells Are Not Prepatterned).

When trunk neural crest cell precursors are substituted for the rostral vagal region of the neural tube (somite levels 1–3), they are unable to supply connective tissue to the heart to form the aorticopulmonary septum (Kirby, 1989). It is possible that, were they implanted as loose aggregates of cells in the heart region in the same manner as for the cartilage induction experiments (McGonnell and Graham, 2002), they would be able to

contribute to the aorticopulmonary septum; however, this remains to be tested.

Most current evidence, therefore, supports the idea that neural crest cells are largely plastic, at least at the population level. This plasticity was, until very recently, hard to reconcile with the classical "prepatterning" model of cranial neural crest cells, which is discussed briefly in the following section. The results that led to this model, though still valid, have been reinterpreted and the idea of prepatterning discarded.

Cranial Neural Crest Cells Are Not Prepatterned

Experiments carried out in the early 1980s led to the view that cranial neural crest cell precursors are extensively prepatterned before they delaminate from the neuroepithelium (Noden, 1983). When mesencephalic neural folds (prospective first arch neural crest) were grafted more caudally to replace hindbrain neural folds (prospective second arch neural crest) (see Fig. 3), a second set of jaw skeletal derivatives developed in place of the normal second (hyoid) arch derivatives (Noden, 1983). Moreover, anomalous first arch-type muscles were associated with the graft-derived first arch skeletal elements in the second arch (Noden, 1983). These experiments were interpreted as suggesting that patterning information for pharyngeal arch-specific skeletal and muscular elements is inherent in premigratory cranial neural crest cells (Noden, 1983).

This model has persisted until very recently. However, accumulating evidence suggests that although the results on which the model is based are valid, the original interpretation is incorrect. Given that this evidence pertains to skeletal patterning, rather than to the development of the PNS, there is insufficient space in this chapter to go into the evidence itself. The main thrust of the new results, however, is that cranial neural crest cells do not carry patterning information into the pharyngeal arches. Rather, they are able to respond to environmental cues from pharyngeal arch tissues, in particular pharyngeal endoderm (reviewed in Richman and Lee, 2003; Santagati and Rijli, 2003). After heterotopic grafts of mesencephalic neural folds to the hindbrain, *Hox* gene expression in the grafted neural crest cells is repatterned by signals from the isthmic organizer at the midbrain–hindbrain border (see Chapter 3), which is included in the graft (Trainor *et al*., 2002a). The changes in *Hox* expression affect the response of neural crest cells to different patterning signals from pharyngeal endoderm in the different arches, resulting eventually in the jaw element duplication (Couly *et al*., 2002).

The idea of a "prepattern" within the premigratory neural crest is now largely untenable, other than as a reflection of axial-specific *Hox* expression profiles that may alter the response of migratory neural crest cells to cranial environmental cues. How, then, can interspecies chimera experiments be explained, in which the size and shape of graft-derived skeletal elements are characteristic of the donor, not the host (e.g., Harrison, 1938; Wagner, 1949; Fontaine-Pérus *et al*., 1997; Schneider and Helms, 2003)? In a striking recent example, interspecies grafts of cranial neural crest between quail and duck embryos resulted in donor-specific beak shapes (Schneider and Helms, 2003). At first sight this may seem to indicate intrinsic patterning information within the grafted premigratory neural crest cells. However, it is clear that reciprocal signaling occurs between neural crest cells and surrounding tissues during craniofacial development. Environmental signals control the size and shape of neural crest-derived skeletal elements (e.g., Couly *et al*., 2002), while skeletogenic neural crest cells regulate gene expression in surrounding tissues (e.g., Schneider and Helms, 2003). Species-specific differences are likely to exist in the interpretation both of environmental signals by neural crest cells, and of neural crest-derived signals by surrounding tissues. This is presumably due to species-specific differences in the upstream regulatory elements of the relevant genes. This may explain why donor-specific skeletal elements are seen in such interspecific chimeras (and also why murine neural crest cells form teeth in response to chick oral epithelium; Mitsiadis *et al*., 2003). However, since our current knowledge of the molecular basis of morphogenesis is scanty, this hypothesis remains to be tested explicitly.

Summary

The general view gained from heterotopic grafting and culture experiments is that, given the right conditions, neural crest cell populations from every level of the neural axis are able to form the derivatives from every other. Hence, the normal restriction in fate that is observed along the neuraxis is not due to a restriction in potential, at least at the population level, but to differences in the environment encountered by the migrating neural crest cells. These experiments do not tell us, however, how the different neural crest lineages are formed at each axial level.

Lineage Segregation at the Same Axial Level

There are two main hypotheses to explain the lineage segregation of the neural crest at a given axial level: instruction and selection. The first (*instruction*) proposes that the emigrating neural crest is a homogeneous population of multipotent cells whose differentiation is instructively determined by signals from the environment. The second (*selection*) proposes that the emigrating neural crest is a heterogeneous population of determined cells (i.e., cells that will follow a particular fate regardless of the presence of other instructive environmental signals), whose differentiation occurs selectively in permissive environments, and which are eliminated from inappropriate environments.

Both of the above hypotheses are compatible with the heterotopic grafting experiments described in the preceding section. Although in their most extreme versions these hypotheses would appear to be mutually exclusive, there is evidence from *in vivo* and *in vitro* experiments to suggest that modified versions of both operate within the neural crest. Multipotent neural crest cells that adopt different fates in response to instructive environmental cues have been identified (reviewed in Anderson, 1997; Le Douarin and Kalcheim, 1999; Sommer, 2001). Conversely, fate-restricted subpopulations of neural crest cells have also been identified, either before or during early stages of migration,

suggesting that the early-migrating neural crest cell population is indeed heterogeneous (reviewed in Anderson, 2000; Dorsky *et al*., 2000a). Interestingly, there is evidence to suggest that at least some of the fate-restriction seen early in neural crest cell migration may result from interactions among neural crest cells themselves (e.g., Raible and Eisen, 1996; Henion and Weston, 1997; Ma *et al*., 1999). However, a restriction in *fate* does not necessarily imply a restriction in *potential*, since the cell under consideration may only have encountered one particular set of differentiation cues. Latent potential to adopt different fates can only be revealed by challenging the cell with different environmental conditions. When isolated in culture in the absence of other environmental signals, a cell that follows its normal fate is defined as *specified* to adopt that fate. However, it may not be *determined*, that is, it may not have lost the potential to adopt a different fate when exposed to different environmental signals. Without knowing all the factors that a cell might encounter *in vivo*, it is difficult to know when the potential of a cell has been comprehensively tested *in vitro*. Hence, the most rigorous assays for cell determination involve grafting cells to different ectopic sites *in vivo*.

Evidence for Both Multipotent and Fate-Restricted Neural Crest Cells: (1) *In Vivo* Labeling

The fate of individual trunk neural crest cell precursors and their progeny has been analyzed *in vivo* by labeling single cells in the neural folds in chick (Bronner-Fraser and Fraser, 1988, 1989; Frank and Sanes, 1991; Selleck and Bronner-Fraser, 1995), mouse (Serbedzija *et al*., 1994), and *Xenopus* (Collazo *et al*., 1993). Two main methods have been used for these clonal lineage analyses. Lysinated rhodamine dextran, a fluorescent, membrane-impermeant vital dye of high molecular weight, can be iontophoretically injected into single cells; it is passed exclusively to the progeny of the injected cell. This technique was used in all the above-cited studies except that of Frank and Sanes (1991). These authors used retroviral-mediated transfection to introduce the gene for β-*galactosidase* (*lacZ*) into the genome of single cells in the dorsal neural tube; the gene is activated on cell division and is transmitted to the progeny of the infected cell (Frank and Sanes, 1991). Similar results were obtained using both marking techniques. In the chick, mouse, and *Xenopus*, many clones contained multiple derivatives, including both neural tube and neural crest derivatives. This showed that neural tube and neural crest cells share a common precursor within the neural folds. Multiple neural crest derivatives were often observed within the same clone, including both neuronal and non-neuronal derivatives, such as glial cells, melanocytes, and in *Xenopus*, dorsal fin cells.

These experiments suggested that individual neural crest precursors are multipotent, but left open the possibility that fate-restricted precursors are generated before the cells leave the neural tube. However, when the lineage of individual neural crest cells migrating through the rostral somite was similarly examined, most labeled clones were found to contain multiple derivatives, including both neuronal and non-neuronal cells (Fraser and Bronner-Fraser, 1991). In extreme cases, clones included both neurons and glia (neurofilament-negative cells) in both sensory and sympathetic ganglia, and Schwann cells along the ventral root (Fraser and Bronner-Fraser, 1991). Hence, at least some individual neural crest cells, early in their migration, are multipotent in the chick. However, some clones were also found that were fate-restricted with respect to a particular neural crest derivative. For example, clones that formed both neurons and glia (neurofilament-negative cells) were found only in the dorsal root ganglia, or only in sympathetic ganglia, while one clone only formed Schwann cells on the ventral root (Fraser and Bronner-Fraser, 1991).

The lineage of individual trunk and hindbrain neural crest cells has also been examined in the zebrafish, which has many fewer neural crest cells than tetrapods (only 10–12 cells per trunk segment) (Raible *et al*., 1992). Trunk neural crest cells were labeled by intracellular injection of lysinated rhodamine dextran just after they segregated from the neural tube (Raible and Eisen, 1994). In contrast to the results in the chick (Fraser and Bronner-Fraser, 1991), most labeled clones in the zebrafish appeared to be fate-restricted; that is, all descendants of the labeled cell differentiated into the same neural crest derivative, for example, dorsal root ganglion neurons, or melanocytes, or Schwann cells (Raible and Eisen, 1994). Nonetheless, about 20% of clones produced multiple-phenotype clones, showing that at least some trunk neural crest cells are multipotent in the zebrafish (Raible and Eisen, 1994). Individual hindbrain neural crest cells in the most superficial 20% of the neural crest cell masses on either side of the neural keel were similarly labeled using fluorescent dextrans (Schilling and Kimmel, 1994). Strikingly, almost all clones were fate-restricted, giving rise to single identifiable cell types, such as trigeminal neurons, pigment cells, or cartilage; the remainder contained unidentified cell types (Schilling and Kimmel, 1994). Whether these results apply to the remaining, deeper 80% of neural crest cells in the cranial neural crest cell masses remains to be determined.

Similar analyses in the zebrafish trunk have also provided an excellent example of how fate-restriction in individual neural crest cells can be explained by regulative interactions between migrating neural crest cells, rather than by restrictions in potential (Raible and Eisen, 1996). Early-migrating neural crest cells along the medial pathway generate all types of trunk neural crest cell derivatives, including dorsal root ganglion neurons. Neural crest cells that migrate later along the same pathway form melanocytes and Schwann cells, but not dorsal root ganglion neurons (Raible *et al*., 1992). When the early-migrating population was ablated, late-migrating cells contributed to the dorsal root ganglion, even when they migrated at their normal time (Raible and Eisen, 1996). This suggests that the fate-restriction of late-migrating cells in normal development is due neither to a restriction in potential, nor to temporal changes in, for example, mesoderm-derived environmental cues, but to regulative interactions between early- and late-migrating neural crest cells that restrict the fate choice of the latter (Raible and Eisen, 1996).

Evidence for Both Multipotent and Fate-Restricted Neural Crest Cells: (2) *In Vitro* Cloning

A wealth of data exists on the fate choices of single neural crest cells and their progeny *in vitro* (reviewed in Le Douarin and Kalcheim, 1999). Migrating neural crest cell populations can be cultured in low-density conditions, followed sometimes by serial subcloning of the primary clones (e.g., Cohen and Königsberg, 1975; Sieber-Blum and Cohen, 1980; Stemple and Anderson, 1992). Alternatively, single neural crest cells can be picked at random from a suspension of migrating neural crest cells and plated individually (e.g., Baroffio *et al*., 1988; Dupin *et al*., 1990). These clonal culture techniques have shown that both fate-restricted and multipotent neural crest cells can be isolated from avian and mammalian embryos. Most clones of migrating quail cranial neural crest cells gave rise to progeny that differentiated into 2–4 different cell types, that is, were multipotent (Baroffio *et al*., 1991). Furthermore, single cells were found (at very low frequency, around 0.3%) that could give rise to neurons, glia, melanocytes, and cartilage, that is, all the major neural crest cell derivatives (Baroffio *et al*., 1991). These highly multipotent founder cells were interpreted as stem cells, although self-renewal of these cells remains to be demonstrated. Self-renewing, multipotent neural crest stem cells have been isolated from the migrating mammalian trunk neural crest, based on their expression of the low-affinity neurotrophin receptor, $p75^{NTR}$ (Stemple and Anderson, 1992). These cells are able to form autonomic neurons, Schwann cells and satellite glia, and smooth muscle cells, though they do not seem able to form sensory neurons (Shah *et al*., 1996; White *et al*., 2001).

As pointed out by Anderson (2000), it is difficult to be sure that the patterns and sequences of lineage restriction seen in these *in vitro* studies accurately reflect the composition of the migrating neural crest cell population *in vivo*. Although different founder cells might give rise to different subsets of neural crest cell derivatives *in vitro* (i.e., under the same culture conditions), this may not reflect intrinsic differences between the founder cells. It is possible that stochastic differences in their behavior, and/or the type and sequence of cell–cell interactions in each clone, might result in very different final outcomes, even if the initial founder cells were equivalent.

Single cell lineage analysis has also been performed on migrating neural crest cell explants *in vitro* (Henion and Weston, 1997). These authors injected lysinated rhodamine dextran intracellularly into random individual neural crest cells, migrating from trunk neural tubes placed in an enriched culture medium that supported the differentiation of melanocytes, neurons, and glia. Crucially, this method, unlike clonal culture, allows normal interactions between migrating neural crest cells to take place. The results showed that even during the first 6 hr of emigration, almost half of the labeled cells were fate-restricted, forming either neurons, glia, or melanocytes (Henion and Weston, 1997). Although the remaining clones formed more than one cell type, most formed neurons and glia, or glia and melanocytes, with only a few forming all three cell types (no cells formed only neurons and melanocytes) (Henion and Weston, 1997). Interestingly, neural crest cells sampled at later times (within a period corresponding to one or two cell divisions) contained no neuronal-glial clones: Almost all the sampled cells that produced neurons were fate-restricted neuronal precursors (Henion and Weston, 1997). Since the medium remained unchanged, and random differentiation would not be expected reproducibly to produce or remove distinct sublineages, the authors suggested that interactions between the neural crest cells themselves are responsible for the sequential specification of neuron-restricted precursors (Henion and Weston, 1997). Again, fate-restriction may not reflect restriction in potential, but it is clear that the early-migrating neural crest cell population is heterogeneous, containing both fate-restricted (as assessed both *in vivo* and *in vitro*) and multipotent precursors.

Other Evidence for Heterogeneity in the Migrating Neural Crest

Some of the earliest evidence for heterogeneity in the migrating neural crest was based on antigenic variation within the migrating population. For example, various monoclonal antibodies raised against dorsal root ganglion cells also recognize early subpopulations of neural crest cells (e.g., Ciment and Weston, 1982; Girdlestone and Weston, 1985). The SSEA-1 antigen is expressed by quail sensory neuroblasts in dorsal root ganglia and in subpopulations of migrating neural crest cells that differentiate into sensory neurons in culture (Sieber-Blum, 1989). A monoclonal antibody raised against chick ciliary ganglion cells, associated with high-affinity choline uptake, also recognizes a small subpopulation of mesencephalic neural crest cells (which normally give rise to the cholinergic neurons of the ciliary ganglion) (Barald, 1988a, b). The progressive restriction of expression of the 7B3 antigen (transitin, a nestin-like intermediate filament) during avian neural crest cell development may reflect glial fate-restriction (Henion *et al*., 2000). However, to show that expression of a particular antigen is related to the adoption of a particular fate, it must either be converted into a permanent lineage tracer, eliminated, or misexpressed ectopically, and this has not yet been achieved.

There is some evidence that late-migrating trunk neural crest cells in the chick may have reduced potential to form catecholaminergic neurons (see Fig. 9). Late-migrating chick trunk neural crest cells (i.e., those emigrating 24 hr after the emigration of the first neural crest cells at the same axial level) do not normally contribute to sympathetic ganglia (Serbedzija *et al*., 1989). When transplanted into an "early" environment, these late-migrating cells are able to form neurons in sympathetic ganglia, but fail to adopt a catecholaminergic fate (Artinger and Bronner-Fraser, 1992). These results may not reflect a loss of all autonomic potential, however, as cholinergic markers were not examined in these embryos.

Neural Crest Cell Precursors are Exposed to Differentiation Cues within the Neural Tube

The dorsal neural tube expresses various signaling molecules known to promote different neural crest cell fates, including Wnt1, Wnt3a, and BMP4 (section Control of Neural Crest Cell

Differentiation in the PNS) (reviewed in Dorsky *et al*., 2000a). Clearly, exposure of premigratory neural crest cell precursors to such factors could lead to at least some of the fate-restrictions and heterogeneity seen within the migrating neural crest cell population. For example, activation of the Wnt signaling pathway has been shown to be necessary and sufficient for melanocyte formation in both zebrafish and mouse (Dorsky *et al*., 1998; Dunn *et al*., 2000), via the direct activation of the *MITF*/*nacre* gene, which encodes a melanocyte-specific transcription factor (Dorsky *et al*., 2000b). Continuous exposure to the neural tube stimulates melanogenesis in cultured neural crest cells (Glimelius and Weston, 1981; Derby and Newgreen, 1982), while Wnt3a-conditioned medium dramatically increases the number of melanocytes in quail neural crest cell cultures (Jin *et al*., 2001). It is possible, therefore, that neural crest cell precursors exposed to Wnt3a in the dorsal neural tube for longer periods of time are more likely to generate progeny that will form into melanocytes, although this has not been directly tested. Wnts in the dorsal neural tube are not the only factors involved in melanocyte formation: For example, extracellular matrix from the subectodermal region specifically promotes neural crest cell differentiation into melanocytes (Perris *et al*., 1988). Nonetheless, these results demonstrate that factors within the neural tube may play important roles in at least some fate decisions.

In summary, therefore, neural crest precursors within the neural tube are exposed to a variety of neural crest cell differentiation cues present within the neural tube (and overlying ectoderm). Although such exposure has not directly been shown to result in the formation of fate-restricted progeny, it may be relevant to at least some of the heterogeneity seen within the migrating neural crest cell population. It is possible that, for example, the early segregation of a subpopulation of sensory-biased progenitors (section Sensory-Biased Neural Crest Cells Are Present in the Migrating Population) and the loss of catecholaminergic potential in late-migrating cells (see preceding section) ultimately result from the exposure of neural crest cell precursors to environmental cues within the neural tube.

Molecular Control of Lineage Segregation: A Paradigm from the Immune System

Relatively little is known in the neural crest field about the downstream effects of transcription factors associated with particular neural crest lineages. The best characterized examples of the molecular control of lineage segregation from multipotent precursors are found in the immune system, for example, the transcriptional control of B-cell development from hematopoietic stem cells (reviewed in Schebesta *et al*., 2002). Results from this field provide a paradigm for thinking about how lineage segregation might occur at the molecular level within the neural crest.

An emerging theme is that hematopoietic lineage segregation reflects not only the activation of lineage-specific genes, but also the suppression of alternative lineage-specific gene programs by negative regulatory networks of transcription factors (see Schebesta *et al*., 2002). For example, the basic helix-loop-helix transcription factors E2A and EBF coordinately activate the expression of B-cell-specific genes, but this is insufficient to determine adoption of a B-cell fate. For B-cell determination (commitment) to occur, the paired-domain homeodomain transcription factor Pax5 must also be present: This factor not only activates some genes in the B-cell program, but also represses lineage-inappropriate genes (Schebesta *et al*., 2002). Indeed, continuous Pax5 expression is required in B-cell progenitors in order to maintain commitment to the B-cell lineage (Mikkola *et al*., 2002).

Much less is known within the neural crest field about the downstream molecular effects of the expression of specific transcription factors. However, it is likely that similar networks of positive regulators activating transcription of lineage-appropriate genes, and negative regulators repressing transcription of lineage-inappropriate genes, are involved in neural crest cell lineage determination.

Segregation of Sensory and Autonomic Lineages

Postmigratory Trunk Neural Crest Cells Are Restricted to Forming Either Sensory or Autonomic Lineages

At postmigratory stages, distinct sensory-restricted and autonomic-restricted neural crest cells can be identified. When embryonic quail autonomic ganglia are "back-grafted" into early chick neural crest cell migration pathways, they are unable to contribute to dorsal root ganglion neurons and glia (reviewed by Le Douarin, 1986). Instead, they only form Schwann cells and autonomic derivatives (catecholaminergic sympathetic neurons, adrenal chromaffin cells, and sometimes enteric ganglia) (reviewed by Le Douarin, 1986). These results suggest that postmigratory neural crest cells in autonomic ganglia are restricted to an autonomic lineage. A similar autonomic restriction is seen in postmigratory neural crest cells in the gut, which normally form enteric ganglia. When these enteric neural precursor cells from rat embryos are grafted into chick neural crest migration pathways, they form neurons and satellite cells in sensory and sympathetic ganglia (White and Anderson, 1999). However, even in the sensory environment, the graft-derived neurons only express parasympathetic neuron markers, suggesting they are not able to form sensory neurons but are restricted to an autonomic lineage (White and Anderson, 1999).

Back-grafted dorsal root ganglia, in contrast, are additionally able to give rise to neurons and glia in the host dorsal root ganglia, provided that sensory neuroblasts are still mitotically active in the back-grafted ganglion (reviewed by Le Douarin, 1986). If sensory ganglia are back-grafted after all their sensory neuroblasts have withdrawn from the cell cycle, the postmitotic neurons die, and the non-neuronal cells within the ganglion differentiate into autonomic (sympathetic and enteric) but not sensory neurons (Ayer-Le Lièvre and Le Douarin, 1982; Schweizer *et al*., 1983). Multipotent postmigratory neural crest progenitors have also been isolated from dorsal root ganglia: These are able to form autonomic neurons, glia, and smooth muscle, but not, apparently, sensory neurons (Hagedorn *et al*., 1999, 2000a).

Hence, the potential to form dorsal root ganglion neurons and glia seems to be restricted, in postmigratory trunk neural crest cells, specifically to dividing sensory neuroblasts within sensory ganglia. Postmigratory neural crest cells in autonomic ganglia, and non-neuronal cells in sensory ganglia, are restricted to forming autonomic derivatives. These results point to a clear sensory vs autonomic lineage restriction within the postmigratory trunk neural crest, and also suggest that this decision occurs prior to any neuronal–glial lineage restriction.

A Model for Sensory–Autonomic Lineage Restriction

Based on the ganglion back-grafting experiments described above, Le Douarin put forward a model for the segregation of sensory and autonomic lineages within the neural crest (Le Douarin, 1986). The model proposed that (1) distinct sensory and autonomic neuronal progenitors are present in the migrating neural crest, as well as progenitors able to give rise to both lineages; (2) the sensory progenitors are only present until all sensory neurons have withdrawn from the cell cycle, while autonomic progenitors persist throughout development; (3) sensory progenitors only survive in sensory ganglia, while autonomic progenitors survive in all types of ganglia, suggesting different trophic requirements. Although the back-grafting data clearly support the existence of a sensory vs autonomic lineage restriction at postmigratory stages, the question of when this lineage restriction takes place has been much debated (see, e.g., Anderson, 2000).

The Le Douarin model proposes that some neural crest cells take the sensory–autonomic lineage decision early in their migration, while others retain the ability to form both lineages. The *in vivo* clonal analysis of migrating neural crest cells in the chick provides some support for this (Fraser and Bronner-Fraser, 1991). Some clones (which included both neurons and glia) were restricted either to dorsal root ganglia or sympathetic ganglia, while others gave rise to neurons and non-neuronal cells in both dorsal root and sympathetic ganglia (Fraser and Bronner-Fraser, 1991).

The ability to adopt a sensory fate may be rapidly lost, however. This is seen not only in postmigratory neural crest cells, as described above, but also in the migrating population. For example, self-renewing (re-plated) rat neural crest stem cells, which make up the bulk of the migrating neural crest cell population, seem to be unable to form sensory neurons, whether tested *in vitro* or *in vivo* (Shah *et al*., 1996; Morrison *et al*., 1999; White *et al*., 2001). Given that neural crest-derived sensory neurons are only found proximal to the neural tube, in dorsal root ganglia and proximal cranial sensory ganglia, such a rapid loss of sensory potential may make some sense, but the underlying mechanism remains obscure.

Sensory-Biased Neural Crest Cells Are Present in the Migrating Population

No evidence as yet supports the existence of determined autonomic progenitors within the migrating neural crest cell population. However, sensory-determined and sensory-biased progenitors are present in the migrating mammalian neural crest (Greenwood *et al*., 1999; Zirlinger *et al*., 2002). When rat trunk neural crest cells are cultured in a defined medium that permits sensory neuron formation, sensory neurons develop from dividing progenitors even in the presence of a strong autonomic neurogenesis cue, BMP2 (section BMPs Induce Both Mash1 and Phox2b in Sympathetic Precursors) (Greenwood *et al*., 1999). These results suggest that at least some dividing progenitors are already determined toward a sensory fate (Greenwood *et al*., 1999).

In another work, an inducible-Cre recombinase system in mice was used to mark permanently a subpopulation of neural crest cells that expresses Neurogenin2 (Ngn2), a basic helix-loop-helix transcription factor required for sensory neurogenesis (sections Proneural Genes: An Introduction; Neurogenins Are Essential for the Formation of Dorsal Root Ganglia) (Zirlinger *et al*., 2002). $Ngn2^+$ progenitors were four times as likely as the general neural crest cell population to contribute to dorsal root ganglia rather than sympathetic ganglia (Zirlinger *et al*., 2002). Within the dorsal root ganglia, the $Ngn2^+$ cells were found to contribute to all the main sensory neuron subtypes, and to satellite glia, without any apparent bias toward a particular lineage (Zirlinger *et al*., 2002). Since some $Ngn2^+$ precursors did contribute to sympathetic ganglia, these results suggest that while Ngn2 expression does not commit neural crest cells to a sensory fate, Ngn2 confers a strong bias toward a sensory fate. Ngn2 expression does not correlate with a bias toward any specific neuronal or glial subtype, however. These results therefore also support the idea that the restriction to sensory or autonomic lineages occurs before the decision to form neurons or glia.

Summary of Sensory/Autonomic Lineage Segregation

There is an autonomic vs sensory lineage restriction in postmigratory trunk neural crest cells in peripheral ganglia, and this seems to occur prior to the neuronal–glial decision. Some migrating neural crest cells may already be determined toward a sensory fate. Expression of the transcription factor Ngn2 in a subpopulation of migrating neural crest cells correlates with a strong bias, though not commitment, toward a sensory neural fate. Within dorsal root ganglia, $Ngn2^+$ cells are not restricted to a specific phenotype, but form multiple sensory neuronal subtypes and satellite glia. Although autonomic-restricted progenitors are found early in development (including, apparently, self-renewing neural crest stem cells), no autonomic-determined progenitors have yet been identified.

Sox10 Is Essential for Formation of the Glial Lineage

Neural crest cells give rise to all peripheral glia. These include satellite cells (glia that ensheathe neuronal cell bodies in peripheral ganglia) and Schwann cells (glia that ensheathe axonal processes of peripheral nerves). These can be distinguished

molecularly: Satellite cells express the Ets domain transcription factor Erm (a downstream target of FGF signaling; Raible and Brand, 2001; Roehl and Nüsslein-Volhard, 2001) and do not express either the POU transcription factor Oct6 or the zinc finger transcription factor Krox20 (see Hagedorn *et al*., 2000b; Jessen and Mirsky, 2002). Schwann cells are Erm-negative, $Oct6^+$, $Krox20^+$, and also express, for example, the surface glycoprotein Schwann cell myelin protein (see Hagedorn *et al*., 2000b; Jessen and Mirsky, 2002). The satellite cell phenotype is maintained by the ganglionic microenvironment; when removed from this environment, satellite cells can adopt a Schwann cell fate, although the reverse does not seem to occur (Dulac and Le Douarin, 1991; Cameron-Curry *et al*., 1993; Murphy *et al*., 1996; Hagedorn *et al*., 2000b). Hence, satellite cells and Schwann cells are closely related.

The HMG-domain transcription factor Sox10 is essential for the formation of all neural crest-derived glia (and melanocytes) (Britsch *et al*., 2001; Dutton *et al*., 2001). In *Sox10*-null mice, all satellite cells and all Schwann cells are missing, leading to eventual degeneration of sensory, autonomic (including all enteric), and motor neurons (Britsch *et al*., 2001). Haploinsufficiency of *Sox10* leads to neural crest defects that cause Waardenburg/Hirschsprung disease in humans (see McCallion and Chakravarti, 2001). Sox10 controls the expression of the *ErbB3* gene (Britsch *et al*., 2001), which encodes one of the high-affinity receptors for the growth factor NRG1, a member of the epidermal growth factor superfamily. (For reviews of NRGs and their receptors, see Adlkofer and Lai, 2000; Garratt *et al*., 2000.)

Sox10 is expressed in migrating neural crest cells (also see section Ap2α and SoxE Transcription Factors), but is downregulated in all lineages except for glial cells and melanocytes. Sox10 function is required for the survival of at least a subpopulation of multipotent neural crest cells, at least in part by regulating their responsiveness to NRG1 (Paratore *et al*., 2001) (also see Dutton *et al*., 2001). Constitutive expression of Sox10 in migrating neural crest stem cells maintains both glial and neuronal differentiation potential, although an additional function of Sox10 is to delay neuronal differentiation (Kim *et al*., 2003). Hence, one role of Sox10 is to maintain multipotency of neural crest stem cells (Kim *et al*., 2003); thus *Sox10* expression does not reflect determination toward the glial lineage.

Sox10 is essential for glial fate acquisition by neural crest stem cells in response to instructive gliogenic signals (Paratore *et al*., 2001). Such gliogenic cues include the type II isoform of NRG1 ("glial growth factor") and perhaps also NRG1 type III (sections Differentiation of DRG Satellite Cells; Neuregulin1 type III Is Essential for Schwann Cell Formation; Differentiation of Satellite Cells in Autonomic Ganglia; Shah *et al*., 1994; Shah and Anderson, 1997; Hagedorn *et al*., 1999, 2000b; Paratore *et al*., 2001; Leimeroth *et al*., 2002). Expression of the transmembrane receptor *Notch1* is also missing from sensory ganglia in *Sox10* mutant mice (Britsch *et al*., 2001): As will be seen in the section on Control of Neural Crest Cell Differentiation in the PNS, Notch activation is also a potent instructive cue for gliogenesis (Morrison *et al*., 2000b).

In summary, Sox10 is expressed in migrating neural crest cells and is maintained and required specifically in the glial lineage within the PNS. The early expression of Sox10 in migrating neural crest cells, as well as glial cells, may be consistent with the evidence (discussed in section Segregation of Sensory and Autonomic Lineages) suggesting that the sensory vs autonomic lineage decision occurs before the neuronal–glial decision.

Summary of Neural Crest Lineage Diversification

Two main hypotheses have been proposed to explain lineage segregation within the neural crest: (1) *instruction*, in which multipotent precursors are instructed by environmental cues to adopt particular fates, and (2) *selection*, in which determined cells, which are only able to adopt one fate, are selected in permissive environments. The available evidence suggests that the migrating population is heterogeneous, containing both highly multipotent cells and fate-restricted cells. However, there is little evidence to correlate fate-restriction with loss of potential to adopt other fates. Neural crest precursors are exposed to multiple environmental cues within the neural tube, and these may underlie at least some of the fate-restrictions seen within the migrating population. Ngn2 expression in a subset of migrating neural crest cells correlates with a strong bias (though not determination) toward a sensory fate. Apart from mitotic sensory neuroblasts in the DRG, postmigratory neural crest cells seem to be restricted to the autonomic lineage. The sensory–autonomic lineage decision seems to occur before the neuronal–glial decision. The transcription factor Sox10, expressed both in migrating neural crest cells and the glial lineage, is essential for, but does not determine, adoption of a glial fate.

CONTROL OF NEURAL CREST CELL DIFFERENTIATION IN THE PNS

A great deal of molecular information is now available concerning the signals and genetic machinery that underpin the differentiation of neural crest cells into specific cell types. Considerable progress has been made in understanding the molecular control of the differentiation of various non-neural and neural crest cell derivatives, for example, melanocytes (reviewed in Le Douarin and Kalcheim, 1999; Rawls *et al*., 2001), smooth muscle (see, e.g., Sommer, 2001), and even cartilage (Sarkar *et al*., 2001) (Fig. 6). However, any detailed discussion of the differentiation of these non-neural derivatives is beyond the scope of this chapter, which will concentrate on differentiation in the PNS. Numerous reviews provide additional information on this topic (e.g., Anderson, 1999; Le Douarin and Kalcheim, 1999; Anderson, 2000; Sieber-Blum, 2000; Morrison, 2001; Sommer, 2001). Chapter 5 should also be consulted for more general information on neuronal differentiation.

Within the PNS, it has become clear that vertebrate homologues of the invertebrate basic helix-loop-helix (bHLH) proneural transcription factors play essential roles in the differentiation of different neural crest cell types. Proneural genes are discussed in more detail in Chapter 5, but a brief introduction is given here for the purposes of this chapter.

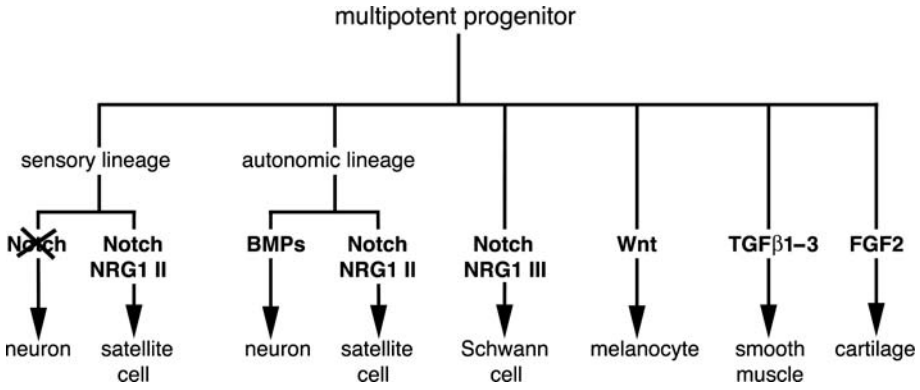

FIGURE 6. Schematic showing known signaling pathways involved in the differentiation of different cell types from multipotent neural crest cells. See the section on Contol of Neural Crest Cell Differentiation in the PNS for details. Modified from Dorsky *et al*. (2000a).

Proneural Genes: An Introduction

In both *Drosophila* and vertebrates, proneural bHLH transcription factors confer neuronal potential and/or specify neural progenitor cell identity (see Chapter 5) (reviewed in Bertrand *et al*., 2002). They act in part by activating the expression of ligands of the Notch receptor, such as Delta. Cells with high levels of Notch activity downregulate Notch ligand expression and adopt a "secondary" (e.g., supporting) cell fate, while cells with low levels of Notch activity adopt a primary (e.g., neuronal) cell fate (see Chapter 5; Gaiano and Fishell, 2002). Two classes of proneural genes are active in the PNS of *Drosophila*: the *achaete-scute* complex and *atonal* (reviewed in Skaer *et al*., 2002). Vertebrate homologues of the *achaete-scute* complex include *ash1* (*Mash1* in mice, *Cash1* in chick, etc.) and additional species-specific genes (e.g., *Mash2* in mice, *Cash4* in chick). The vertebrate *atonal* class contains many more genes, divided into various families based on the presence of specific residues in the bHLH domain (reviewed in Bertrand *et al*., 2002). The *neurogenin*s (*ngn*s), which were briefly introduced in the section on Segregation of Sensory and Autonomic Lineages, make up one of these *atonal*-related gene families. In neural crest cells, the *atonal*-related *neurogenin* family is particularly important for the sensory lineage (section Neurogenins Are Essential for the Formation of Dorsal Root Ganglia), while the *achaete-scute* homologue *ash1* (*Mash1*) is important for aspects of autonomic neurogenesis (section *Mash1* Is Essential for Noradrenergic Differentiation).

Dorsal Root Gangliogenesis

Trunk neural crest cells that remain within the somite, in the vicinity of the neural tube, aggregate and eventually differentiate to form the sensory neurons and satellite glia of the dorsal root ganglia. Similar differentiation processes presumably occur within proximal neural crest-derived cranial sensory ganglia, but most information is available for dorsal root ganglia.

Neurogenins Are Essential for the Formation of Dorsal Root Ganglia

As described in the section Sensory-Biased Neural Crest Cells Are Present in the Migrating Population, Ngn2 expression biases (but does not determine) neural crest cells toward the sensory lineage, including both neurons and satellite glia (Zirlinger *et al*., 2002). Ngn2 and a related factor, Ngn1, are expressed in complementary patterns in peripheral sensory neurons derived from neural crest and placodes (reviewed in Anderson, 1999) (sections Sense Organ Placodes; Trigeminal and Epibranchial Placodes). Knockout experiments in mice have shown that the Ngns are essential for the formation of sensory ganglia (Fode *et al*., 1998; Ma *et al*., 1998, 1999).

In the mouse, Ngn2 is expressed in cells in the dorsal neural tube, and in a subpopulation of migrating mammalian trunk neural crest cells, continuing into the early stages of dorsal root ganglion (DRG) condensation (Ma *et al*., 1999). In contrast, Ngn1 is first expressed only after DRG condensation has begun (Ma *et al*., 1999). In the chick, both Ngns are expressed in the dorsal neural tube, and in a subset of migrating neural crest cells (Perez *et al*., 1999). Chick Ngn2 is transiently expressed during chick dorsal root gangliogenesis, while Ngn1 is maintained until late stages in non-neuronal cells and/or neuronal precursors at the DRG periphery (Perez *et al*., 1999).

Normal Ngn2 expression in the mouse correlates with a strong bias toward the sensory lineage, but not toward any particular neuronal or glial phenotype within the sensory lineage (Zirlinger *et al*., 2002) (section Sensory-Biased Neural Crest Cells Are Present in the Migrating Population).

In contrast, Ngn1 overexpression studies suggest that Ngn1 may act to promote a specifically sensory neuronal phenotype. Retroviral-mediated overexpression of mouse Ngn1 in premigratory neural crest precursors in the chick leads to a significant bias toward population of the DRG, and to ectopic sensory neuron formation in neural crest derivatives, and even in the somite (Perez *et al*., 1999). Similar overexpression of Ngn1 in dissociated rat neural tube cultures, which are competent to

form sensory and autonomic peripheral neurons, also leads to increased sensory neurogenesis (Lo *et al*., 2002). However, permanent genetic labeling experiments, like those performed for Ngn2 (Zirlinger *et al*., 2002), are needed to show whether this correlation holds true during normal development.

Differentiation of DRG Neurons Depends on Inhibition of Notch Signaling

There is accumulating evidence that the decision to follow a sensory vs autonomic lineage occurs before the neuronal–glial decision (section Segregation of Sensory and Autonomic Lineages). Hence, sensory precursors within the DRG give rise to both sensory neurons and satellite glia. How are both neurons and satellite glia produced from the same precursors within the same ganglionic environment? It is now clear that neuronal and glial differentiation within the DRG depend on inhibition and activation, respectively, of signaling by the transmembrane receptor Notch (see Chapter 5; Fig. 7) (Wakamatsu *et al*., 2000; Zilian *et al*., 2001).

Notch1 is expressed by most migrating chick trunk neural crest cells and is downregulated on differentiation of both neurons and glia. In the DRG, Notch1 is initially preferentially expressed by cycling cells in the periphery, while one of its ligands, Delta1, is expressed by differentiating neurons located in the core of the ganglion (Wakamatsu *et al*., 2000) (Fig. 7). If Notch signaling is activated in cultured quail trunk neural crest cells (by overexpression of the Notch1 cytoplasmic domain), neuronal differentiation is inhibited and cell proliferation is transiently increased, suggesting that in order for neurons to form, Notch activity must be inhibited (Wakamatsu *et al*., 2000).

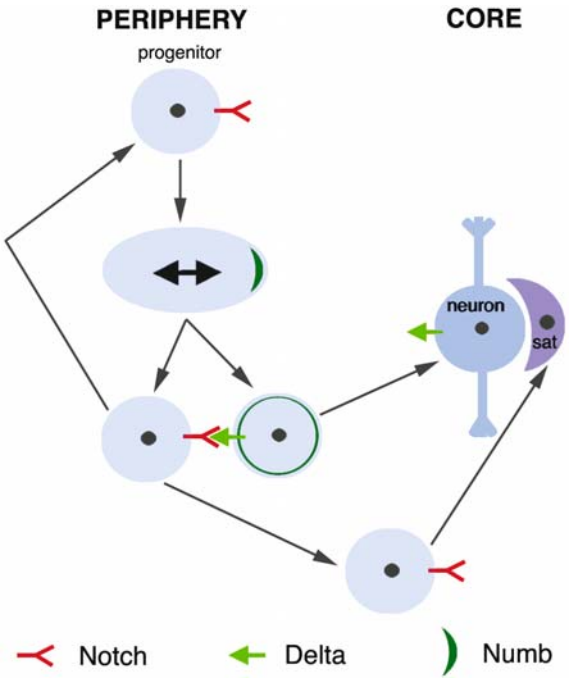

FIGURE 7. Schematic showing a model for neurogenesis within the dorsal root ganglion. The Notch inhibitor Numb is inherited asymmetrically by daughters of proliferating progenitors in the periphery of the ganglion. Cells with high levels of Numb have low levels of Notch activity: They upregulate the Notch ligand Delta, move to the core of the ganglion, and differentiate as neurons. Cells with low levels of Numb have high levels of Notch activity: They either divide again or differentiate into satellite cells (sat). Modified from Wakamatsu *et al*. (2000).

The Notch antagonist, Numb (see Chapter 5), is expressed asymmetrically in about 40% of the cycling cells at the periphery of the chick DRG (Wakamatsu *et al*., 2000). It is not known how this asymmetrical expression is established, but, after these cells divide, Numb will be inherited in high concentrations by only one of the daughter cells. In the Numb-inheriting daughter cell, high levels of Numb will inhibit Notch signaling; Delta1 will be upregulated, and the cell will differentiate as a neuron. The daughter cell that does not inherit Numb will have high levels of Notch signaling, probably activated by Notch ligands (e.g., Delta1) expressed on differentiating neurons in the core. This daughter cell will therefore be able to divide again, and/or form a satellite cell (see the following section) (Fig. 7). In agreement with this model, knockout experiments in mice have shown that Numb is essential for the formation of DRG sensory neurons (but not for, e.g., sympathetic neurons, although it is expressed in sympathetic ganglia) (Zilian *et al*., 2001).

As will be seen later, autonomic neuronal differentiation is promoted by instructive growth factors. Similar instructive sensory neuronal differentiation cues that act on multipotent progenitors have not been identified, although neural tube-derived neurotrophins, such as brain-derived neurotrophic factor (BDNF), are required for the survival and proliferation of DRG progenitors (reviewed in Kalcheim, 1996). Since the trigger for neuronal differentiation in the DRG seems to be the asymmetric expression of Numb in some of the cycling cells at the DRG periphery, understanding how this asymmetry is set up will shed light on how DRG neuronal differentiation is controlled.

Differentiation of DRG Satellite Cells Depends on Notch Activation and Instructive Gliogenic Cues

The above results give some insight into how neurogenesis occurs within the DRG. How, though, do satellite cells form in the same environment? Neuronal differentiation always occurs before glial differentiation in the DRG (Carr and Simpson, 1978), and it is likely that signals from the differentiating neurons instruct non-neuronal cells within the ganglion to form satellite cells. A model for how glial differentiation is controlled is emerging from studies of cultured neural crest stem cells and multipotent progenitors from cultured DRGs in the rat embryo (Hagedorn *et al*., 1999, 2000b; Morrison *et al*., 2000a; Leimeroth *et al*., 2002). This model proposes a combinatorial action of Notch-mediated neurogenic repression and gliogenic instruction, triggered by Notch ligands on differentiating neurons, together with additional gliogenic growth factors expressed or secreted by differentiating neurons.

Notch activation, as well as inhibiting neurogenesis (Wakamatsu *et al*., 2000), also instructively promotes a glial fate in cultured rat neural crest stem cells (Morrison *et al*., 2000b;

Kubu *et al*., 2002). This is discussed more fully in the section on Notch Activation Leads to Gliogenesis by Neural Crest Stem Cells. Although these rat neural crest stem cells seem to lack sensory potential (Shah *et al*., 1996; Morrison *et al*., 1999; White *et al*., 2001), it is likely that Notch activation is also involved in DRG satellite glial differentiation, probably in association with other instructive cues. Notch activation is presumably triggered by the Notch ligands, such as Delta1, expressed on differentiating neurons in the DRG core (Wakamatsu *et al*., 2000). *Delta1*-null mice have reduced numbers of satellite glia and Schwann cells, providing some corroborating evidence for this (De Bellard *et al*., 2002).

An independent instructive cue for satellite gliogenesis was also initially identified in studies of cultured rat neural crest stem cells (Shah *et al*., 1994). These authors showed that the type II isoform ("glial growth factor") of the growth factor Neuregulin1 (NRG1) both inhibits neuronal differentiation and instructively promotes a glial fate in rat neural crest stem cells (Shah *et al*., 1994; Shah and Anderson, 1997). Several NRG1 isoforms are expressed in DRG neurons (Meyer *et al*., 1997; Wakamatsu *et al*., 2000). NRG1 type II specifically induces the formation of satellite cells (as opposed to Schwann cells) in migrating neural crest stem cells and in DRG-derived progenitor cells *in vitro* (Hagedorn *et al*., 2000b; Leimeroth *et al*., 2002). However, knockout experiments in mice have failed to reveal a role either for NRG1 isoforms, or for one of their high-affinity receptors, ErbB3, in the DRG (Meyer *et al*., 1997). Additional gliogenic signals, therefore, may also operate in the DRG.

Summary of Dorsal Root Gangliogenesis

Ngns are essential for the formation of sensory ganglia, including dorsal root ganglia. Mouse Ngn2 biases neural crest cells toward the sensory lineage, while Ngn1 may be involved in sensory neurogenesis within the DRG. Differentiation of DRG neurons requires inhibition of Notch signaling, mediated in part by asymmetric inheritance of Numb. Differentiation of satellite cells involves two instructive gliogenic cues: Notch activation, and gliogenic growth factors. Differentiating neurons in the core of the DRG express Notch ligands, which activate Notch signaling in cycling non-neuronal cells at the periphery of the DRG. Notch activation instructively promotes a glial cell fate. NRG1 type II, produced by differentiating DRG neurons, also instructively promotes a satellite cell fate.

Schwann Cell Differentiation

The differentiation of Schwann cells has been intensively studied (for reviews, see Le Douarin and Kalcheim, 1999; Jessen and Mirsky, 2002). As for satellite cells, Schwann cell differentiation may involve the combination of two independent pathways: Notch activation, and instructive gliogenic cues from neurons.

Notch Activation Leads to Gliogenesis by Neural Crest Stem Cells

Even transient activation of Notch signaling (using a soluble clustered form of its ligand, Delta) inhibits neuronal differentiation and instructively promotes glial differentiation, in cultures of postmigratory neural crest stem cells isolated from fetal rat sciatic nerve (Morrison *et al*., 2000b; Kubu *et al*., 2002). While Notch activation also instructively promotes the glial differentiation of migrating neural crest stem cells, it is less efficient at inhibiting neuronal differentiation than in postmigratory cells, suggesting that glial promotion and neuronal inhibition are independent effects (Kubu *et al*., 2002).

Neuregulin1 Type III Is Essential for Schwann Cell Formation

Knockout experiments in mice have shown that NRG1 type III, the major NRG1 isoform produced by sensory neurons and motor neurons, is essential for Schwann cell formation (Meyer *et al*., 1997) (reviewed in Garratt *et al*., 2000; Jessen and Mirsky, 2002). Migrating neural crest cells express ErbB3, a high-affinity NRG1 receptor that is downregulated in most lineages but maintained in glial lineages. As described in the section on Sox10 Is Essential for Formation of the Glial Lineage, *ErbB3* gene expression is at least partly controlled by Sox10, which is essential for the formation of all peripheral glia, including Schwann cells (Britsch *et al*., 2001). Schwann cell precursors lining peripheral axons are missing in mice lacking NRG1 type III (see Meyer *et al*., 1997). It was originally unclear whether this effect of NRG1 type III was solely due to its support of the survival and/or proliferation of Schwann cell precursors (reviewed in Garratt *et al*., 2000; Jessen and Mirsky, 2002). However, membrane-bound NRG1 type III has now been shown to act as an instructive Schwann cell differentiation cue (Leimeroth *et al*., 2002). Cultured rat neural crest stem cells and multipotent progenitors isolated from DRGs are specifically induced to form Schwann cells (as opposed to satellite cells) by membrane-bound NRG1 type III (Leimeroth *et al*., 2002). Soluble NRG1 type III is unable to promote Schwann cell differentiation (Leimeroth *et al*., 2002). Hence, locally presented NRG1 type III (e.g., on axons) may regulate Schwann cell differentiation. Signaling by membrane-bound NRG1 type III seems to be dominant over NRG1 type II, which induces satellite cell differentiation (see section Differentiation of DRG Satellite Cells) (Leimeroth *et al*., 2002). This may underlie the apparent inability of Schwann cells to adopt a satellite cell fate (Hagedorn *et al*., 2000b).

Differences in the Sensitivity of Different Neural Crest Stem Cells to Gliogenic Cues

In the rat, postmigratory neural crest stem cells from fetal sciatic nerves do not differentiate into neurons as readily as migrating neural crest stem cells, as shown by transplantations to chick neural crest cell migratory pathways (White and Anderson, 1999; White *et al*., 2001). These fetal nerve neural crest stem cells express significantly higher levels of Notch1, and lower levels of the Notch antagonist Numb, than migrating neural crest stem cells (Kubu *et al*., 2002). Postmigratory cells on the sciatic nerve are therefore more sensitive to Notch activation than migrating cells and hence more likely to differentiate into glia (Kubu *et al*., 2002). The changes in Notch1 and Numb expression levels, and

the sensitivity to Notch activation, require neural crest cell–cell interactions. These are probably mediated, at least in part, by Delta (or other Notch ligand) expression on differentiating neurons and peripheral nerves (Bixby *et al*., 2002; Kubu *et al*., 2002).

Similar intrinsic differences in the sensitivity of different neural crest stem cell populations to gliogenic signals have been observed in neural crest stem cells isolated from the rat gut (Bixby *et al*., 2002; Kruger *et al*., 2002). Fetal gut neural crest stem cells are highly resistant to gliogenic signals and form neurons, rather than glia, on chick peripheral nerves (probably in response to local BMPs; see the section BMPs Induce Both Mash1 and Phox2b in Sympathetic Precursors) (Bixby *et al*., 2002). Conversely, postnatal gut neural crest stem cells are much more sensitive to gliogenic factors (including both NRG1 and Delta) than to neurogenic factors like BMPs and form glia on chick peripheral nerves (Kruger *et al*., 2002). It remains to be seen whether differences in the expression levels of Notch and Numb also underlie these differences in sensitivity to gliogenic and neurogenic cues.

Summary of Schwann Cell Differentiation

Schwann cell differentiation, like satellite cell differentiation, involves two instructive gliogenic cues: activation of Notch signaling, and gliogenic growth factors. Notch activation, by Notch ligands present on differentiating neurons and axons, instructively promotes gliogenesis. Membrane-bound NRG1 type III, which is probably present on axons, instructively promotes Schwann cell differentiation. Different neural crest stem cell populations, isolated from different locations and developmental stages, show instrinsic differences in their sensitivity to gliogenic signals. These may be related to differences in the levels of expression of Notch and Numb, probably triggered by local neural crest cell–cell interactions involving Notch ligands. Such differences may help promote appropriate glial (or neuronal) fate decisions by multipotent neural crest progenitors.

Autonomic Gangliogenesis

The peripheral autonomic nervous system is by far the most complex division of the PNS. In order to aid the discussion of the control of differentiation of various autonomic cell types, the subdivisions of the autonomic nervous system are introduced below.

The Autonomic Nervous System: An Introduction

The autonomic nervous system has three major divisions: sympathetic, parasympathetic, and enteric. The sympathetic and parasympathetic subdivisions innervate smooth muscle, cardiac muscle, and glands (Fig. 8), and mediate various visceral reflexes. The enteric nervous system controls the motility and secretory function of the gut, pancreas, and gall bladder.

All peripheral autonomic neurons and glia are derived from the neural crest. These include the postganglionic motor neurons and satellite glia of the sympathetic and parasympathetic divisions, which are collected together in peripheral ganglia

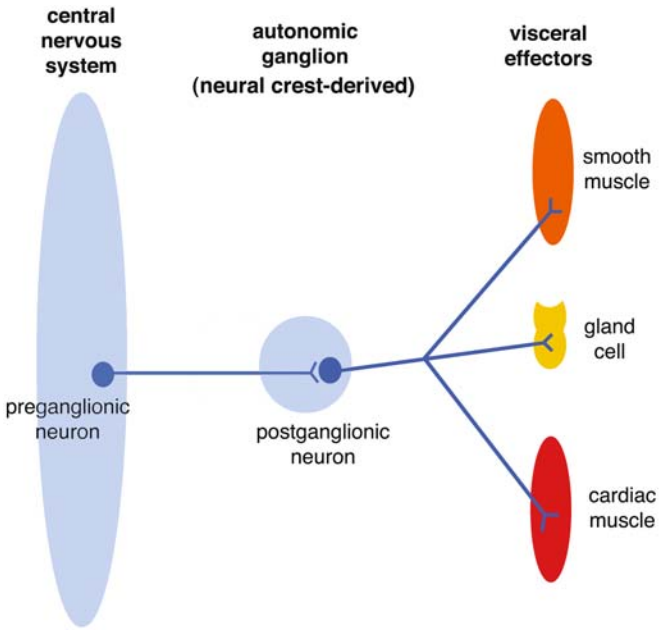

FIGURE 8. Schematic showing the structure of the autonomic nervous system. All peripheral autonomic neurons (sympathetic, parasympathetic, and enteric) are derived from the neural crest. Modified from Iversen *et al*. (2000).

(Fig. 8). The neurons in these ganglia are activated by preganglionic efferent neurons located in the brainstem and spinal cord (Fig. 8). Sympathetic ganglia are found in chains on either side of the spinal cord and hence are some considerable distance from their targets, while parasympathetic ganglia lie close to or are embedded in their target tissues. Enteric ganglia are located within the gut itself; they function relatively autonomously with respect to central nervous system input.

Preganglionic sympathetic neurons extend from the first thoracic spinal segment to upper lumbar segments; they innervate the bilateral chains of sympathetic ganglia. The postganglionic sympathetic neurons in these ganglia are derived from trunk neural crest cells that settle near the dorsal aorta to form the primary sympathetic chains. They innervate the glands and visceral organs, including the heart, lungs, gut, kidneys, bladder, and genitalia. Most of these neurons are noradrenergic, that is, release noradrenaline, a catecholamine derived from tyrosine via dopamine (Fig. 9). Some mature postganglionic sympathetic neurons, however, are cholinergic, that is, release acetylcholine. The endocrine (chromaffin) cells of the adrenal medulla, which are derived from a specific level of the trunk neural crest (somite levels 18–24 in the chick), are developmentally and functionally related to postganglionic sympathetic neurons (reviewed in Anderson, 1993). Adrenal chromaffin cells are adrenergic: They release adrenaline, another catecholamine, in turn derived from noradrenaline (Fig. 9).

Preganglionic parasympathetic neurons are found in various brain stem nuclei and in the sacral spinal cord. The brain stem nuclei innervate postganglionic neurons in cranial

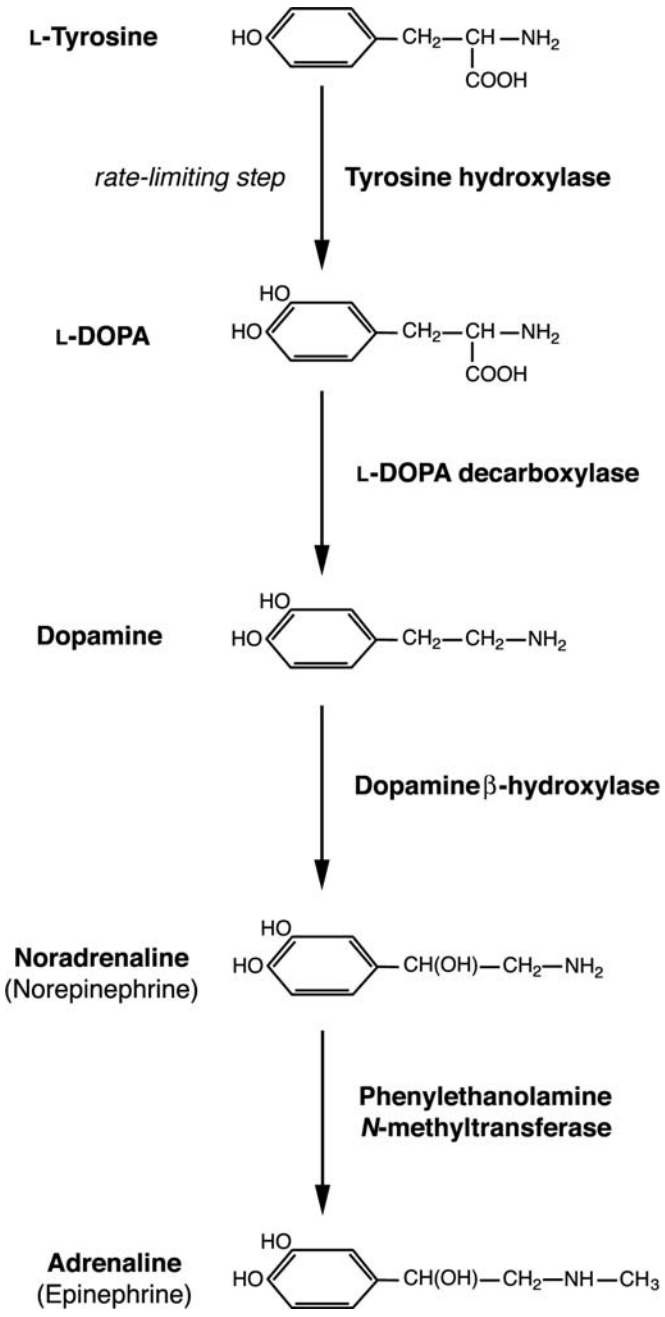

FIGURE 9. Catecholamine biosynthesis pathway: Intermediate stages in the formation of adrenaline. Redrawn from Blaschko (1973).

parasympathetic ganglia, including the ciliary, otic, sphenopalatine, and submandibular ganglia. These postganglionic neurons are derived from the cranial neural crest (Table 1), and innervate the eye, and lacrimal and salivary glands. Preganglionic parasympathetic axons exiting in the vagal nerve (cranial nerve X) innervate postganglionic neurons in cardiac ganglia and are embedded in the visceral organs of the thorax and abdomen. These postganglionic neurons are derived from vagal neural crest cells (Table 1). Preganglionic parasympathetic neurons in the sacral spinal cord innervate the pelvic ganglion plexus, which is derived from sacral neural crest cells (Table 1). The neurons in this plexus innervate the colon, bladder, and external genitalia. Most of these postganglionic parasympathetic neurons are cholinergic, that is, release acetylcholine.

The enteric nervous system, which is entirely derived from vagal and sacral levels of the neural crest (Table 1), contains local sensory neurons (responding to specific chemicals, stretch, and tonicity), interneurons, and motor neurons, together with their associated glia. Enteric neurons innervate smooth muscle, local blood vessels, and mucosal secretory cells. They use a variety of neurotransmitters: Catecholaminergic, cholinergic, and serotonergic neurons can all be identified within the enteric nervous system.

Phox2b is Essential for the Formation of all Autonomic Ganglia

The paired-like homeodomain transcription factor Phox2b is expressed in all autonomic neural crest cell precursors (reviewed in Brunet and Pattyn, 2002; Goridis and Rohrer, 2002). *Phox2b* expression begins in prospective sympathetic neural crest cells as they aggregate at the aorta, and in enteric neural crest cells as they invade the gut (Pattyn *et al*., 1997, 1999). In *Phox2b*-null mice, all these autonomic precursor cells die by apoptosis, so mutant animals lack all autonomic neurons and glia, that is, all sympathetic, parasympathetic, and enteric ganglia (Pattyn *et al*., 1999).

Intriguingly, Phox2b is also expressed in and required for the development of visceral sensory neurons derived from the epibranchial placodes (Pattyn *et al*., 1997, 1999) (Fig. 11; section Neurogenesis in the Epibranchial Placodes). These neurons provide autonomic afferent innervation to the visceral organs. Hence, Phox2b seems to be a pan-autonomic marker, despite the enormous variety of peripheral autonomic neural phenotypes. These include not only postganglionic neurons and satellite glia, but also autonomic sensory neurons, for example, enteric sensory neurons, and epibranchial placode-derived visceral sensory neurons. *Phox2b*-null mice lack the neural circuits underlying medullary autonomic reflexes (for a discussion of Phox2b in the CNS, see Brunet and Pattyn, 2002; Goridis and Rohrer, 2002).

Phox2b Is Required for Development of the Noradrenergic Phenotype

Within sympathetic and enteric precusors, Phox2b is required for expression of the *tyrosine hydroxylase* and *dopamine β-hydroxylase* (*DBH*) genes; these encode two enzymes in the catecholamine biosynthesis pathway (Fig. 9) (Pattyn *et al*., 1999). Hence, Phox2b is an essential determinant of the catecholaminergic (particularly noradrenergic) phenotype. Several transcription factors that act downstream of Phox2b in sympathetic neurons to control noradrenergic differentiation have been identified. These include the closely related protein Phox2a (which functions upstream of Phox2b in epibranchial placode-derived neurons; see Brunet and Pattyn, 2002), the bHLH protein dHAND (HAND2), and the zinc finger protein Gata3 (reviewed

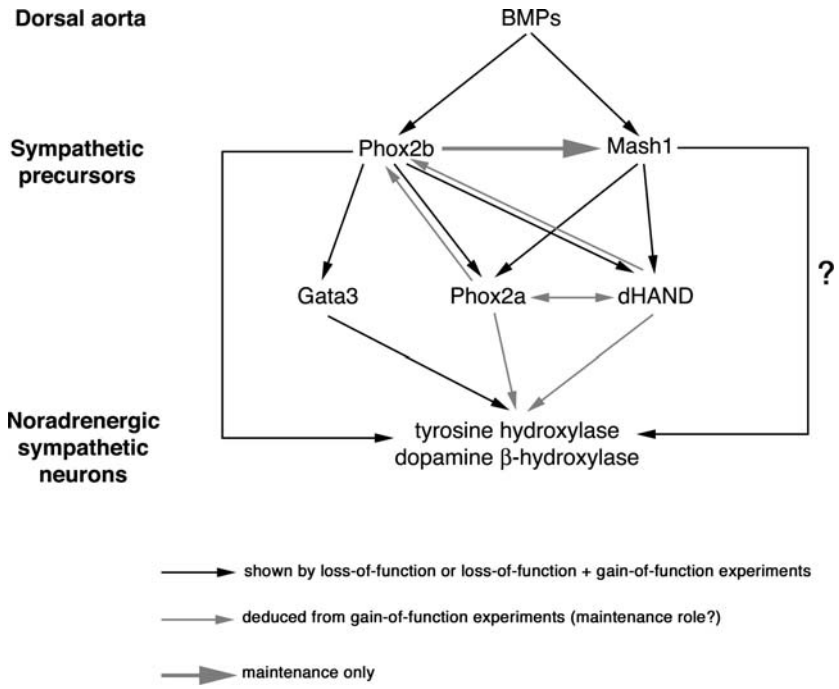

FIGURE 10. Regulatory network of transcription factors controlling sympathetic neuron development. See the section on Automatic Gangliogenesis for details. Question mark on arrow from Mash1 to *tyrosine hydroxylase* and *dopamine β-hydroxylase* indicates current uncertainty as to whether Mash1 acts on their promoters only through dHAND. BMPs, bone morphogenetic proteins. Modified from Goridis and Rohrer (2002).

in Brunet and Pattyn, 2002; Goridis and Rohrer, 2002). Although these factors are genetically downstream of Phox2b in sympathetic ganglia, together they form a complex regulatory network, in which most actions seem to be reciprocal (e.g., forced expression of dHAND can ectopically activate Phox2b) (Fig. 10) (reviewed in Brunet and Pattyn, 2002; Goridis and Rohrer, 2002).

Phox2b and Phox2a can each directly activate the *DBH* promoter, either alone or in conjunction with activation of the cyclic AMP second-messenger pathway (reviewed in Brunet and Pattyn, 2002; Goridis and Rohrer, 2002). There is some evidence that Phox2a can directly activate the *tyrosine hydroxylase* promoter, but again, cyclic AMP signaling may be required (see Goridis and Rohrer, 2002). Ectopic retroviral-mediated expression of either *Phox2b* or *Phox2a* in chick embryos promotes the formation of ectopic sympathetic neurons from trunk neural crest cells (Stanke *et al*., 1999). These neurons express pan-neuronal markers, noradrenergic markers (tyrosine hydroxylase and *DBH*), and also cholinergic markers (e.g., *choline acetyltransferase*) (Stanke *et al*., 1999). Hence, Phox2 proteins are sufficient to specify the differentiation of sympathetic neurons (including expression of both pan-neuronal and subtype-specific markers) *in vivo*.

In similar overexpression experiments in the chick, Phox2 proteins were found to be sufficient to induce expression of the bHLH transcription factor dHAND in trunk neural crest cells (Howard *et al*., 2000). Expression of dHAND alone is likewise sufficient to elicit the formation of catecholaminergic sympathetic neurons, both *in vitro* and *in vivo* (Howard *et al*., 1999, 2000). Indeed, dHAND and Phox2a act synergistically to enhance DBH transcription (Xu *et al*., 2003).

The zinc finger transcription factor Gata3 is also genetically downstream of Phox2b (Goridis and Rohrer, 2002). In *Gata3*-null mice, sympathetic ganglia form but the neurons fail to express tyrosine hydroxylase and have reduced levels of DBH, suggesting that *Gata3* is also essential for the noradrenergic phenotype (Lim *et al*., 2000).

This complex network of transcriptional regulation (Fig. 10) is perhaps the best characterized example of how neurotransmitter identity is controlled at the molecular level. One important gene in this network that has not yet been discussed, however, is the *achaete-scute* homologue *ash1* (*Mash1*) (sections Proneural Genes: An Introduction; *Mash1* Is Essential for Noradrenergic Differentiation). Although Phox2b is required to maintain *Mash1* expression, *Mash1* is induced independently of Phox2b in autonomic precursors, and itself induces a number of the same downstream genes (section *Mash1* Is Essential for Noradrenergic Differentiation).

Phox2b Is Required for Ret Expression in a Subset of Neural Crest Cells

Phox2b is required for expression of the receptor tyrosine kinase Ret in a subset of enteric precursors and in the most rostral sympathetic ganglion, the superior cervical ganglion (SCG) (Pattyn *et al*., 1999). These cells are completely absent in Ret-deficient mice (Durbec *et al*., 1996). One of the family of ligands

that signal through Ret, glial cell line-derived neurotrophic factor (GDNF), is essential for the development of the entire enteric nervous system (Moore *et al*., 1996) (section The Differentiation of Enteric Neurons; reviewed in Young and Newgreen, 2001; Airaksinen and Saarma, 2002).

Mash1 Is Essential for Noradrenergic Differentiation

Mash1 (mouse Ash1), a bHLH transcription factor related to the invertebrate proneural Achaete-Scute complex (section Proneural Genes: An Introduction; Chapter 5), was the first transcription factor found to be necessary for sympathetic development. Like Phox2b, Mash1 is expressed in all neural crest-derived autonomic precursors (sympathetic, parasympathetic, and enteric). Unlike Phox2b, however, it is not expressed in epibranchial placode-derived visceral sensory neurons. Mash1 is first expressed in sympathetic precursors shortly after they settle near the dorsal aorta. Like Phox2b, Mash1 is essential for *DBH* expression in all cell types except epibranchial placode-derived neurons; that is, Mash1 is a noradrenergic determinant, independent of Phox2b (Hirsch *et al*., 1998).

In *Mash1*-null mice, sympathetic and parasympathetic ganglia form (and express Phox2b), but pan-neuronal markers, Phox2a, tyrosine hydroxylase, and *DBH* are all lacking, and most (but not all) sympathetic and parasympathetic neuroblasts subsequently degenerate (Guillemot *et al*., 1993; Hirsch *et al*., 1998). *dHAND* expression is also reported to be missing in these embryos (Anderson and Jan, 1997). If Mash1 is constitutively expressed in cultured neural crest stem cells, it induces both *Phox2a* and *Ret*, together with pan-neuronal markers and morphological neuronal differentiation (Lo *et al*., 1998). Hence, *Phox2a*, *dHAND*, and *Ret* expression are induced not only by Phox2b, but also by Mash1. Mash1, like Phox2b, therefore, couples expression of pan-neuronal and neuronal subtype-specific markers (Fig. 10) (reviewed in Goridis and Rohrer, 2002). However, this linkage can be uncoupled experimentally: Floorplate ablation in the chick abolishes *Phox2a* and *tyrosine hydroxylase* expression, but not *Cash1* (chick Ash1) or pan-neuronal marker expression, in neural crest cells near the dorsal aorta (Groves *et al*., 1995). This suggests that a floorplate-derived signal, in addition to Mash1, is required for noradrenergic identity in prospective sympathetic neurons (section Floorplate-Derived Signals). Hence, Mash1 expression is not sufficient, in all contexts, to promote noradrenergic identity. Indeed, Mash1 alone does not promote autonomic neurogenesis *in vitro* in the absence of BMP2; hence it must interact with other factors induced by BMP2, such as Phox2b (Lo *et al*., 2002) (section BMPs Induce Both Mash1 and Phox2b in Sympathetic Precursors).

Interestingly, given the requirement of Gata3 for noradrenergic development (section *Phox2b* Is Required for Development of the Noradrenergic Phenotype), the *Drosophila* Gata factor Pannier can either activate or repress *achaete-scute* complex genes, in association with various transcriptional cofactors (Ramain *et al*., 1993; Skaer *et al*., 2002). This suggests a mechanism whereby Gata3 might also interact with *Mash1*, as well as being downstream of Phox2b, although currently there is no evidence for this (Goridis and Rohrer, 2002).

A subset of enteric neurons, including apparently all serotonergic enteric neurons, is also missing in *Mash1*-null mice (Blaugrund *et al*., 1996; Hirsch *et al*., 1998). Since serotonergic enteric neurons seem to develop from tyrosine hydroxylase-expressing precursors, this loss is perhaps to be expected (Blaugrund *et al*., 1996).

Mash1 Also Plays Roles in Sensory Neurogenesis

Mash1 is not only required for the development of autonomic neurons, and it does not always function by inducing *Phox2a*. The mesencephalic nucleus of the trigeminal nerve, which was introduced in the section on Neural Crest Derivatives as a (somewhat controversial) neural crest derivative within the brain, also depends on Mash1, but never expresses Phox2a (Hirsch *et al*., 1998). Mash1 is also essential for the development of olfactory neuron progenitors in the olfactory placode, which likewise do not express Phox2a (Guillemot *et al*., 1993; Cau *et al*., 1997) (section A bHLH Transcription Factor Cascade Controls Olfactory Neurogenesis). Hence, different neuronal subtype-specific factors must cooperate with Mash1 in the formation of these cell types.

BMPs Induce Both Mash1 and Phox2b in Sympathetic Precursors

Neural crest cells that migrate past the notochord and stop in the vicinity of the dorsal aorta (section Migration Arrest at Target Sites) will form the neurons and satellite cells of the sympathetic ganglia. Transplantation, rotation, and ablation experiments in the chick suggest that catecholaminergic neuronal differentiation only occurs near the aorta/mesonephros and also requires the presence of either the ventral neural tube or the notochord (Teillet and Le Douarin, 1983; Stern *et al*., 1991; Groves *et al*., 1995).

As described above, both *Phox2b* and *Mash1* are first expressed shortly after neural crest cells arrive at the dorsal aorta. At this time, the dorsal aorta expresses *Bmp2*, *Bmp4*, and *Bmp7* (Reissmann *et al*., 1996; Shah *et al*., 1996). All three factors induce increased numbers of catecholaminergic cells in neural crest cell cultures, as does forced expression of a constitutively active BMP receptor (reviewed in Goridis and Rohrer, 2002). BMP2 induces *Mash1* and *Phox2a* in cultured neural crest stem cells (Shah *et al*., 1996; Lo *et al*., 1998). Overexpression of BMP4 near the developing sympathetic ganglia leads to the ectopic formation of catecholaminergic cells *in vivo* (Reissmann *et al*., 1996). Conversely, when beads soaked in the BMP inhibitor Noggin are placed near the dorsal aorta in the chick, sympathetic ganglia initially form, but sympathetic neurons do not develop (Schneider *et al*., 1999). In these Noggin-treated embryos, sympathetic ganglia lack expression of pan-neuronal markers, and of *Phox2b*, *Phox2a*, *DBH*, and tyrosine hydroxylase, while *Cash1* is strongly reduced (Schneider *et al*., 1999). Together, these results

provide overwhelming evidence that dorsal aorta-derived BMPs induce expression of both *Phox2b* and *Mash1*, thus initiating the regulatory network of transcription factors that leads eventually to sympathetic neuron differentiation. However, these cues may be insufficient for catecholaminergic differentiation *in vivo*, as discussed in the following section.

Floorplate-Derived Signals Are Also Required for Catecholaminergic Differentiation

In addition to signals from the dorsal aorta, the presence of floorplate and/or notochord is also required for catecholaminergic differentiation (Teillet and Le Douarin, 1983; Stern *et al*., 1991; Groves *et al*., 1995). In particular, although neurons differentiate in the sympathetic ganglia in the absence of floorplate, they do not express catecholaminergic markers (Groves *et al*., 1995). This suggests that in addition to BMPs from the dorsal aorta (which induce *Phox2b* and *Mash1*), floorplate-derived signals are also required to induce or maintain subtype-specific markers in the sympathetic ganglia (Groves *et al*., 1995). Sonic hedgehog (see Chapter 3) seems to have little effect on catecholaminergic differentiation (Reissmann *et al*., 1996), and the molecular nature of the floorplate-derived signal(s) remains unclear. It may be relevant in this context that enhanced cyclic AMP signaling is required for efficient activation of the *tyrosine hydroxylase* promoter by Phox2a *in vitro* (reviewed in Goridis and Rohrer, 2002). Also, activation of the mitogen-activated protein (MAP) kinase signaling cascade in avian neural crest cells causes catecholaminergic differentiation independently of BMP4 (Wu and Howard, 2001). Clearly, there is still much to learn about the control of sympathetic neuron differentiation.

BMPs and Parasympathetic vs Sympathetic Differentiation

The differentiation of parasympathetic vs sympathetic autonomic neurons may be determined by local concentrations of BMPs at different neural crest target sites, as well as, perhaps, differential sensitivities of responding neural crest cells to BMPs (White *et al*., 2001). Postmigratory rat neural crest stem cells, isolated from fetal sciatic nerve, are more likely to differentiate as cholinergic parasympathetic neurons than as catecholaminergic sympathetic neurons when back-grafted into chick neural crest migratory pathways (White *et al*., 2001). After such grafts, they form cholinergic neurons in both sympathetic ganglia and parasympathetic ganglia, such as the pelvic plexus (White *et al*., 2001). In culture, they respond to BMP2 by differentiating as both cholinergic and noradrenergic autonomic neurons. However, they are significantly less sensitive to the neuronal differentiation-inducing activity of BMP2 than are migrating neural crest stem cells (section Differences in the Sensitivity of Different Neural Crest Stem Cells to Gliogenic Cues), and differentiate as cholinergic neurons at lower BMP2 concentrations (White *et al*., 2001). The molecular basis for this cholinergic bias is unknown.

BMPs are expressed at some sites of parasympathetic gangliogenesis. For example, the caudal cloaca, located proximal to the forming parasympathetic pelvic plexus, expresses BMP2 at an appropriate time to be involved in inducing parasympathetic neuronal differentiation (White *et al*., 2001).

The Differentiation of Enteric Neurons

BMP2, which is expressed in gut mesenchyme, promotes the neuronal maturation of postmigratory enteric neural precursors isolated from the rat gut (Pisano *et al*., 2000). However, several other growth factors have also been found to affect enteric neuronal differentiation.

Glial cell line-derived neurotrophic factor (GDNF) is the founding member of a family of ligands that act via a common signal transducer, the receptor tyrosine kinase Ret, complexed with ligand-specific receptors, the GDNF family receptor-α (GFRα) receptors (reviewed in Airaksinen and Saarma, 2002). GDNF is expressed in gut mesenchymal cells, and the entire enteric nervous system is missing in *GDNF*-deficient mice (Moore *et al*., 1996). In *Ret*-deficient mice, all enteric neurons and glia are missing from the gut below the level of the esophagus and the immediately adjacent stomach (Durbec *et al*., 1996). GDNF and Neurturin, another GDNF family ligand, promote the *in vitro* survival, proliferation, and neuronal differentiation of migrating and postmigratory Ret^+ enteric precursors from the rat gut (Taraviras *et al*., 1999).

The growth factor Endothelin3 (Edn3), conversely, seems to inhibit the neuronal differentiation of enteric precursors, thus maintaining a sufficiently large pool of migratory, undifferentiated precursors to colonize the entire gut (Hearn *et al*., 1998; Shin *et al*., 1999). Endothelin3 prevents the neurogenic activity of GDNF on migrating enteric neural precursors isolated from the quail embryo gut (Hearn *et al*., 1998).

Mutations that affect the Ret or Endothelin signaling pathways cause Hirschsprung's disease in humans, in which enteric ganglia are missing from the terminal colon (reviewed in Gershon, 1999; Manie *et al*., 2001; McCallion and Chakravarti, 2001).

Differentiation of Satellite Cells in Autonomic Ganglia

Strong autonomic neurogenic cues, such as BMP2, are clearly present at sites of autonomic gangliogenesis. How, then, do satellite glia form within autonomic ganglia? Exposure to gliogenesis-promoting factors such as NRG1 type II (section Differentiation of DRG Satellite Cells) is insufficient. Cultured rat neural crest stem cells rapidly commit to an autonomic neuronal fate on exposure to BMP2, but only commit to a glial fate after prolonged exposure to NRG1 type II (Shah and Anderson, 1997). Furthermore, saturating concentrations of BMP2 are dominant over NRG1 type II (although at low BMP2 concentrations, NRG1 type II can attenuate *Mash1* induction by BMP2) (Shah and Anderson, 1997). These results may explain why, *in vivo*, neurons differentiate before glia in autonomic ganglia. What, then, prevents all autonomic progenitors from differentiating into neurons?

Activation of the Notch signaling pathway seems to be essential for adoption of a glial fate in the presence of BMP2

(Morrison *et al*., 2000b). As discussed in the section on Notch Activation Leads to Gliogenesis by Neural Crest Stem Cells, even transient activation of Notch signaling inhibits neuronal differentiation and instructively promotes glial differentiation, in cultures of postmigratory neural crest stem cells isolated from fetal rat sciatic nerve (Morrison *et al*., 2000b). This action of Notch is dominant over that of BMP2, blocking neurogenesis at a point upstream of *Mash1* induction (Morrison *et al*., 2000b). It is likely that a similar mechanism of Notch activation acts within autonomic ganglia to promote satellite cell differentiation in the presence of BMP2. One model suggested by these results is that differentiating autonomic neurons express Notch ligands; these then activate Notch signaling in neighboring non-neuronal cells, which are then able to differentiate as glia (Morrison *et al*., 2000b). Other gliogenesis-promoting factors, such as NRG1 type II, may also act in concert with, or reinforce, the gliogenic action of Notch in peripheral autonomic ganglia (Hagedorn *et al*., 2000b). It is possible that once Notch is activated, preventing a neuronal fate and promoting a glial fate, NRG1 type II may then be able to promote a satellite cell fate (Hagedorn *et al*., 2000b; Leimeroth *et al*., 2002).

Summary of Autonomic Gangliogenesis

Phox2b is required for the formation of the entire peripheral autonomic nervous system. It is also necessary and sufficient for catecholaminergic (particularly noradrenergic) neuronal differentiation. Mash1 is necessary, but not sufficient, for noradrenergic differentiation. Phox2b and Mash1 interact in a complex regulatory network of transcription factors to induce noradrenergic differentiation. They are independently induced in sympathetic precursors by BMPs from the dorsal aorta; however, additional floorplate-derived signals are also required for catecholaminergic differentiation of sympathetic neurons. BMPs may also induce parasympathetic fates: The choice between parasympathetic and sympathetic fates may depend on local BMP concentrations and intrinsic differences in the sensitivity of different postmigratory neural crest cell populations to BMPs. BMPs and GDNF promote the differentiation of enteric neurons, while Edn3 may prevent enteric neuronal differentiation. Satellite cell differentiation requires Notch activation, which is dominant to the neurogenesis-promoting activity of BMPs. The gliogenic activity of NRG1 type II is subordinate to BMPs, but may be able to promote satellite cell differentiation once Notch has been activated.

Community Effects Alter Fate Decisions

A multipotent neural crest cell may adopt one fate in response to a given instructive growth factor when it is alone, but a different fate when it is part of a cluster ("community") of neural crest cells (reviewed in Sommer, 2001). Individual postmigratory multipotent cells isolated from embryonic rat DRG respond to BMP2 by forming both autonomic neurons and smooth muscle cells, while clusters of the same multipotent cells form significantly more autonomic neurons, at the expense of smooth muscle cells (Hagedorn *et al*., 1999, 2000a). This "community effect" (Gurdon *et al*., 1993) may prevent neural crest cells in autonomic ganglia from adopting an aberrant (smooth muscle) fate in response to BMP2 *in vivo*.

Different concentrations of the same factor can also have different effects when local neural crest cell–cell signaling is allowed to occur. Individual postmigratory progenitors from rat DRG respond to TGFβ by adopting a predominantly smooth muscle fate; they never form neurons (Hagedorn *et al*., 1999, 2000a). Although high doses of TGFβ cause some cell death, the predominant fate choice is still smooth muscle (Hagedorn *et al*., 2000a). Clusters of these progenitors, in contrast, respond to high TGFβ doses by dying, and to low TGFβ doses by forming autonomic neurons (Hagedorn *et al*., 1999, 2000a).

Similar community effects may underlie the results discussed in the section on Axial Fate-Restriction, in which individual trunk neural crest cells form cartilage in the head when surrounded by host cartilage cells, but coherent masses of trunk neural crest cells do not (McGonnell and Graham, 2002). Community effects also help to maintain neural crest cell regional identity: Individual neural crest cells will change their *Hox* gene expression patterns in response to environmental cues, while large groups of neural crest cells do not (e.g., Golding *et al*., 2000; Trainor and Krumlauf, 2000; Schilling *et al*., 2001).

In summary, local neural crest cell–cell interactions may reinforce fate choice in particular environmental contexts, and prevent inappropriate fate choices in response to environmental cues.

NEURAL CREST SUMMARY

Since the last edition of this book, in 1991, there has been an explosion of information about the genes and signaling pathways important for neural crest cell development. Molecular cues involved in neural crest cell induction at the neural plate border have now been identified. These include BMPs, which are important for setting up the neural plate border itself and, later, for neural crest cell delamination, and Wnts, which are both necessary and sufficient for neural crest precursor cell induction within the neural plate border. Numerous repulsive guidance cues, including ephrins, are now known to play essential roles in sculpting the migration pathways of both cranial and trunk neural crest cells, and some progress has been made in understanding migration arrest at target sites. The migrating neural crest cell population is heterogeneous, containing multipotent and fate-restricted cells; however, the latter do not seem to be determined; that is, they retain the potential to adopt other fates when challenged experimentally. There is a greater molecular understanding of lineage diversification, and it is becoming apparent that the sensory–autonomic lineage decision is taken before the neuronal–glial fate decision. Various transcription factors are known to be essential for the formation of particular neural crest lineages, including Phox2b for the autonomic lineage and Sox10 for the glial lineage. Several instructive differentiation cues that act on multipotent neural crest cells, including BMPs and NRGs, have been identified. Finally, an emerging theme is that neural crest cell–cell interactions, including community effects, are

important in determining neural crest cell fate choice. Clearly, a great deal has been learned about neural crest cell induction, migration, and differentiation. However, many questions still remain, and there are many fruitful avenues for future research into the development of these fascinating cells.

OVERVIEW OF CRANIAL ECTODERMAL PLACODES

Cranial ectodermal placodes (Greek root $\pi\lambda\alpha\kappa$, i.e., flat plate, tablet) are discrete patches of thickened ectoderm that appear transiently in the head of all vertebrate embryos (reviewed in Webb and Noden, 1993; Baker and Bronner-Fraser, 2001; Begbie and Graham, 2001a). They were discovered 120 years ago (van Wijhe, 1883) and given the name "placode" by von Kupffer (1894). Placodes give rise to the bulk of the peripheral sensory nervous system in the head. The olfactory, otic, and lateral line placodes give rise to the paired peripheral sense organs (olfactory epithelium, inner ears, and lateral line system of anamniotes) together with their afferent innervating neurons. The lens placodes give rise to the lenses of the eye. The trigeminal placodes form many of the cutaneous sensory neurons that innervate the head, including the jaws and teeth. The epibranchial placodes give rise to visceral sensory neurons that provide afferent innervation for tastebuds, and afferent autonomic innervation for the visceral organs. Finally, the hypophyseal (or adenohypophyseal) placode gives rise to all of the endocrine cells and supporting cells of the adenohypophysis (anterior pituitary gland). Although the molecular mechanisms underlying the induction and development of the hypophyseal placode are perhaps the best understood of all the placodes, its development will not be discussed here (for detailed reviews of hypophyseal placode induction and development, see Baker and Bronner-Fraser, 2001; Dasen and Rosenfeld, 2001; Scully and Rosenfeld, 2002).

Like the neural crest, therefore, placodes give rise to a very diverse array of cell types, including sensory receptors, sensory neurons, supporting cells, secretory cells, glia, neuroendocrine, and endocrine cells (Table 3). Figure 11 shows a fate-map of the placode-forming ectoderm in the head of the 8-somite stage chick embryo, together with the respective neuronal contributions to cranial sensory ganglia of placodes and the neural crest. Figure 12 shows the location of the different placodes in the head of the 19-somite stage *Xenopus* embryo. It is evident from these schematics that a relatively large proportion of dorsal head ectoderm contributes to placodal tissue.

Also like the neural crest, cranial ectodermal placodes are usually considered to be a defining characteristic of the craniates (vertebrates plus hagfish) (Gans and Northcutt, 1983; Northcutt and Gans, 1983; Baker and Bronner-Fraser, 1997). However, molecular analyses suggest that at least some vertebrate placodes may have homologues in non-vertebrate chordates (e.g., Boorman and Shimeld, 2002; Christiaen *et al*., 2002). Placodes

TABLE 3. Cell Types and Cells Derived from Cranial Ectodermal Placodes

Placode	General cell type	Cells
Olfactory	Sensory ciliary receptor	Chemoreceptive olfactory receptor neurons
	Sensory neurons	Olfactory receptor neurons
	Glia	Olfactory ensheathing glia
	Neuroendocrine cells	Gonadotropin-releasing hormone (GnRH)-producing neurons
	Secretory/support cells	Sustentacular cells (secrete mucus; provide support)
Otic	Sensory ciliary receptor	Mechanosensory hair cells
	Sensory neurons	Otic hair cell-innervating neurons, collected in vestibulo-cochlear ganglion of cranial nerve VIII
	Secretory cells	Cupula-secreting cells; endolymph-secreting cells; cells secreting biomineralized matrix of otoliths/otoconia
	Supporting cells	Hair cell support cells; non-sensory epithelia
Lateral line	Sensory ciliary receptor	Mechanosensory hair cells in neuromasts Electroreceptive cells in ampullary organs
	Sensory neurons	Lateral line hair cell-innervating neurons, collected in lateral line ganglia
	Secretory cells	Cupula-secreting cells in neuromasts
	Supporting cells	Hair cell support cells in neuromasts
Lens	Specialized epithelium	Lens fiber cells
Ophthalmic and maxillomandibular trigeminal	Sensory neurons	Cutaneous sensory neurons (pain, touch, temperature), collected in trigeminal ganglion of cranial nerve V
Epibranchial	Sensory neurons	Afferent neurons for taste buds and visceral organs, collected in geniculate, petrosal, and nodose ganglia (distal ganglia of cranial nerves VII, IX, and X, respectively)
Hypophyseal	Endocrine cells	All endocrine cells of the adenohypophysis (anterior pituitary gland)
	Supporting cells	Support cells of the adenohypophysis

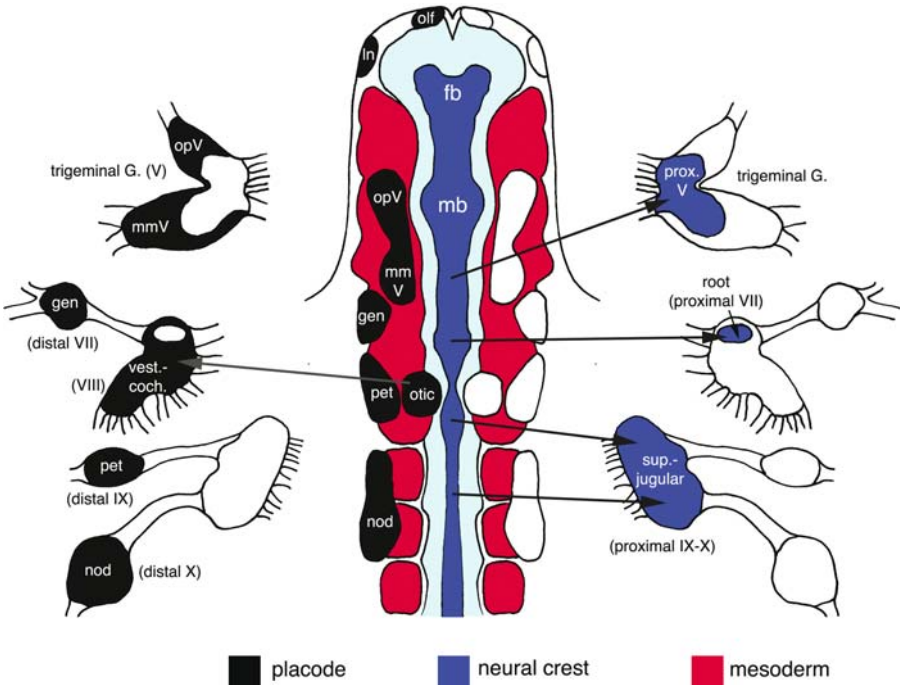

FIGURE 11. Fate-map of placodes (black ovals) and neural crest (dark blue) in the head of an 8-somite stage chick embryo, and their neuronal contribution to the sensory ganglia of the cranial nerves (Roman numerals). All satellite cells in cranial sensory ganglia are derived from the neural crest. fb, forebrain; G., ganglion; gen, geniculate; ln, lens; mb, midbrain; mmV, maxillomandibular trigeminal; nod, nodose; olf., olfactory; opV, ophthalmic trigeminal; pet, petrosal; prox., proximal; sup., superior; vest.-coch., vestibulocochlear. Redrawn from D'Amico-Martel and Noden (1983).

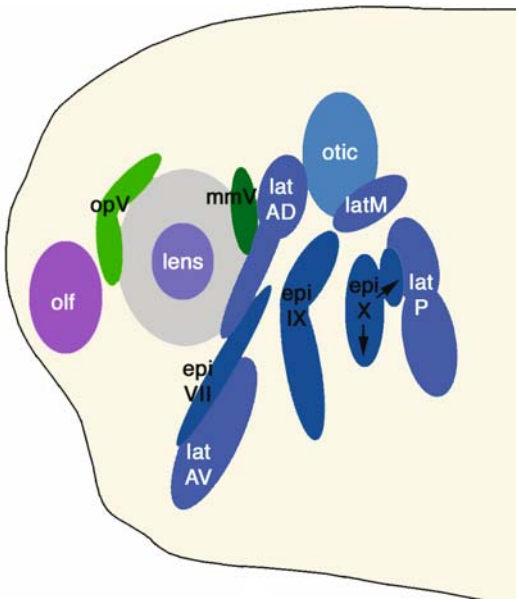

FIGURE 12. Location of placodes in the head of a 19-somite stage *Xenopus* embryo. epi, epibranchial placode; epi VII, facial/geniculate placode; epi IX, glossopharyngeal/petrosal placode; epi X, vagal/nodose placodes; lat, lateral line placode; latAD, anterodorsal lateral line placode; latAV, anteroventral lateral line placode; latM, middle lateral line placode; latP, posterior lateral line placode; mmV, maxillomandibular trigeminal placode; olf., olfactory placode; opV, ophthalmic trigeminal placode. Redrawn from Schlosser and Northcutt (2000).

have been studied in all craniate classes, including hagfish (e.g., Wicht and Northcutt, 1995) and lamprey (e.g., Bodznick and Northcutt, 1981; Neidert *et al*., 2001; McCauley and Bronner-Fraser, 2002). Although most research has been done on the sense organ placodes, in particular the lens and otic placodes, as well as the hypophyseal placode, molecular information has also enabled closer investigation of the development of the trigeminal and epibranchial placodes. Here, a relatively brief summary is provided of the current state of knowledge of the induction and development of the different placodes. For a more detailed review of classical and modern research into placode induction, the reader is referred to Baker and Bronner-Fraser (2001).

A PREPLACODAL FIELD AT THE ANTERIOR NEURAL PLATE BORDER

All fate-mapping studies to date have shown that placodes arise from ectoderm at the neural plate border in the prospective head region (Baker and Bronner-Fraser, 2001). Older fate maps suggest that placodes originate from ectoderm lying lateral to the neural crest-forming area, except in the most rostral region, where no neural crest cells form and olfactory and hypophyseal placodes directly abut prospective neural plate territories (Baker and Bronner-Fraser, 2001). However, cell lineage analysis shows that placodal precursors, like neural crest precursors (section Embryonic Origin of the Neural Crest), do not exist

as a segregated population (Streit, 2002). Although prospective placodal territory extends more laterally than prospective neural crest territory, placodal and neural crest precursors are mingled together more medially (Streit, 2002).

Molecular evidence supports some early morphological observations in suggesting that there is a preplacodal field, or panplacodal anlage, around the anterior neural plate. This field is morphologically visible in the frog, *Rana*, which has a continuous band of thickened ectoderm around the edge of the anterior neural plate, from which most placodes originate (Knouff, 1935) (Fig. 13A). Molecularly, this field seems to be characterized in multiple species by the expression of various genes in a horseshoe-shaped band around the anterior neural plate border (Figs 13B, C). These genes, which primarily encode transcription factors, are often subsequently maintained in all or multiple placodes. They include the homeodomain transcription factors Six1, Six4, Dlx3, Dlx5, and Dlx7, the HMG-domain transcription factor Sox3 (which is also expressed in the neural plate), and the transcription cofactors Eya1 and Eya2 (for original references, see Baker and Bronner-Fraser, 2001; also David *et al*., 2001; Ghanbari *et al*., 2001). See the section on Establishment of the Neural Plate Border for a discussion of the role of Dlx genes in positioning the neural plate border. In the chick, the expression domains of these genes are not coincident; rather, they are expressed in a series of overlapping domains that shift both spatially and temporally with the position of placodal precursors (Streit, 2002).

It is clear that several of these genes play important roles in the development of multiple placodes. For example, *dlx3*, acting in concert with *dlx7*, is necessary for the formation of both olfactory and otic placodes in the zebrafish (Solomon and Fritz, 2002). Ectopic expression of *Sox3* in another teleost fish, medaka, causes ectopic lens and otic vesicle formation in ectodermal regions relatively close to the endogenous lens and otic placodes (Köster *et al*., 2000). Sox3 may, therefore, act as a competence factor, enabling ectopic ectoderm to respond to placode-inducing signals (Köster *et al*., 2000).

However, the precise significance of the preplacodal domain of gene expression remains unclear: It does not seem to correlate either with the site of origin of all placodal precursor cells, or with determination toward a placodal fate. A cell lineage analysis in the chick showed that some otic placode precursors arise from ectoderm lying medial to the *Six4* expression domain (Streit, 2002). Hence, not all placodal precursors originate from the *Six4*$^+$ domain. Furthermore, cells within the *Six4*$^+$ domain form not only placodal derivatives, but also neural crest and epidermis (and neural tube until the 2-somite stage, at the level of the future otic placode) (Streit, 2002). Hence, cells within the preplacodal domain are not all determined toward a placodal fate.

Some insight into the function of the preplacodal domain may come from observations showing that there is a large degree of ectodermal cell movement in the neural plate border region (Whitlock and Westerfield, 2000; Streit, 2002). These studies combined cell lineage analysis (using DiI or fluorescent dextrans) with time-lapse analysis and *in situ* hybridization. Precursors of a particular placode, such as the olfactory placode in zebrafish (Whitlock and Westerfield, 2000) or the otic placode in chick (Streit, 2002), originate from a fairly large region of ectoderm at the anterior neural plate border, and subsequently converge to form the final placode. This may suggest a model whereby cells that move into the preplacodal gene expression domain upregulate the genes defining the domain, while cells that move out of the domain downregulate these genes. Cells that express the "preplacodal" genes may be rendered competent to respond to specific placode-inducing signals. However, the fate of a given cell within the preplacodal domain will depend on the precise combination of signals it subsequently receives. Hence, although it is competent to form placode, it may give rise to neural crest or epidermis instead.

The Pax/Six/Eya/Dach Regulatory Network

The overlapping expression of various *Six* and *Eya* genes in the preplacodal domain is of particular interest. Six and Eya

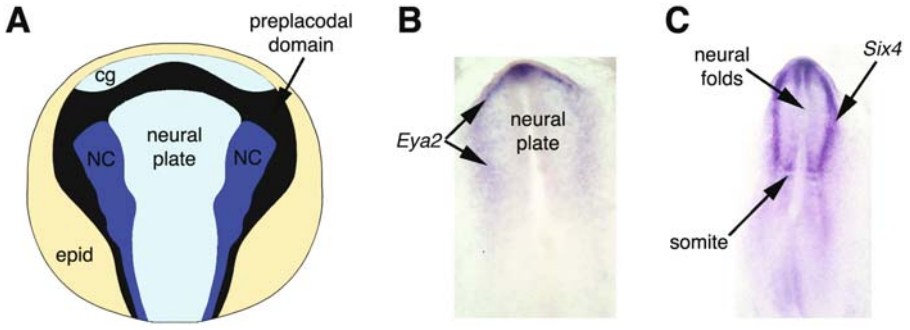

FIGURE 13. A preplacodal domain of ectoderm can be recognized around the anterior neural plate border, occasionally by morphology alone, more often by specific gene expression. (A) Fate map of open neural plate stage *Rana* embryo (dorsal view), showing the preplacodal domain, recognizable morphologically as a continuous strip of thickened ectoderm around the prospective neural crest (NC) domain. cg, prospective cement gland; epid, epidermis; NC, neural crest. Redrawn from Knouff (1935). (B) *Eya2* expression (dark staining) around the anterior neural plate border in a stage 6 (neurula-stage) chick embryo (dorsal view). (C) *Six4* expression (dark staining) around the anterior neural plate border in a 2-somite stage chick embryo (dorsal view). Both *Eya2* and *Six4* are subsequently maintained in most placodes (section A Preplacodal Field at the Anterior Neural Plate Border for details). Photographs courtesy of Dr. Andrea Streit and Anna Litsiou, King's College, London, United Kingdom. Chick staging after Hamburger and Hamilton (1951).

family members function in a complex cross-regulatory network with Pax transcription factors and the transcription cofactor Dachshund (Dach, also a multimember family in vertebrates), in a variety of developmental contexts. These include eye development in *Drosophila* (reviewed in Wawersik and Maas, 2000) and vertebrate muscle development (Heanue *et al*., 1999). Dach family members are expressed in part of the preplacodal domain and in various placodes (Davis *et al*., 2001; Hammond *et al*., 2002; Heanue *et al*., 2002; Loosli *et al*., 2002). While *Pax* genes are not expressed in the preplacodal domain, most individual placodes are characterized by a particular combination of *Pax* gene expression. For example, *Pax6* is expressed in the olfactory and lens placodes, *Pax3* in the ophthalmic trigeminal placode, *Pax2/5/8* in the otic placode, and *Pax2* in the epibranchial placodes (reviewed in Baker and Bronner-Fraser, 2001). Mouse knockout studies have shown that *Pax* gene expression within the placodes is important for their proper development. For example, *Pax6* is essential for olfactory and lens placode formation, while *Pax2* is important for aspects of otic placode development (reviewed in Baker and Bronner-Fraser, 2001).

Given the above, it is possible that expression of *Six, Eya*, and perhaps also *Dach* genes within the preplacodal domain may represent a molecular framework common to all placodes. This network might then be able to interact with different *Pax* genes, induced in different regions of the preplacodal domain by specific placode-inducing signals, to specify individual placode identities. Although this model is attractive, further supporting evidence is required.

Models of Individual Placode Formation in the Preplacodal Domain

The active convergence of cells at the neural plate border to form specific placodes (Whitlock and Westerfield, 2000; Streit, 2002) suggests two possible models for the formation of individual placodes from ectoderm in this region (Streit, 2002). The first model proposes that cells in a large region of ectoderm receive a specific placode-inducing signal: Those cells that respond to the signal "sort out" from non-responding cells and actively migrate to the site of formation of the placode. The second model proposes that ectodermal cells move at random: Those that come within range of localized placode-inducing signals adopt a specific placodal fate. Evidence exists to support the existence of both localized and widespread placode-inducing signals (e.g., sections Induction of the Otic Placodes; Induction of the Trigeminal Placodes). However, it is currently unknown whether active sorting processes occur within the ectoderm to cause the aggregation of specific placode precursors.

Summary

All placodes originate from ectoderm at the anterior neural plate border. A horseshoe-shaped domain of ectoderm at the anterior neural plate border expresses numerous specific transcription factors, such as Six, Eya, and Dlx family members, all of which have roles in placode development. Cells expressing an appropriate combination of these genes may be competent to adopt a placodal fate in response to placode-inducing signals. Cells that respond to specific placode-inducing signals may sort out and aggregate to form the placode. Alternatively, cells may randomly move within range of specific placode-inducing signals, cease migrating, and differentiate accordingly. Further evidence is required to distinguish between these models.

In the following sections, the induction and some aspects of the development of individual placodes are discussed in turn, beginning with the sense organ placodes (olfactory, otic, lateral line, and lens), and ending with the trigeminal and epibranchial placodes.

SENSE ORGAN PLACODES

Olfactory Placodes

Olfactory Placode Derivatives

The paired olfactory placodes, which invaginate toward the telencephalon to form olfactory pits, give rise to the entire olfactory (odorant-sensing) and, where present, vomeronasal (pheromone-sensing) epithelia, together with the respiratory epithelium that lines the nasal passages. The olfactory and vomeronasal epithelia contain ciliated sensory receptor neurons, each of which bind odorants via a single member of an enormous family of G-protein-coupled, seven-transmembrane domain receptor molecules (reviewed in Mombaerts, 2001; Ronnett and Moon, 2002). The epithelia also contain basal cells, which generate olfactory sensory neurons throughout life (for a review on stem cells in the olfactory epithelium, see Calof *et al*., 1998), and supporting sustentacular cells, which share some characteristics with glia (reviewed in Ronnett and Moon, 2002). All of these cells are derived from the olfactory placode.

The cell bodies of the olfactory receptor neurons remain in the placode, while their axons extend into the brain to form the olfactory, vomeronasal, and terminal nerves (for reviews of olfactory axon pathfinding, see Mombaerts, 2001; St. John *et al*., 2002). These nerves are ensheathed by olfactory placode-derived glial cells (reviewed in Wewetzer *et al*., 2002) that leave the placode and migrate along the nerves into the brain. In the zebrafish, pioneer neurons, distinct from olfactory receptor neurons, differentiate early within the placode and send their axons to the telencephalon (Whitlock and Westerfield, 1998). Axons from the olfactory receptor neurons follow this initial scaffolding, and the pioneer neurons subsequently die by apoptosis (Whitlock and Westerfield, 1998). Olfactory axons are the first peripheral input to reach the brain during development. The axons of pioneer neurons in the rat induce formation of the olfactory bulbs (Gong and Shipley, 1995), which fail to form if the olfactory placodes are missing or if olfactory axons fail to reach the brain (reviewed in Baker and Bronner-Fraser, 2001). The olfactory epithelium is also required for induction of the cartilaginous nasal capsule, which is derived from the neural crest.

The olfactory placode also forms neuroendocrine cells that migrate along the olfactory nerve into the forebrain and diencephalon. These neurons produce gonadotropin-releasing hormone (GnRH) and form the terminal nerve-septo-preoptic GnRH system (reviewed in Dubois *et al*., 2002). This system regulates gonadotropin release from the adenohypophysis (anterior pituitary), another placodal derivative (section Overview of Cranial Ectodermal Placodes). Hence, the olfactory placode is not only essential for olfaction, but also for reproduction. This is seen clinically in Kallmann's syndrome, in which olfactory axons and GnRH neurons fail to migrate into the brain, resulting in anosmia and sterility (hypogonadism) (reviewed in MacColl *et al*., 2002). An early-stage fate-map in zebrafish, however, challenges the olfactory placode origin of GnRH neurons, suggesting that terminal nerve GnRH neurons originate from the neural crest, and hypothalamic GnRH neurons from the hypophyseal placode (adenohypophysis) (Whitlock *et al*., 2003). More early-stage fate-map data are needed from multiple species to resolve this controversy.

Olfactory Placode Formation Involves the Convergence of Cellular Fields

In the 4-somite stage zebrafish embryo, the olfactory placodes fate-map to bilateral regions of *Dlx3*$^+$ ectoderm at the lateral borders of the anterior-most neural plate, much longer in rostrocaudal extent than the final placodes, abutting prospective telencephalic territory rostrally and prospective neural crest caudally (Whitlock and Westerfield, 2000). *Dlx3*, acting in concert with *Dlx7*, is essential for the formation of the olfactory placode in the zebrafish, as shown both by mutant and knockdown analysis using antisense morpholino oligonucleotides (Solomon and Fritz, 2002). Each of these long bilateral *Dlx3*$^+$ cellular fields converges to form an olfactory placode (Whitlock and Westerfield, 2000).

In neurula-stage *Xenopus* embryos, and 3-somite stage chick and mouse embryos, the olfactory placodes fate-map to the outer edge of the anterior neural ridge (the rostral boundary of the neural plate) (Couly and Le Douarin, 1988; Eagleson and Harris, 1990; Osumi-Yamashita *et al*., 1994). Future olfactory placode and olfactory bulb tissues are contiguous within the anterior neural plate. It is currently unknown whether olfactory placode formation in these species also involves cellular convergence, as in the zebrafish.

Induction of the Olfactory Placodes

Classical grafting and coculture experiments in amphibian embryos (reviewed in Baker and Bronner-Fraser, 2001) suggested that anterior mesendoderm is an important source of olfactory placode-inducing signals. This tissue is also important for forebrain induction (reviewed in Foley and Stern, 2001) (Chapter 3). Forebrain tissue is also important for olfactory placode induction and/or maintenance (reviewed in Baker and Bronner-Fraser, 2001). Nothing is currently known about which molecular signals from these tissues, or others, might be involved in the induction of the olfactory placode.

In the chick, FGF8 from the midfacial ectoderm is necessary and sufficient to induce the genes *erm* and *pea3* in the olfactory pits (Firnberg and Neubüser, 2002). (The induction and maintenance of these two Ets-domain transcription factors is generally tightly coupled to FGF signaling; Raible and Brand, 2001; Roehl and Nüsslein-Volhard, 2001.) FGFs stimulate the proliferation of olfactory receptor neuron progenitors *in vitro* (reviewed in Calof *et al*., 1998) (see next section), hence, FGF8 may play a similar role in promoting olfactory neurogenesis *in viv*o. However, this remains to be demonstrated.

Experiments in mice and zebrafish have shown that the transcription factors Otx2, Pax6, Dlx5, and Dlx3 (acting in concert with Dlx7), all of which are expressed in the anterior neural ridge and in the olfactory placodes, are required for olfactory placode development (reviewed in Baker and Bronner-Fraser, 2001) (also Solomon and Fritz, 2002). However, their precise roles are currently undefined.

A bHLH Transcription Factor Cascade Controls Olfactory Neurogenesis

Mice lacking the Achaete-Scute homologue Mash1 (section *Mash1* Is Essential for Noradrenergic Differentiation) have a drastically reduced number of olfactory receptor neurons, due to the death of most olfactory neuron progenitors (Guillemot *et al*., 1993; Cau *et al*., 1997). *Mash1* is required for the expression of the *atonal*-related bHLH genes *Neurogenin1* (*Ngn1*) (section Neurogenins Are Essential for the Formation of Dorsal Root Ganglia) and *NeuroD* (Cau *et al*., 1997), and for activation of the Notch signaling pathway (Cau *et al*., 2000, 2002). *Ngn1* is required for neuronal differentiation; it does not affect either *Mash1* expression or the Notch signaling pathway (Cau *et al*., 2002).

Mash1 is not expressed in the earliest-differentiating neurons in the olfactory placode, whose formation is unaffected in *Mash1*-null mice (Cau *et al*., 1997, 2002). These *Mash1*-independent progenitors express *Ngn2* as well as *Ngn1*, but their differentiation is blocked in *Ngn1*-mutant mice (Cau *et al*., 1997, 2002). Given their early differentiation, these could represent the pioneer neurons whose axons set up the initial scaffold for the olfactory nerve (section Olfactory Placode Derivatives). Interestingly, vomeronasal sensory neurons are relatively unaffected in mice that are double mutant for both *Mash1* and *Ngn1* (Cau *et al*., 2002). This suggests both that a different gene controls their development, and that vomeronasal and olfactory sensory neuron progenitors are molecularly distinct (Cau *et al*., 2002).

As described in the previous section, FGF8, which is expressed in the epithelium around the placodes, may stimulate the proliferation of olfactory neuron stem cells and neuronal progenitors (reviewed in Calof *et al*., 1998) (also see LaMantia *et al*., 2000). Treatment of olfactory placode explants with a function-blocking FGF8 antibody causes a reduction in the numbers of neurons relative to controls, though not a complete loss, suggesting that FGF8 is not only sufficient but also necessary for olfactory neurogenesis (LaMantia *et al*., 2000).

BMPs also play an important role in olfactory neurogenesis: In fact, they can both promote and inhibit neurogenesis in cultures of olfactory epithelium, depending on the concentration, the specific ligand, and the cellular context (Shou *et al*., 1999, 2000). Exposure of Mash1^{+} olfactory neuron progenitors to high concentrations of BMP4 or BMP7 in culture leads to the degradation of Mash1 protein via the proteasome pathway, and hence to inhibition of neuronal differentiation (Shou *et al*., 1999). However, treatment of olfactory epithelium cultures with the BMP antagonist Noggin inhibits neuronal differentiation, showing a requirement for BMP signaling in neurogenesis (Shou *et al*., 2000). This requirement is explained by the observation that low concentrations of BMP4, but not BMP7, promote the survival of newly born olfactory receptor neurons (Shou *et al*., 2000). Hence, BMP4 inhibits the production of olfactory receptor neurons at high concentrations and promotes the survival of differentiated neurons at low concentrations. This may provide a feedback mechanism to maintain an appropriate number of olfactory receptor neurons in the epithelium, particularly as BMP4 may be produced by the olfactory receptor neurons themselves (Shou *et al*., 2000).

Retinoic acid is produced by the neural crest-derived frontonasal mesenchyme between the olfactory placode and the ventrolateral forebrain (LaMantia *et al*., 1993, 2000). *In vitro*, retinoic acid stimulates the maturation of olfactory receptor neurons from immortalized clonal cell lines derived from the mouse olfactory placode, suggesting a possible role in this process *in vivo* (Illing *et al*., 2002). However, retinoic acid treatment of olfactory placode explants leads to reduced neuronal differentiation (LaMantia *et al*., 2000). Further evidence is therefore required to establish the precise role of retinoic acid.

Lateral Line Placodes

Lateral Line Placode Derivatives

The lateral line system is a mechanosensory and electroreceptive sensory system in which individual sense organs are arranged in characteristic lines along the head and trunk of fish and amphibians (Fig. 14C) (Coombs *et al*., 1989). The entire lateral line system seems to have been lost in amniotes, presumably in association with the transition to a primarily terrestrial lifestyle; it is also often lost in amphibians at metamorphosis. Lateral line electroreception was lost in most teleost fish and in anuran amphibians; indeed, different elements of the lateral line system have been lost independently in multiple vertebrate lineages (reviewed in Northcutt, 1997; Schlosser, 2002b).

There are two types of lateral line sense organs: Mechanosensory neuromasts (Fig. 14B) that respond to local disturbances in the water surrounding the animal, and electroreceptive ampullary organs that respond to weak electric fields. They are used in various behaviors, including schooling, obstacle avoidance, and prey detection. The sense organs themselves, and the neurons that provide their afferent innervation, are derived from a series of paired lateral line placodes on the head (reviewed in Winklbauer, 1989; Baker and Bronner-Fraser, 2001; Schlosser, 2002a) (Fig. 12). The same lateral line placode can form both mechanosensory neuromasts and electroreceptive ampullary organs (Northcutt *et al*., 1995). Primitively, there were probably at least three pre-otic and three post-otic lateral line placodes (Northcutt, 1997). One pole of each lateral line placode gives rise to neuroblasts, which exit the placode and aggregate nearby to form the sensory neurons of a lateral line ganglion (the satellite

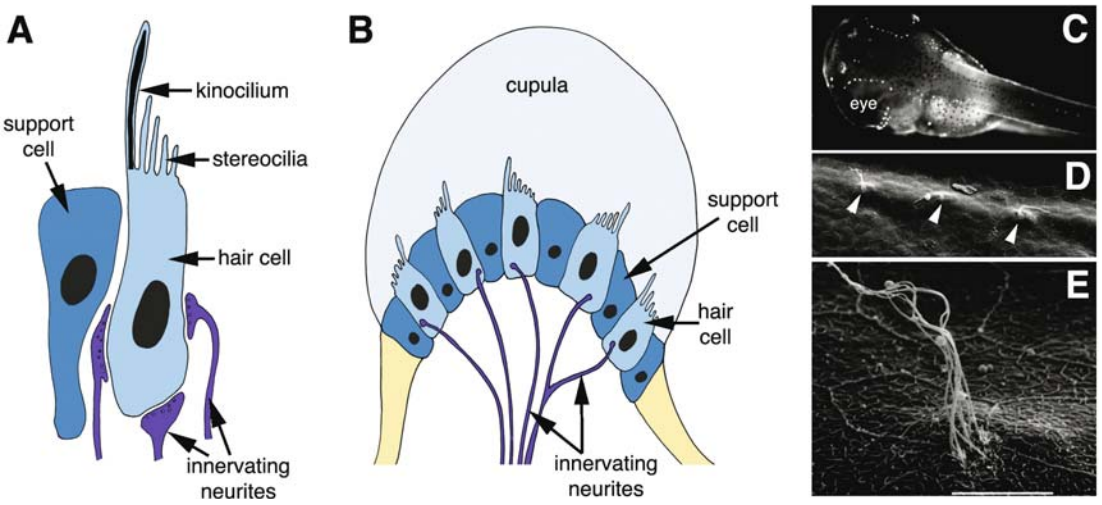

FIGURE 14. The mechanosensory lateral line system. (A) Schematic showing an individual unit of a lateral line neuromast: A mechanosensory hair cell bearing a true cilium (kinocilium) and stereocilia, innervated by lateral line neurites, with an adjacent support cell. Redrawn from Kardong (1998). (B) Schematic section through a neuromast organ: The cilia and stereocilia of each hair cell project into a gelatinous cupula, secreted by the supporting cells. Redrawn from Kardong (1998). (C) Lateral line neuromasts on the head (particularly visible in a ring around the eye) and along the trunk of a live stage 45 (4-day) *Xenopus* tadpole, visualized with the vital mitochondrial stain DASPEI. (D) Scanning electron micrograph of lateral line neuromasts along the trunk of a stage 49 (12-day) *Xenopus* tadpole. (E) High-power view of individual neuromast from (D), showing bundle of long kinocilia with smaller stereocilia at its base. *Xenopus* staging after Nieuwkoop and Faber (1967).

cells of the ganglion are derived from the neural crest). Neurites from these neurons, followed and ensheathed by neural crest-derived glial cells (Gilmour *et al*., 2002), track the remaining non-neurogenic part of the placode as it elongates to form a lateral line primordium and undergoes a remarkable migration through the epidermis, depositing clusters of cells in its wake. These cells give rise to the supporting and mechanosensory hair cells of the lateral line sense organ (neuromast). Hence, the placode that forms a given line of sense organs also typically forms their afferent innervating neurons. In the zebrafish, the atonal-related bHLH transcription factor Neurogenin1 (Ngn1; section Proneural Genes: An Introduction) is required for the formation of lateral line neurons, but its loss has no effect on the migration of the lateral line primordia, or on neuromast development (Andermann *et al*., 2002). Hence, as previously demonstrated in amphibian embryos (Tweedle, 1977), lateral line sense organ development is independent of innervation.

A single mechanosensory neuromast is composed of several hair cells, together with supporting cells; each hair cell has a single true cilium (kinocilium) with a bundle of stereocilia at its base (Figs. 14A, E). Lateral line hair cells are very similar in structure to inner ear hair cells derived from the otic placode (section Otic Placode Derivatives). The kinocilia and sterocilia of the hair cells in each lateral line neuromast are embedded in a gelatinous sheath, or cupula, which is secreted by the supporting cells of the neuromast (Fig. 14B). Water movements deflect the cupula. If the cupula movement bends the stereocilia toward the kinocilium, mechanosensitive ion channels in the hair cell open, depolarizing the hair cell and stimulating the afferent fibers of the lateral line nerve which synapses onto its basal surface. If the stereocilia are bent away from the kinocilium, this closes the few ion channels that are open at rest, causing hyperpolarization of the hair cell and neuronal inhibition (reviewed in Winklbauer, 1989; Pickles and Corey, 1992).

Electroreceptor cells are structurally similar to neuromast hair cells, although they are more variable across taxa (Bodznick, 1989). They are secondary sense cells (i.e., they require afferent innervation) with apical microvilli and/or a kinocilium. Interestingly, while teleost electroreceptors, where present, seem to have secondarily evolved from neuromast hair cells, nonteleost electroreceptors (i.e., those found in lamprey, nonteleost fish, and amphibia) are phylogenetically as old or older than neuromast hair cells (Bodznick and Northcutt, 1981; Bodznick, 1989). From the phylogenetic evidence, it is equally likely that neuromast hair cells evolved from electroreceptors, that electroreceptors evolved from mechanosensory hair cells, or that both evolved independently from a common ancestral ciliated cell type (Bodznick, 1989).

Induction of the Lateral Line Placodes

Relatively little is currently known about the sources of lateral line placode-inducing signals, and nothing of their molecular nature (reviewed in Baker and Bronner-Fraser, 2001; Schlosser, 2002a). Lateral line placodes are induced separately from the otic placodes (section Otic Placodes), despite their proximity and even (in some species) their apparent derivation from a common $Pax2^+$ "dorsolateral placode area" (Schlosser and Northcutt, 2000; Schlosser, 2002a). Lateral line and otic placodes can be induced independently in grafting experiments, and ectodermal competence to form lateral line placodes persists much longer than that to form otic placodes (reviewed in Baker and Bronner-Fraser, 2001; Schlosser, 2002a). Furthermore, mutations in zebrafish can affect otic placodes but not lateral line placodes, and *vice versa* (Whitfield *et al*., 1996), and lateral line placodes have been lost multiple times in evolution with no effect on the otic placodes (e.g., Schlosser *et al*., 1999).

In the axolotl, lateral line placodes are determined (i.e., develop autonomously in ectopic locations) by late neural fold stages (Schlosser and Northcutt, 2001). Hence, lateral line placode-inducing signals must act before this time, although they persist until relatively late stages, as shown by grafts of non-placodal ectoderm to the placode-forming region of tailbud stage embryos (Schlosser and Northcutt, 2001). Grafting experiments in amphibians have implicated both mesoderm and neural plate as sources of lateral line placode-inducing signals (reviewed in Baker and Bronner-Fraser, 2001; Schlosser, 2002a). Their molecular nature is currently unknown. Migrating lateral line primordia in zebrafish express the FGF target genes, *erm* and *pea3* (Münchberg *et al*., 1999; Raible and Brand, 2001; Roehl and Nüsslein-Volhard, 2001), the Wnt receptor gene *Frizzled7a* (Sumanas *et al*., 2002), and the BMP inhibitor follistatin (Mowbray *et al*., 2001). Hence, FGF, Wnt, and BMP signaling may all be involved in aspects of lateral line placode development; however, any role for these signaling pathways in lateral line placode induction remains to be demonstrated. Early development and migration of lateral line placodes in *fgf8* mutant zebrafish appears normal, although the number of neuromasts formed is strongly reduced, suggesting an involvement of FGF8 at later stages (Léger and Brand, 2002).

Individual lateral line placodes differ in the number and type of sense organs that they form, and also in their gene expression patterns (e.g., only the middle lateral line placode expresses *Hoxb3* in the axolotl; Metscher *et al*., 1997). Additional inducing signals are presumably involved, therefore, in specifying individual lateral line placode identity, but these are wholly unknown.

Migration of Lateral Line Primordia

Cells of the lateral line primordia in amphibian embryos actively migrate through and displace the inner cells of the bilayered epidermis. Cell division also occurs within the primordia as they migrate (Winklbauer, 1989; Schlosser, 2002a). In zebrafish, lateral line primordia migrate just beneath the epidermis; time-lapse analysis of living embryos shows that each cell migrates independently, rather than the whole primordium moving as a solid block of cells, but they generally retain their neighbor relationships (Gompel *et al*., 2001). The clusters of undifferentiated cells that will form the neuromasts are deposited when a group of cells at the trailing edge of the primordium progressively slows down relative to the rest of the primordium (Gompel *et al*., 2001).

During normal development, lateral line primordia migrate along invariant pathways. When pre-otic and post-otic lateral line placodes are exchanged, they migrate along the pathway appropriate for their new position (reviewed in Schlosser, 2002a), suggesting that they are following extrinsic guidance cues. Several such guidance cues have now been identified in zebrafish. The chemokine SDF1 is expressed in a trail along the migration pathway of the posterior lateral line primordium (David *et al*., 2002). The migrating cells of the lateral line primordium express the SDF1 receptor, CXCR4, and inactivation of either the receptor or its ligand blocks migration (David *et al*., 2002). Also, the posterior lateral line primordium migrates along the trunk at the level of the horizontal myoseptum that divides the axial muscles into dorsal and ventral halves. Semaphorin3A1 (Sema3A1) is expressed throughout the somites except in the horizontal myoseptum, and when the myoseptum is missing, as in certain mutant strains, the lateral line primordium migrates aberrantly (Shoji *et al*., 1998). These results suggest that in addition to the SDF1-CSCR4 system, the primordium may be directed along the myoseptum by repulsive Sema3A1 migration cues from the dorsal and ventral somites (Shoji *et al*., 1998). Finally, the posterior lateral line primordium also expresses *robo1* (Lee *et al*., 2001), which encodes a receptor for the repulsive migration cue Slit (reviewed in Ghose and Van Vactor, 2002). Hence, Slit-Robo signaling may also be involved in guiding lateral line primordia along specific migration pathways.

The lateral line axons that track the migrating primordium are ensheathed in neural crest-derived glial cells, which lag slightly behind the axonal growth cones (Gilmour *et al*., 2002). These are not required for the growth or pathfinding of the lateral line axons, but their genetic ablation leads to defasciculation of the lateral line nerves (Gilmour *et al*., 2002). Hence, neural crest-derived glia are required for the organization of the lateral line nerves (Gilmour *et al*., 2002).

Cell Fate Determination Within Lateral Line Neuromasts

The bHLH *atonal* homologue *zath1* (section Proneural Genes: An Introduction) is progressively restricted to prospective mechanosensory hair cells in lateral line neuromasts in the zebrafish, suggesting that its expression defines the cells with the potential to form hair cells (Itoh and Chitnis, 2001). The mouse *ath1* homologue, *Math1*, is Specifically required for inner ear (otic placode-derived) hair cell formation (Bermingham *et al*., 1999) (section Hair Cell Specification Requires Notch Inhibition and *Math1*), so it is likely that *zath1* may similarly be required for lateral line hair cell formation. Determination of lateral line hair cell vs support cell fate probably involves Notch-mediated lateral inhibition (e.g., sections Differentiation of DRG Neurons Depends on Inhibition of Notch Signaling; Hair Cell Specification Requires Notch Inhibition and *Math1*): Expression of the Notch ligand DeltaB correlates with *zath1* expression in prospective hair cells, while *Notch3* expression is excluded from prospective hair cells (Itoh and Chitnis, 2001).

Otic Placodes

Otic Placode Derivatives

The paired otic placodes, which form in the ectoderm adjacent to the hindbrain (Figs. 3 and 10), invaginate to form closed otic vesicles (otocysts). In zebrafish, the placodes sink beneath the surface ectoderm and the vesicles form by cavitation (Whitfield *et al*., 2002). Each simple hollow epithelial ball undergoes profound morphogenetic changes to produce the highly complex, three-dimensional structure of the inner ear, or vestibular apparatus (membranous labyrinth) (Fig. 15). A neurogenic region

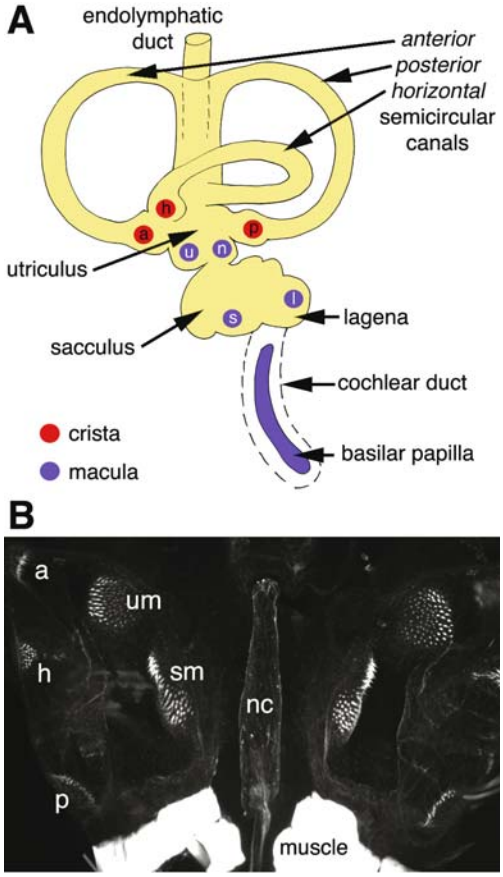

FIGURE 15. The complex structure of the inner ear. (A) Schematic showing a generalized vertebrate vestibular apparatus, with the three semicircular canals and major compartments: utriculus, sacculus, and lagena. The cochlear duct is an extension of the lagena that forms only in terrestrial vertebrates; hence it is shown as a dashed line. Specialized auditory hair cells are collected in a strip in the cochlear duct called the basilar papilla in birds, and the organ of Corti in the coiled mammalian cochlea. See section Otic Placode Derivatives for details. a, anterior crista; h, horizontal crista; l, lagenar macula; n, macula neglecta; p, posterior crista; s, saccular macula; u, utricular macula. Redrawn from Kardong (1998). (B) Confocal image of a 5-day zebrafish larva in dorsal view (rostral to the top), dissected to show the two ears on either side of the notochord (nc). The hair cells are visualized using fluorescent phalloidin, which binds the actin-rich stereocilia on the surface of each hair cell. Photograph courtesy of Dr. Tanya Whitfield, University of Sheffield, United Kingdom. a, anterior crista; h, horizontal (lateral) crista; nc, notochord; p, posterior crista; sm, saccular macula; um, utricular macula.

in each otic vesicle also gives rise to the sensory neurons that provide afferent innervation for the mechanosensory hair cells of the inner ear; these neurons are collected in the VIIIth cranial ganglion (vestibulocochlear/statoacoustic) (Fig. 11). The otic vesicle itself induces the formation of the cartilaginous otic capsule, which surrounds and protects the vestibular apparatus, from adjacent head mesenchyme (Frenz *et al*., 1994).

The vestibular apparatus contains three semicircular canals, oriented roughly in the three planes of space, and two or three relatively distinct chambers, the utriculus, sacculus and lagena (the latter being an extension of the sacculus) (Fig. 15A). These compartments all contain both non-sensory epithelium, and sensory vestibular epithelium containing neuromasts, that is, collections of mechanosensory hair cells and supporting cells. These are similar to those found in the lateral line system (section Lateral Line Placode Derivatives), except that otic neuromasts are usually much larger and contain many more hair cells than lateral line neuromasts. Each hair cell has a true cilium (kinocilium) with a bundle of stereocilia at its base; these are all embedded in a gelatinous cupula secreted by the supporting cells of the neuromast (Figs. 14A, B). Deflection of the cupula in a particular direction triggers depolarization of the hair cell, stimulating the afferent fibers of the sensory neurons that synapse onto the hair cell base. The high K^+/low Na^+ concentration of the endolymph filling the vestibular apparatus is essential for sensory tranduction by inner ear hair cells. Specialized epithelial cells within the vestibular apparatus (stria vascularis in mammals; tegmentum vasculosum in birds) secrete the endolymph.

Dilated ampullae at the base of each semicircular canal contain expanded neuromast organs, the cristae (Figs. 15A, B). When the head is turned, the semicircular canals are accelerated, but fluid inertia causes the endolymph to lag behind, relative to the semicircular canal itself, deflecting the cupula and stimulating the hair cells. The cristae therefore detect angular acceleration (rotation). Their afferent innervation is from otic placode-derived neurons in the vestibular part of the VIIIth ganglion (Fig. 11).

The utriculus and sacculus also contain large, modified neuromast organs, the maculae (Figs. 15A, B). The utricular macula in the adult zebrafish contains approximately 6,000 hair cells (Platt, 1993), which gives some idea of the size of these neuromasts. Each macula has dense crystals composed of protein and calcium carbonate, called otoliths or otoconia, embedded in the cupular surface (Riley *et al*., 1997). Otoliths intensify the displacements of the hair cells in response to linear acceleration. The maculae therefore detect gravity and linear acceleration. Like the cristae, their afferent innervation is provided by otic placode-derived vestibular ganglion neurons.

The maculae in the sacculus and lagena are also involved in hearing in all vertebrates, including fish, where compression waves cause movement of the maculae in relation to the otoliths resting on them. The lagena is lengthened in terrestrial vertebrates to form the cochlear duct, which is coiled in mammals (Fig. 15A). Cochlear auditory hair cells, which are often highly modified in structure, are collected in a specialized strip called the basilar papilla in birds and the organ of Corti in mammals. The afferent innervation for auditory hair cells is provided by otic placode-derived neurons in the auditory (cochlear) part of the VIIIth ganglion.

In summary, the entire vestibular apparatus, together with its afferent neurons, is derived from a simple epithelial ball, the otic vesicle, which in turn is derived from the otic placode. The formation of the inner ear is clearly a highly complex process, and only selected aspects will be discussed here. For more detailed analysis of otic morphogenesis, the reader is referred to several recent reviews (Torres and Giraldez, 1998; Rinkwitz *et al*., 2001; Whitfield *et al*., 2002).

Otic Placode Formation Involves Cell Movement and Convergence

Cell lineage analysis in the chick has shown that up to the 1-somite stage, otic placode precursors are found in a large region of ectoderm at the neural plate border, intermingled with precursors of neural tube, neural crest, epibranchial placodes (section Epibranchial Placodes), and epidermis (Streit, 2002). By the 4-somite stage, otic placode precursors extend from the level of the anterior hindbrain to the level of the first somite (Streit, 2002). By the 8-somite stage, a few hours before the otic placode is morphologically visible, quail-chick chimera analysis shows that prospective otic placode cells are found in a relatively small area adjacent to rhombomeres 5 and 6, just rostral to the first somite (Fig. 11) (D'Amico-Martel and Noden, 1983). A few hours later, at the 10-somite stage, the otic placode becomes morphologically visible.

Time-lapse video analysis of DiI-labeled ectodermal cells shows that the convergence of otic placode precursors to the final placode area results from extensive cell movement within the ectoderm (Streit, 2002). It is currently unclear whether this reflects active migration of otic-specified cells to the location of the future placode, or capture of randomly moving cells by progressively more localized otic placode-inducing signals (section Models of Individual Placode Formation in the Preplacodal Domain). *Pax2*, which is essential for proper otic placode development (Torres and Giraldez, 1998) is induced at the 4-somite stage in the broad region of ectoderm that contains otic precursors (Groves and Bronner-Fraser, 2000; Streit, 2002). However, not all cells within the *Pax2*$^+$ domain contribute to the otic placode: Even at the 7–10-somite stage, some cells in this domain contribute to epidermis, or to the epibranchial placodes, which also express *Pax2* (Fig. 11; section Epibranchial Placodes) (Streit, 2002). Hence, Pax2 expression does not correlate with determination toward an otic fate (also see Groves and Bronner-Fraser, 2000). Nonetheless, it remains to be seen whether there is directed migration of otic precursor cells toward the site of the future otic placode.

Interestingly, a fate-map of different sensory areas within the otic placode in *Xenopus* suggests that extensive cell movement continues within the otic placode and vesicle until fairly late stages (Kil and Collazo, 2001).

Induction of the Otic Placodes

More detailed information on the induction of the otic placodes can be found in various reviews (Baker and Bronner-Fraser,

2001; Noramly and Grainger, 2002; Whitfield *et al*., 2002; Riley and Phillips, 2003). Ablation and grafting experiments in a variety of species suggested that both mesendoderm and hindbrain are sources of otic placode-inducing signals. They led to a model in which the earliest otic placode-inducing signals are derived from mesendoderm, with later signals from the hindbrain. This model has been supported by experiments that have identified some of the signals involved in otic placode development.

Fgf3 is dynamically expressed in several vertebrates in rhombomeres 4–6 (r4–6), adjacent to the site of formation of the otic placodes (Fig. 11); however, otic vesicles form normally in *Fgf3*-null mice (Mansour *et al*., 1993). Experiments in zebrafish and mouse suggest that FGF3 acts redundantly with a second FGF family member to induce the otic placodes (Phillips *et al*., 2001; Maroon *et al*., 2002; Wright and Mansour, 2003). In zebrafish, simultaneous knockdown of FGF3 and FGF8 function using antisense morpholino oligonucleotides results in the loss of early otic markers such as *pax2.1* and *dlx3* (see previous section and section A Preplacodal Field at the Anterior Neural Plate Border) and of the otic vesicles (Maroon *et al*., 2002; Phillips *et al*., 2001). *Fgf3* and *Fgf8* are co-expressed in r4 in the zebrafish, but *Fgf8* is not expressed in the hindbrain in chick or mouse. However, at neural plate stages in the chick, *Fgf4* is co-expressed with *Fgf3* in prospective hindbrain neuroectoderm (Maroon *et al*., 2002), while *Fgf19* is expressed in paraxial mesoderm at same axial level (Ladher *et al*., 2000) (see below). In the mouse, *Fgf10* is expressed in the mesenchyme underlying the prospective otic placode, and mice lacking both *Fgf3* and *Fgf10* fail to form otic vesicles and show abnormal otic placode marker expression patterns (Wright and Mansour, 2003). Hence, FGF3 may cooperate with other FGF family members in different vertebrate species to induce expression of early otic markers such as Pax2, and the otic vesicles themselves.

Nonetheless, abrogation of FGF3 and FGF8 function in zebrafish does not affect expression of *Pax8*, the earliest known specific marker for prospective otic placode ectoderm (Maroon *et al*., 2002). *Pax8* expression, which normally appears at late gastrula/neural plate stages, is delayed in mutant zebrafish embryos that lack cranial mesendoderm (Mendonsa and Riley, 1999; Phillips *et al*., 2001). It is possible, therefore, that early signals from cranial mesendoderm normally induce *Pax8*, but that in the mutant embryos, *Pax8* expression is rescued by later, as-yet unidentified hindbrain-derived signals.

Foxi1, a member of the forkhead family of winged-helix transcription factors, is expressed prior to *pax8* and is required for *pax8* expression in zebrafish (Solomon *et al*., 2003). *Foxi1* mutant zebrafish show a severe reduction or loss both of *pax8* expression and the otic placodes, and *foxi1* misexpression induces ectopic *pax8* expression (Solomon *et al*., 2003). However, while necessary for *pax8* expression, *foxi1* is not in fact sufficient, because *pax8* is not expressed in every cell that expresses *foxi1* (Solomon *et al*., 2003). Hence, *pax8* expression requires additional regulatory factors besides Foxi1. Also, *foxi1* is expressed in and required for the development of the epibranchial placodes (section A Common Primordium for Epibranchial and Otic Placodes?), so *foxi1* is not specific to the otic placodes (Lee *et al*., 2003).

In the chick, Wnt8c from the hindbrain and FGF19 from the paraxial mesoderm have been suggested to be involved in otic placode induction (Ladher *et al*., 2000). However, loss of Wnt8c (via inhibition of retinoic acid signaling) does not affect otic vesicle formation in the chick, and FGF19 acts specifically through FGF receptor 4, whose loss does not affect otic placode development in the mouse (see discussion in Maroon *et al*., 2002).

In summary, signals from cranial mesendoderm and the hindbrain are involved in induction of the otic placodes. FGF3 from the hindbrain, acting redundantly with another FGF family member, is required for otic vesicle formation: In their absence, Pax8 expression in prospective otic territory is unaffected, but Pax2 is not expressed and subsequent otic development is blocked. Wnts may also be involved in otic placode development, but a requirement for Wnt signaling has not yet been demonstrated.

Neurogenesis in the Otic Vesicle Requires *Neurogenin1* and Notch Inhibition

The neuroblasts that will form the neurons of the vestibulocochlear (VIIIth) ganglion delaminate from the ventromedial region of the otic vesicle and aggregate nearby to form the ganglion (Fig. 11). All satellite glia within the ganglion are derived from the neural crest.

In the mouse, Atonal-related neural bHLH factors such as Neurogenin1 (Ngn1; section Proneural Genes: An Introduction) and NeuroD are expressed in epithelial cells within the otic vesicle prior to delamination (Ma *et al*., 1998). Vestibulocochlear neurons are entirely missing in *Ngn1*-null mice (Ma *et al*., 1998). *Ngn1* is required prior to the delamination of otic neuroblasts from the otic vesicle, since *NeuroD* and the expression of the Notch ligand Delta-like1 are both missing from the otic epithelium in *Ngn1*-null mice (Ma *et al*., 1998). Evidence that Notch inhibition (see section Proneural Genes: An Introduction) is involved in selection of neuronal cell fate within the otic vesicle comes from zebrafish carrying the *mindbomb* mutation: These embryos, in which Notch activation is blocked, have double the wildtype number of statoacoustic ganglion neurons (Haddon *et al*., 1998).

Hair Cell Specification Requires Notch Inhibition and *Math1*

Cell fate specification in the inner ear is discussed in more detail in several recent reviews (Fekete and Wu, 2002; Whitfield *et al*., 2002; Riley and Philllips, 2003). Cell fate specification within the sensory patches of the inner ear (the areas containing mechanosensory hair cells and supporting cells) depends on Notch signaling (section Proneural Genes: An Introduction). Well before hair cell differentiation occurs, prospective sensory patches are prefigured by their expression of Notch (initially expressed throughout the otic placode and later restricted to sensory epithelium) and its ligands Delta and Serrate. Delta expression eventually becomes restricted to nascent hair cells. Cells with low levels of Notch activity differentiate as hair cells, while

Notch activation leads to supporting cell differentiation (cf. glial differentiation, e.g., section Differentiation of DRG Satellite Cells Depends on Notch Activation and Instructive Gliogenic Cues) (reviewed in Eddison *et al*., 2000; Fekete and Wu, 2002; Whitfield *et al*., 2002). Numerous lines of evidence support this model. For example, in *mindbomb* mutant zebrafish (where Notch activation is blocked and cells are "deaf" to Delta signaling), sensory patch cells differentiate as hair cells at the expense of supporting cells (Haddon *et al*., 1998). The Notch effector Hes1, a bHLH transcriptional repressor (Davis and Turner, 2001), negatively regulates hair cell production: *Hes1*-null mice have extra inner ear hair cells (Zheng *et al*., 2000). Finally, the Notch antagonist Numb is expressed at high levels in hair cells in the chick (Eddison *et al*., 2000).

The mouse Atonal homologue Math1 (section Proneural Genes: An Introduction) is both necessary and sufficient for hair cell differentiation, as shown by knockout and overexpression studies (Bermingham *et al*., 1999; Zheng and Gao, 2000). Math1 expression first begins in nascent hair cells; it is not required to set up the area that will form a sensory patch (Chen *et al*., 2002). Hence, Math1 may specify hair cell identity in the inner ear.

Lens Placodes

Lens Placode Derivatives

The lens placodes are unusual among the cranial ectodermal placodes, as they do not produce either sensory receptor cells or neurons. Where the evaginating optic vesicles approach the overlying surface ectoderm, it thickens to form the lens placodes; these invaginate and pinch off to form the eye lenses. The newly formed lenses have a distinct polarity, maintained throughout life, with proliferating cuboidal cells covering the anterior surface, and terminally differentiated lens fiber cells making up the bulk of the lens. Successive layers of lens fiber cells differentially accumulate highly stable, soluble proteins called crystallins, giving a smooth decreasing gradient of refractive index from the center to the periphery.

The Importance of Pax6 for Lens Placode Development

Pax6 has been implicated in eye and anterior head development in all major animal groups. The Pax/Six/Eya/Dach regulatory network (section The Pax/Six/Eya/Dach Regulatory Network) was first identified in studies of the *Pax6* homologue *eyeless* in *Drosophila* eye development (Wawersik and Maas, 2000). In vertebrates, Pax6 is essential for both lens placode and retinal development (reviewed in Ashery-Padan and Gruss, 2001; Baker and Bronner-Fraser, 2001). Pax6 is initially expressed in a broad region of head ectoderm and is eventually restricted to the lens placode itself. The homeobox transcription factors Meis1 and Meis2 are direct upstream regulators of Pax6 expression in lens ectoderm (Zhang *et al*., 2002). Knockout experiments in mice have shown that Pax6 is required in head ectoderm both for competence to respond to a lens-inducing signal from the optic vesicle (see next section) and also for subsequent steps in lens placode development (reviewed in Ashery-Padan and Gruss, 2001; Baker and Bronner-Fraser, 2001). Pax6 is required for the upregulation of the HMG-domain transcription factor Sox2 (and/or Sox1, Sox3) in prospective lens ectoderm after it is contacted by the optic vesicles: These genes are essential for lens differentiation (Baker and Bronner-Fraser, 2001). Sox2 and Pax6 act together in subsequent steps of lens differentiation, by cooperatively binding *crystallin* gene enhancers and activating their expression (Kamachi *et al*., 2001). Pax6 can induce ectopic lenses (and eyes) in *Xenopus* head ectoderm (Altmann *et al*., 1997; Chow *et al*., 1999), although in chick head ectoderm, ectopic lens induction requires both Pax6 and Sox2 (Kamachi *et al*., 2001). Hence, Pax6 is necessary, though not sufficient, for lens formation.

Induction of the Lens Placodes

More detailed descriptions of lens placode induction can be found in recent reviews (Ogino and Yasuda, 2000; Ashery-Padan and Gruss, 2001; Baker and Bronner-Fraser, 2001). Classical grafting experiments demonstrated that anterior mesendoderm, anterior neural plate, and the optic vesicles may all play roles in lens placode induction (reviewed in Baker and Bronner-Fraser, 2001). Some of the signals from the optic vesicles have now been identified. Knockout experiments in mice have shown that optic vesicle-derived BMP7 is required for *Sox2* expression and *Pax6* maintenance in presumptive lens ectoderm (Wawersik *et al*., 1999). FGF8 is expressed in the optic vesicles in the chick and can induce lens placode markers (Vogel-Höpker *et al*., 2000), and genetic block of FGF signaling in prospective lens ectoderm in the mouse leads to defects in lens formation (Faber *et al*., 2001). Presumptive lens ectoderm in the mouse also receives retinoic acid signals, as demonstrated by the activation of retinoic acid-responsive transgenes (Baker and Bronner-Fraser, 2001). Finally, optic vesicle-derived BMP4 is involved in a somewhat later phase of lens placode formation, downstream of Pax6 (Furuta and Hogan, 1998).

Lens Fiber Differentiation

Factors that induce lens fiber differentiation are found in the vitreous and aqueous humors of the eye, and several different families of growth factors have been implicated in lens fiber differentiation. FGFs can stimulate the differentiation of lens fiber cells from lens epithelial cells (Govindarajan and Overbeek, 2001), and transgenic expression of dominant negative FGF receptors in mouse lenses or eyes leads to delayed lens fiber differentiation and apoptosis (Robinson *et al*., 1995; Govindarajan and Overbeek, 2001). Transgenic expression of dominant negative TGFβ receptors in the mouse lens also disrupts lens fiber differentiation, suggesting a role for TGFβ family members as well as FGFs (de Iongh *et al*., 2001). Finally, retroviral-mediated overexpression of the BMP antagonist Noggin in chick eyes delays lens fiber development and results in lens cell death, suggesting that BMPs are also involved in lens fiber differentiation and survival (Belecky-Adams *et al*., 2002).

Transgenic expression of a dominant negative BMP type I receptor in the mouse eye also leads to defects in lens fiber formation (Faber *et al*., 2002). Hence, FGF, TGFβ, and BMP family members may all be involved in triggering lens fiber differentiation.

TRIGEMINAL AND EPIBRANCHIAL PLACODES

The trigeminal and epibranchial placodes (Figs. 10 and 11) do not contribute to the paired sense organs. However, trigeminal placode-derived neurons are important for touch, pain, and temperature sensations from the head, including the jaws and teeth, while epibranchial placode-derived neurons provide afferent innervation for taste buds, and autonomic afferent innervation for the visceral organs. The trigeminal placodes form in the surface ectoderm adjacent to the midbrain and rostral hindbrain, while the epibranchial placodes form above each pharyngeal (branchial) cleft (Figs. 3 and 10).

Trigeminal Placodes

Trigeminal Placode Derivatives

The sensory trigeminal ganglion complex of cranial nerve V is formed in most craniates by the fusion of two separate ganglia during development: the ophthalmic trigeminal (opV; sometimes called profundal) and maxillomandibular trigeminal (mmV; sometimes called gasserian) ganglia. The neurons in the trigeminal ganglion are of mixed origin, being derived both from neural crest and from the two separate trigeminal placodes (opV and mmV) (see Figs. 10 and 11). All satellite glial cells in the ganglion are derived from the neural crest. In the chick, both the opV lobe and the mmV lobe of the trigeminal ganglion contain large-diameter placode-derived neurons distally, and small-diameter neural crest-derived neurons proximally (Hamburger, 1961; D'Amico-Martel and Noden, 1983) (Fig. 11).

Trigeminal ganglion neurons are primary sensory neurons, like those in the dorsal root ganglia, transmitting cutaneous (touch, pain, and temperature) information from the skin and proprioceptive information from muscles. Neurons in the opV lobe/ganglion innervate the head, including the nose and eyeballs, while neurons in the mmV lobe/ganglion innervate the lower face, jaws, tongue, and teeth. Cutaneous neurons are derived from both the neural crest and the two placodes, while proprioceptive neurons seem only to be derived from the neural crest, at least in the chick (Noden, 1980). (Most of the proprioceptive neurons that innervate the jaws are found in the mesencephalic nucleus of the trigeminal nerve (mesV), which seems to be a neural crest-derived sensory ganglion within the brain; see section Neural Crest Derivatives.)

In fish and amphibians, trigeminal neurons are born very early and make up part of the primary nervous system that mediates swimming reflexes. Judging by their position, lateral to the *FoxD3*$^{+}$ neural crest domain in zebrafish (e.g., Kim *et al*., 2000; Andermann *et al*., 2002; Itoh *et al*., 2002), these early-born trigeminal neurons are placode-derived. Like all other placode-derived neurons in the zebrafish, they express the *atonal*-related proneural bHLH gene *ngn1* (section Proneural Genes: An Introduction) (Andermann *et al*., 2002). Their early differentiation is consistent with the early birth of placode-derived trigeminal neurons relative to that of neural crest-derived neurons in other vertebrates, such as the chick (D'Amico-Martel and Noden, 1980).

Induction of the Trigeminal Placodes

The trigeminal placodes form in the surface ectoderm adjacent to the midbrain and rostral hindbrain (Figs. 3 and 10; D'Amico-Martel and Noden, 1983). For more detailed information about classical experiments on induction of the trigeminal placodes, see Baker and Bronner-Fraser (2001). Very little is known about the formation of the mmV placode. More information is available on induction of the opV placode in the chick, which begins to express Pax3 from the 4-somite stage (Stark *et al*., 1997). Pax3 expression correlates with the determination of opV placode-derived cells to adopt a cutaneous sensory neuron fate (Baker and Bronner-Fraser, 2000; Baker *et al*., 2002). The importance of Pax3 is shown by the severe reduction of the opV lobe of the trigeminal ganglion in mice carrying a mutated *Pax3* gene (Tremblay *et al*., 1995). Barrier implantation and coculture experiments in the chick have shown that Pax3 is induced in head ectoderm by an unidentified neural tube-derived signal (Stark *et al*., 1997; Baker *et al*., 1999). The Pax3-inducing signal is produced along the entire length of the neuraxis; however, restriction of Pax3 expression to the forming opV placode may result, at least in part, from spatiotemporal changes in ectodermal competence to respond to this signal (Baker *et al*., 1999).

Experiments in the zebrafish have shown that homologues of the Iroquois family of homeodomain transcription factors, which are required for the expression of proneural *achaete-scute* genes in *Drosophila* (section Proneural Genes: An Introduction), are involved in the formation of the trigeminal placodes (Itoh *et al*., 2002). Zebrafish *iro1* and *iro7* are expressed at neural plate stages in a region of neuroectoderm extending from the midbrain to r4 (Itoh *et al*., 2002). As somitogenesis begins, the expression of both genes expands into the ectoderm where the trigeminal placodes form, as defined by expression of the *atonal* homologue *neurogenin1* (*ngn1*) (Itoh *et al*., 2002) (see next section). Functional knockdown of Iro7 (though not Iro1) using antisense morpholino oligonucleotides leads to loss of *ngn1* expression in the trigeminal placode (Itoh *et al*., 2002). *Ngn1* in the mouse is essential for neurogenesis in the trigeminal placodes (see next section). Hence, *iro7* is required for trigeminal placode-derived neurogenesis.

The rostral border of *iro1* and *iro7* expression in the trigeminal placode ectoderm is expanded rostrally in zebrafish mutants with increased Wnt signaling (Itoh *et al*., 2002); this correlates with the rostral expansion of *ngn1*$^{+}$ trigeminal placode-derived neurons seen in such mutants (Kim *et al*., 2000; Itoh *et al*., 2002). Wnt signaling may, therefore, be involved in trigeminal placode induction and/or neurogenesis. Several Wnt receptors are expressed broadly in rostral head ectoderm at appropriate stages in the chick (Stark *et al*., 2000).

In the chick, the FGF receptor FREK is expressed in the opV placode, but only from the 10-somite stage, well after initial induction of Pax3 (Stark *et al*., 1997). It continues to be expressed in delaminating neuroblasts, but is not maintained after gangliogenesis (Stark *et al*., 1997). FGF family members may, therefore, play a role in trigeminal placode-derived cell migration.

Neurogenesis in the Trigeminal Placodes Requires *Neurogenin1*

In zebrafish, the trigeminal placodes are first detectable by *ngn1* expression in lateral patches of ectoderm at late gastrula stages; antisense morpholino-mediated functional knockdown of Ngn1 completely abrogates formation of the trigeminal ganglia (Andermann *et al*., 2002). In the mouse, *Ngn1* is expressed in subsets of cells in the trigeminal placodes, in delaminating trigeminal neuroblasts, and in condensing trigeminal ganglion neurons in the mouse (Ma *et al*., 1998). *Ngn2* is weakly expressed in the trigeminal ganglion well after *Ngn1* (Fode *et al*., 1998; Ma *et al*., 1998), and *Ngn2*-null mice have no trigeminal ganglion defects (Fode *et al*., 1998). In contrast, the trigeminal ganglia are totally absent in *Ngn1*-null mice (Ma *et al*., 1998). *Ngn1* is required in the trigeminal placodes for neuroblast delamination, and for expression of downstream neural bHLH genes, such as the *atonal*-related *NeuroD* family members *NeuroD* and *Math3*, the *achaete-scute*-related gene *NSCL1*, and *Ngn2* (Ma *et al*., 1998) (for family relationships of proneural genes, see Bertrand *et al*., 2002). *Ngn1* is also required for expression of the Notch ligand *Delta-like1* in the trigeminal placodes (Ma *et al*., 1998); Delta–Notch signaling is presumably also involved in the selection of neuronal fate (section Proneural Genes: An Introduction). *Notch* expression within the trigeminal placodes is seen at the same time as *Ngn1* expression (Reaume *et al*., 1992). However, abrogation of Notch signaling (in mice with mutations in a transcriptional effector of the Notch signaling pathway) has no effect on the initial expression of *Ngn1* in the trigeminal placodes (Ma *et al*., 1998). Hence, the establishment of *Ngn1* expression in the trigeminal placodes is independent of Notch signaling.

The total absence of the trigeminal ganglion in *Ngn1*-null mice is due not only to trigeminal placode defects: Neural crest cells condense to form the trigeminal ganglionic primordium in *Ngn1*-null mice, but fail to form neurons (Ma *et al*., 1998). The other proximal cranial sensory ganglia, whose neurons are all derived from the neural crest (Fig. 11), also fail to form in *Ngn1*-null mice (Ma *et al*., 1998).

Interactions Between Neural Crest-Derived and Placode-Derived Trigeminal Cells in Gangliogenesis

Placode-derived neurons differentiate before neural crest-derived neurons in the trigeminal ganglion (D'Amico-Martel and Noden, 1980). However, the first ganglionic condensation is made up of neural crest cells, which are only later joined by placode-derived neurons (Covell and Noden, 1989). Neural crest cells are not required for induction at least of the opV placodes (Stark *et al*., 1997), and their ablation delays, but does not abolish, gangliogenesis and pathfinding by placode-derived trigeminal neurons (Hamburger, 1961; Moody and Heaton, 1983b). In the absence of neural crest cells, the placode-derived ganglia tend to remain as two separate ganglia, suggesting the neural crest cells act as an aggregation center for ganglionic fusion (Yntema, 1944; Hamburger, 1961). In contrast, when the trigeminal placodes are ablated, neural crest-derived trigeminal neurons do not make appropriate peripheral projections (Hamburger, 1961; Lwigale, 2001). Furthermore, the central projections of trigeminal placode-derived neurons are required for trigeminal motor neuron migration and axonal projection (Moody and Heaton, 1983a, b).

Epibranchial Placodes

Epibranchial Placode Derivatives

The epibranchial placodes form above the pharyngeal (branchial) clefts (section Pharyngeal Arches and Neural Crest Streams; Figs. 3 and 10). The first epibranchial placode (facial or geniculate) forms above the first pharyngeal cleft, and gives rise to sensory neurons in the distal (geniculate) ganglion of cranial nerve VII (facial) (Fig. 11). These neurons primarily provide afferent innervation for the taste buds. The second epibranchial placode (glossopharyngeal or petrosal) forms above the second pharyngeal cleft and gives rise to sensory neurons in the distal (petrosal) ganglion of cranial nerve IX (glossopharyngeal) (Fig. 11). These neurons provide afferent innervation for taste buds, and afferent autonomic innervation for visceral organs such as the heart. The third epibranchial placode (vagal or nodose) forms above the third pharyngeal cleft, and gives rise to sensory neurons in the distal (nodose) ganglion of cranial nerve X (vagal) (Fig. 11). These neurons primarily provide afferent autonomic innervation for the heart and other visceral organs. Additional vagal epibranchial placodes form above more posterior pharyngeal clefts and contribute neurons to the nodose ganglion or ganglia (see Baker and Bronner-Fraser, 2001). Satellite cells in all of these ganglia are derived from the neural crest (Narayanan and Narayanan, 1980).

The geniculate placode in nonteleost fish and birds has also been described as giving rise to a pouch-like sense organ associated with the first pharyngeal cleft, lined with mechanosensory hair cells (Vitali, 1926; Yntema, 1944; D'Amico-Martel and Noden, 1983; Baker and Bronner-Fraser, 2001). This organ appears to have been lost in teleosts, amphibians, reptiles, and mammals. In nonteleost fish, this "spiracular organ" is considered to be a specialized lateral line organ (reviewed in Barry and Bennett, 1989). However, if it is indeed derived from the geniculate placode and not from a lateral line placode, then it would appear that epibranchial placodes are able to form not only sensory neurons, but also mechanosensory hair cells like those of the inner ear and lateral line.

A Common Primordium for Epibranchial and Otic Placodes?

In the 10-somite stage chick embryo, the HMG-domain transcription factor Sox3, which labels the thickened ectoderm of the neural plate and cranial ectodermal placodes, is expressed in two narrow domains near the otic placode (Ishii *et al*., 2001). One of these contains the otic placode itself plus prospective geniculate placode ectoderm; the other, more ventrocaudal domain, fate-maps to the petrosal and nodose placodes (Fig. 11) (Ishii *et al*., 2001). Ectoderm that will eventually form the epibranchial placodes remains thickened and retains *Sox3* expression, while the ectoderm between the placodes thins and loses *Sox3* expression (Ishii *et al*., 2001). These results suggest that a broad domain of thickened ectoderm is partitioned into the different epibranchial placodes in the chick.

Intriguingly, the broad domain of *Sox3*$^+$ ectoderm that eventually forms the geniculate placode also contains the otic placode (Ishii *et al*., 2001). Pax2 is also expressed in a broad region of ectoderm that includes precursors of both the otic and epibranchial placodes in the chick (Groves and Bronner-Fraser, 2000; Streit, 2002) (section Otic Placode Formation). It has been suggested in *Xenopus* that the Pax2$^+$ "dorsolateral placode area," which includes otic and lateral line placodes (section Lateral Line Placode Derivatives), may also include the epibranchial placodes (Schlosser, 2002a), although this remains to be demonstrated. Furthermore, the winged-helix transcription factor Foxi1, which is required for otic placode formation (Solomon *et al*., 2003; section Induction of the Otic Placodes) is also expressed in and required for epibranchial placode development (Lee *et al*., 2003) (section Neurogenesis in the Epibranchial Placodes). The domain of *foxi1* expression in the zebrafish has been described as a "lateral cranial placodal domain" that encompasses otic and epibranchial placodes (Lee *et al*., 2003). As described in the previous section, the geniculate placode may form mechanosensory hair cells, like otic and lateral line hair cells, during normal development in chick and nonteleost fish. It is possible, therefore, that the close spatial association of epibranchial placodes with the otic placodes, together with their shared expression of Pax2, might reflect previously unrecognized embryonic and, potentially, evolutionary relationships. However, additional evidence is required to support this hypothesis.

Induction of the Epibranchial Placodes

In all vertebrate species, epibranchial placode formation occurs in close spatiotemporal association with (1) contact between the outpocketing pharyngeal endoderm and the overlying surface ectoderm, and (2) migrating neural crest streams (Fig. 3; Baker and Bronner-Fraser, 2001). Mechanical and genetic ablation experiments have shown that neural crest cells are not required for the formation of the epibranchial placodes (Yntema, 1944; Begbie *et al*., 1999; Gavalas *et al*., 2001). Instead, signals from the pharyngeal endoderm seem to be important, at least for the induction of neurogenesis within the epibranchial placodes in the chick (Begbie *et al*., 1999). Pharyngeal endoderm is sufficient to induce epibranchial neurons (*Phox2a*$^+$; see next section) from non-placode-forming chick head ectoderm *in vitro* (Begbie *et al*., 1999). BMP7, which is produced by pharyngeal endoderm, is also sufficient to induce epibranchial neurons from this ectoderm *in vitro* (Begbie *et al*., 1999). Furthermore, the BMP7 inhibitor follistatin reduces neuronal induction by pharyngeal endoderm *in vitro*, suggesting that BMP7 might be the pharyngeal endoderm-derived signal *in vivo* (Begbie *et al*., 1999). Nonetheless, pharyngeal endoderm cannot induce epibranchial neurons from trunk ectoderm (Begbie *et al*., 1999), which is competent to make nodose placode neurons when grafted to the nodose placode region (Vogel and Davies, 1993). Hence, additional signals in the pharyngeal region must enable trunk ectoderm to form epibranchial neurons in response to signals from pharyngeal endoderm.

Neurogenesis in the Epibranchial Placodes Requires *Neurogenin2, Phox2b,* and *Phox2a*

The bHLH proneural transcription factor Neurogenin2 (Ngn2) (section Proneural Genes: An Introduction) is expressed in epibranchial placodes and delaminating cells prior to overt neuronal differentiation in the mouse (Fode *et al*., 1998). In *Ngn2*-mutant mice, geniculate and petrosal placode-derived cells fail to delaminate, migrate, or differentiate (Fode *et al*., 1998). In the nodose placode, which develops normally in *Ngn2* mutants, *Ngn2* may act redundantly with *Ngn1* (Fode *et al*., 1998; Ma *et al*., 1998). In all three epibranchial placodes, Ngn2 is required for *Delta-like1* expression, suggesting that Notch–Delta signaling is also involved in epibranchial placode-derived neurogenesis (Fode *et al*., 1998).

In the zebrafish, Ngn1 seems to encompass all functions of murine Ngn1 and Ngn2, and *ngn1* is expressed in the epibranchial placodes (Andermann *et al*., 2002). All peripheral ganglia, including the epibranchial placode-derived ganglia, are missing after antisense morpholino-mediated functional knockdown of Ngn1 (Andermann *et al*., 2002). The winged-helix transcription factor Foxi1, which is expressed prior to *ngn1* in prospective epibranchial placode ectoderm, is required for *ngn1* expression in the epibranchial placodes (Lee *et al*., 2003).

As described in the section Phox2b Is Essential for the Formation of All Autonomic Ganglia, the paired-like homeodomain transcription factor Phox2b is required for the development of all autonomic ganglia, including the epibranchial placode-derived ganglia (Pattyn *et al*., 1999). The neurons in these ganglia provide autonomic afferent innervation to the visceral organs and transiently express the noradrenergic markers tyrosine hydroxylase and dopamine β-hydroxylase (DBH) (Fig. 9) (e.g., Katz and Erb, 1990; Morin *et al*., 1997). As described in section *Phox2b* Is Required for Development of the Noradrenergic Phenotype, Phox2b and the related factor Phox2a directly activate the *DBH* promoter (reviewed in Brunet and Pattyn, 2002; Goridis and Rohrer, 2002). *Phox2b*-mutant mice show severe apoptotic atrophy of all three epibranchial placode-derived ganglia (Pattyn *et al*., 1999).

In epibranchial placode-derived ganglia, unlike sympathetic ganglia (section *Phox2b* Is Required for Development of the Noradrenergic Phenotype), *Phox2a* lies genetically upstream of *Phox2b*, which is in turn required for *DBH* expression (Pattyn *et al*., 1999, 2000). *Phox2a* is not required for delamination or aggregation of epibranchial placode-derived cells, or for the expression of certain neuronal markers, but is required for *DBH* and *Ret* expression (hence probably for neuronal survival in response to the Ret ligand GDNF) (Morin *et al*., 1997). *Phox2a*-mutant mice show severe atrophy of the petrosal and nodose ganglia, while the geniculate ganglion is relatively unaffected (Morin *et al*., 1997), perhaps via redundancy with Phox2b. Like sympathetic neurons (section BMPs Induce Both Mash1 and Phox2b in Sympathetic Precursors), a BMP family member, in this case BMP7, is able to induce *Phox2a* expression in head ectoderm (see previous section) (Begbie *et al*., 1999).

Interactions Between Neural Crest-Derived and Epibranchial Placode-Derived Cells in Gangliogenesis

Although neural crest cells are not required for epibranchial placode formation or neurogenesis (Yntema, 1944; Begbie *et al*., 1999; Gavalas *et al*., 2001), they seem to play an important role in guiding the migration and projection patterns of epibranchial placode-derived neurons (Begbie and Graham, 2001b). After neural crest ablation, epibranchial placode-derived neurons remain subectodermal and make aberrant projections (Begbie and Graham, 2001b).

It is possible that some neural crest cells initially form neurons in the epibranchial ganglia in the chick (Kious *et al*., 2002), although these presumably die, as they are not seen at later stages of development (D'Amico-Martel and Noden, 1983). Neural crest cells can also compensate to some extent for loss of the epibranchial placodes. Neural crest cells may form neurons in the geniculate ganglion in *Ngn2*-mutant mice, which lack epibranchial placode-derived neurons (Fode *et al*., 1998). Also, neural crest cells from the same axial level as the nodose placode can form neurons in the nodose ganglion after the nodose placode is ablated (Harrison *et al*., 1995). However, these neurons may not substitute functionally for nodose placode-derived neurons, as nodose placode-ablated embryos have abnormal cardiac function (Harrison *et al*., 1995).

PLACODE SUMMARY

Cranial ectodermal placodes are, at first sight, a disparate collection of embryonic structures, united by their early-thickened morphology and association with the paired sense organs and/or cranial sensory ganglia. Each individual placode gives rise to very different derivatives, from mechanosensory hair cells to lens fibers to visceral sensory neurons. However, some early steps in placode induction may be common to all placodes. They share a common origin from a preplacodal field of ectoderm around the anterior border of the neural plate that can be identified molecularly and, in some species, morphologically. The Pax/Six/Eya/Dach genetic regulatory network seems to be active in all placodes, with different combinations of Pax genes, in particular, expressed in different placodes and possibly serving to determine placode identity. Recent evidence suggests that there is a substantial degree of cell movement within the pre-placodal field. Individual placodes may form within this field either by differential cellular responses to widespread inducing signals and active convergence to the forming placode, or by the "trapping" of randomly moving cells by localized placode-inducing signals. Current evidence cannot distinguish between these two hypotheses. Each individual placode seems to be induced by a different combination of tissues (neural tube, pharyngeal endoderm, paraxial mesoderm, etc) and molecules: where identified, the latter include members of the BMP, FGF, and Wnt families. Neurogenesis within all neurogenic placodes involves one or both Ngns, and probably Delta–Notch signaling, showing clear parallels with sensory neurogenesis in the neural crest. As is the case for autonomic neural crest-derived neurons, Phox2a and Phox2b are required for the transient expression of the catecholaminergic phenotype within epibranchial placode-derived neurons, which provide afferent autonomic innervation to the visceral organs.

As should be evident from this section of the chapter, great strides have been made in our understanding of placode induction and development, particularly with the application of molecular techniques. However, there is much still to learn, from the earliest stages of placode induction at the neural plate border, to the final patterning and morphogenesis of their diverse derivatives.

OVERALL SUMMARY

Hopefully, this chapter has succeeded in giving a flavor of the complexity that underlies the induction and development of the neural crest and cranial ectodermal placodes. The neural crest forms the entire PNS in the trunk, while placodes are essential for the formation of the paired peripheral sense organs and most cranial sensory neurons. Although for the most part they have been treated separately, it is important to realize that neural crest and placodes do not develop in isolation from one another. As discussed in the preceding sections, placode-derived neurons in cranial sensory ganglia are supported by neural crest-derived satellite glia. Neural crest-derived trigeminal neurons need placode-derived trigeminal neurons in order to make appropriate peripheral projections. Migrating streams of cranial neural crest cells are required for proper migration of epibranchial placode-derived neurons. Hence, both the formation and interaction of placodes and neural crest cells are essential for the development of a fully functional peripheral nervous system. The mutual interdependence of these two cell populations reflects their long evolutionary history together: Both neural crest and placodes are present in hagfish, the most primitive extant craniate.

Since the last edition of this book, in 1991, our understanding of the induction and development of both neural crest and cranial ectodermal placodes has advanced in leaps and bounds. It is to be hoped that the next decade will prove similarly fruitful.

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