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Molecular Regulation of Neural Crest Development

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Abstract

The neural crest is a transient embryonic structure that gives rise to a multitude of different cell types in the vertebrate. As such, it is an ideal model to study the processes of vertebrate differentiation and development. This review focuses on two major questions related to neural crest development. The first question concerns the degree and time of commitment of the neural crest cells to different cell lineages and the emerging role of the homeobox containing genes in regulating this process. Evidence from the cephalic crest suggests that the

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commitment process does start before the neural crest cells migrate away from the neural tube and gene ablation experiments suggest that different homeobox genes are required for the development of neural and mesenchymal tissue derivatives. However, clonal analysis of neural crest cells before migration suggests that many of the cells remain multi-potential indicating that the final determinative steps occur progressively during migration and in association with environmental influences. The second question concerns the nature of the environmental factors that determine the differentiation of neural crest cells into discrete lineages. Evidence is provided, mainly from in vitro experiments, that purified growth factors selectively promote the differentiation of neural crest cells down either sympathetic, adrenal, sensory, or melanocytic cell lineages.

Index Entries: Neural crest, cell differentiation, cell proliferation, homeobox genes, *Hox* genes, *Pax* genes, sympathetic neurons, sensory neurons, melanocytes, fibroblast growth factor, leukemia inhibitory factor, *Steel* factor.

Introduction

The neural crest is a transient structure that arises from the dorsolateral aspect of the closing neural tube. The cells migrate along several discrete tissue pathways and give rise to the majority of cells of the peripheral nervous system, both neuronal and glial, melanocytes, and adrenal medullary cells. In the cephalic region, crest cells give rise to additional cell types, including facial mesenchyme derivatives and branchial mesenchymal structures. In addition to forming structures it also influences the development of structures such as the thymus. Thus, studying the regulation of neural crest development has not only profound relevance to neural development but also to the development of the whole animal. As a consequence many of the major questions raised in crest development are identical to those concerning broader aspects of developmental biology.

In this review, two of what we consider to be the major questions in crest development will be discussed. First, we explore whether the crest cells are multipotential or committed to a particular cell lineage; if committed, then we explore what may influence this state. Second, we discuss the role of environmental influences, in particular growth factors, in regulating the proliferation and subsequent differentiation of migrating neural crest cells into discrete cell types.

Many of the more general issues concerning crest development have been discussed previously in a number of excellent books and review articles, including Horstadius, 1950; Le Douarin, 1982,1986; Le Douarin and Smith, 1988; Weston, 1986,1991; Noden, 1986; Newgreen and Erikson, 1986; Hall and Horstadius, 1988; and Anderson, 1989.

Fate Map of the Neural Crest

One early problem in the study of neural crest ontogeny was tracing the neural crest cells as they migrated through the embryo. Early studies involving extirpation of the neural crest in a variety of experimental animals led to the identification of some of the neural crest derivatives (*see* Le Douarin, 1982). Later, Weston and Johnston pioneered the technique of tagging the neural crest cells, in this case with ^{3}H-thymidine, and following their fate through the embryo (Weston, 1963 and review in Weston, 1986). Perhaps the most definitive studies of the fate of the neural crest came from the use of the chick-quail marker system (reviewed in Le Douarin, 1982,1986; Noden, 1978,1980; Le Douarin and Smith, 1988). In this system, chimeras were made by replacing a particular region of an embryo of one species with the same region from the other species. These chimeras remain viable at least until birth, and the cells of the donor can be identified on the basis of structural differences in the interphase nuclei between the two species.

Le Douarin and colleagues transplanted fragments of either the entire neural primordium (that is, fragments of the neural tube with associated neural crest) or, at the cephalic level, the neural folds alone containing the neural crest cells. This approach, over a period of 10 yr or more has led to the construction of a fate map of the neural crest. This map showed that there are discrete regions of the crest that give rise to particular ganglia and other neural crest derived structures. The fate map demonstrates that the direction of most neural crest cell migration is lateral to the neural tube and thus the resultant neural crest

derivatives correspond to their original position along a rostro-caudal axis. For example, the adrenal medullary cells originate from the spinal neural crest between the level of somites 18–24; the spinal neural crest caudal to somite 5 gives rise to the ganglia of the sympathetic chains; the ciliary ganglion is derived from the mesencephalic neural crest; and the mesectodermal derivatives are derived from the rostral regions of the neural crest and are mainly located in the head and neck.

Commitment vs Multipotentiality

Grafting Experiments Show Populations of Neural Crest Cells Are Multipotential

The fate map described above refers to the normal developmental potential of the neural crest as the grafting experiments described were both isotopic and isochronic. However, in other experiments neural primordia or neural folds were transplanted to different regions (heterotopic) of the host embryo to determine their full developmental potential (*see* Le Douarin, 1982). The results showed that in general, the location of the grafted cells in the chimeric embryo and not their origin determined their developmental fate. For example, vagal crest cells (which normally contribute to parasympathetic innervation of the gut) grafted to the level of somites 18–24 differentiated into adrenergic cells in the sympathetic ganglia and adrenal medulla (the normal derivatives of the crest of this region). In the reverse experiment, where the presumptive adrenomedullary neural crest cells were transplanted into the vagal region, enteric ganglia containing both cholinergic and peptidergic neurons were formed. A range of such experiments established that in most cases it is the embryonic environment of the neural crest cells that determines their differentiated phenotype and that the crest cells are multipotential. It is important to note, however, that this type of analysis reflects the differentiation potential of a population of cells and can be explained as either the selection of different populations of partially committed cells or the multipotentiality of individual crest cells.

There are some significant exceptions to the perceived multipotentiality of neural crest cells. It is only the cephalic regions of the neural crest that can give rise to ectomesenchymal derivatives, such as bone, smooth muscle, adipose tissue, meninges, and endothelial cells, that are exclusively located in the head and upper body. Further, if chick midbrain neural crest, which normally migrates to the first (mandibular) arch, is grafted to the second (hyoid) arch, normal migration into the arch occurs, but first arch structures are formed (Noden, 1983,1986). These results show that not only are there regional variations in the potential of the neural crest, but also that, to a limited extent, some neural crest cells may already be committed to a particular fate.

In addition to these obvious differences there are some subtle differences in the capacity of different regions of the crest to replace other normal crest regions. For example, replacement of the mesencephalic neural crest with trunk results in the development of an abnormal trigeminal ganglion (Noden, 1978); and the potential for adrenergic differentiation and melanocyte formation is greater in the trunk neural crest than in the cephalic crest (Newgreen et al., 1980). Also, when cephalic crest is transplanted to the trunk, the neural crest cells migrate into the dorsal mesentery and colonize the gut, which does not normally happen (Le Douarin and Teillett, 1973).

It is these exceptions that suggest that there are regional differences in the composition of the crest along its rostral-caudal axis. The cephalic regions of the crest appear to have the potential to give rise to all neural crest derivatives, whereas the trunk neural crest is restricted to PNS and melanocytic derivatives. This restriction may apply only after a particular developmental stage, as Lumsden has found that trunk neural crest cells from the mouse can participate in tooth formation when combined with mandibular epithelium (Lumsden, 1987,1988), but only if the cells are taken from very early neural crest (6-12 somite stage). Thus, the restriction process presumably occurs after this time. The rostral-caudal gradient and segmentation pattern observed in verte-

brate neural development may be primarily the result of mesenchymal influence, although the inductive agents are as yet unknown (*see* Dodd, 1992 for review). As will be discussed later this pattern may influence the neural crest differentiation repertoire directly by regulating homeobox expression, or it could influence the environment in which neural crest cells migrate.

Neural Crest Cells Display Multipotential and Committed Characteristics When Grown In Vitro: Defining the Differentiative Pathway of the Neural Crest

The above transplantation experiments cannot give a clear picture of the differentiation potential of individual neural crest cells within a regional population. To do this, clonal analysis, either in vitro or in vivo, is required. In vitro, progeny of single neural crest cells (clones) can be influenced or manipulated relatively simply by adjusting the components of the medium. In this way, the differentiation potential of neural crest cells may be examined. An important bonus of this approach is that it also provides an assay to assess putative factors that may influence the development of the crest derivatives. A number of workers have developed these clonal cultures, and their results suggest that there are both committed and multipotential cells within the neural crest.

Sieber-Blum and Cohen (1980) first used clonal analysis to study quail neural crest cells and found a proportion of clones that contained both catecholaminergic (neuronal lineage) and pigmented cells. More recent studies (Sieber-Blum 1989; Ito and Sieber-Blum, 1991) have revealed three classes of clones: clones exclusively of the melanogenic lineage, clones that were unpigmented, and clones containing both pigmented and unpigmented cells (mixed). The unpigmented and mixed clones all contained both catecholaminergic and sensory neurons. Thus, in this system, there is evidence for tripotent cells; cells restricted to two lineages; and fully committed cells. In the latter study (Ito and Sieber-Blum, 1991), a clonal analysis of the cardiac neural crest, pluripotential (mesenchymal, neuronal, and melanocytic), bipotential (mesenchymal, neuronal), and fully restricted clones were found.

Studies from the laboratory of Le Douarin found evidence for a similarly heterogeneous range of clones (Baroffio et al., 1988,1991; Dupin et al., 1990). In these studies, besides the fully restricted clones, multipotent clones comprising neurons, pigmented cells, and nonneuronal cells were found, as well as partially more restricted clones that contained Schwann cells, satellite cells, and neurons, but not pigmented cells. This pattern may indicate that neural and melanocytic cell precursors segregate early in the differentiation process. In one study (Baroffio et al., 1991), some multipotential clones were found to contain either the full array of neural crest derivatives, including mesenchymal elements, or were restricted to either neural and melanocytic, or neural and mesenchymal.

These studies thus support the idea that within any crest population there are both multipotential and committed cells at the migratory stage. The observation of considerable heterogeneity in the clones is not necessarily an indication that there is innate heterogeneity in the neural crest cell's repertoire. It may be that all neural crest cells are initially multipotent, however, at the time the cells are isolated they have reached different stages of differentiation. If this is the case, then the actual lineage pathways, or commitment steps, may be inferred from the segregation pattern observed in the clones. Within the multipotent clones, there are some that are either neural/melanocytic or neural/mesenchymal, which suggests that this may be the first restrictive choice the neural crest cells make. Neurons, melanocytes, and mesenchymal cells segregate from each other in the more restricted clones. Glial cells also segregate from the other cell types, but a significant number of clones show cosegregation of neurons and glial cells indicating that there is a common glial/neuronal precursor that retains its bipotentiality late into the differentiation process. These findings, especially with the many intermediate, or partially committed clones,

strongly supports the concept of sequential differentiation from a multipotential cell. However, the concept of identical pluripotential stem cells in all regions of the neural crest may be an oversimplification given the differing potential found between cephalic and trunk crest to give rise to mesectodermal derivatives.

A complication in these studies is that the clones are normally analyzed after a number of weeks when there can be thousands of cells in each clone. Under these conditions the microenvironment of each clone might itself vary; there might be endogenous production of different growth factors that could influence cell phenotype. One extremely labor intensive approach to this problem would be to subclone daughter cells as soon as they arise and to examine the resultant clonal phenotype. This would also provide a more detailed account of the sequence of lineage restrictions occuring during crest development.

In spite of these drawbacks, it is still conceptually useful to use the available data to compose a parsimonius diagram of the temporal development of the neural crest lineages, as in Fig. 1. In general, the flow diagram represents the concepts of a common stem cell for most of the neural crest derivatives; segregation of mesenchymal precursors, which only pertains to the cranial crest, occuring very early in development; followed by the separation of the melanocytic and neural lineages. The parasympathetic lineage is not included here mainly for simplicity, but it is clear that this lineage, at least in the cephalic crest, is delineated early in development (Barald, 1989). As indicated, the sympathoadrenal lineage, which has been extensively studied (*see* Anderson, 1989; Patterson, 1990), appears to share a common precursor with the enteric lineage, based on recent studies by Carnahan et al. (1991) demonstrating shared antigen expression.

In this diagram we have indicated that the melanocyte and Schwann cell lineage diverge early in development. This is based both on the aforementioned clonal analysis studies and on our recent findings that melanocyte precursors, identified by their expression of the *c-kit* protein (the receptor for *Steel* factor) by immunohistochemistry, do not survive in vitro when *Steel* factor is removed from the culture by a neutralizing antibody (unpublished observations). However, in spite of the total loss of melanocytes, Schwann cells formed normally. Thus, it appears that if melanocytes and Schwann cells do have a common ancestry, as suggested (*see* Weston, 1991 for discussion) this must precede their migration. The findings that the peripheral nervous system contains satellite cells that can be converted to melanocytes in vitro (Ciment, 1986), seems contrary to this assertion but may be explained by two independent lineages that can give rise to melanocytes. The first is the *c-kit* bearing population, which is dependent on *Steel* factor for its survival in vitro (Murphy et al. 1992; *see below* for details), and is reponsible for the generation of skin melanocytes; as evident by lack of melanocytes in *W/W* and *Steel* mice (*see below* for further discussion). The second, without *c-kit*, are primarily destined to differentiate into Schwann cells, but retain the ability to produce melanin under appropriate conditions that include both the removal of a neuronal influence and a positive differentiating signal. It would be predicted that these latter cells would not be absent in the *Steel* or *W/W* mutants.

The other clonal approach used to determine the degree of multipotentiality or commitment of the neural crest cells has been undertaken in vivo. In these studies, single neural crest cells have been microinjected with a flourescent dye prior to migration from the neural tube (Bronner-Fraser and Fraser, 1988, 1989; Fraser and Bronner-Fraser, 1991). The clonal progeny of the cells after 2 d were sometimes found to be distributed in many regions to which neural crest cells normally migrate. Although the phenotype of these cells could not be definitively ascribed, it was found on the basis of morphology and antibody binding that individual clones contained sensory neurons, presumptive melanoblasts, satellite cells in dorsal root ganglia, adrenomedullary cells, and neural tube cells. Thus, these findings support the idea that there are multipotential neural crest cells in vivo. These studies also purported to show that some cells in the neural tube can give rise to both neural crest cells and neural tube cells destined to become mature CNS cells (Bronner-Fraser and

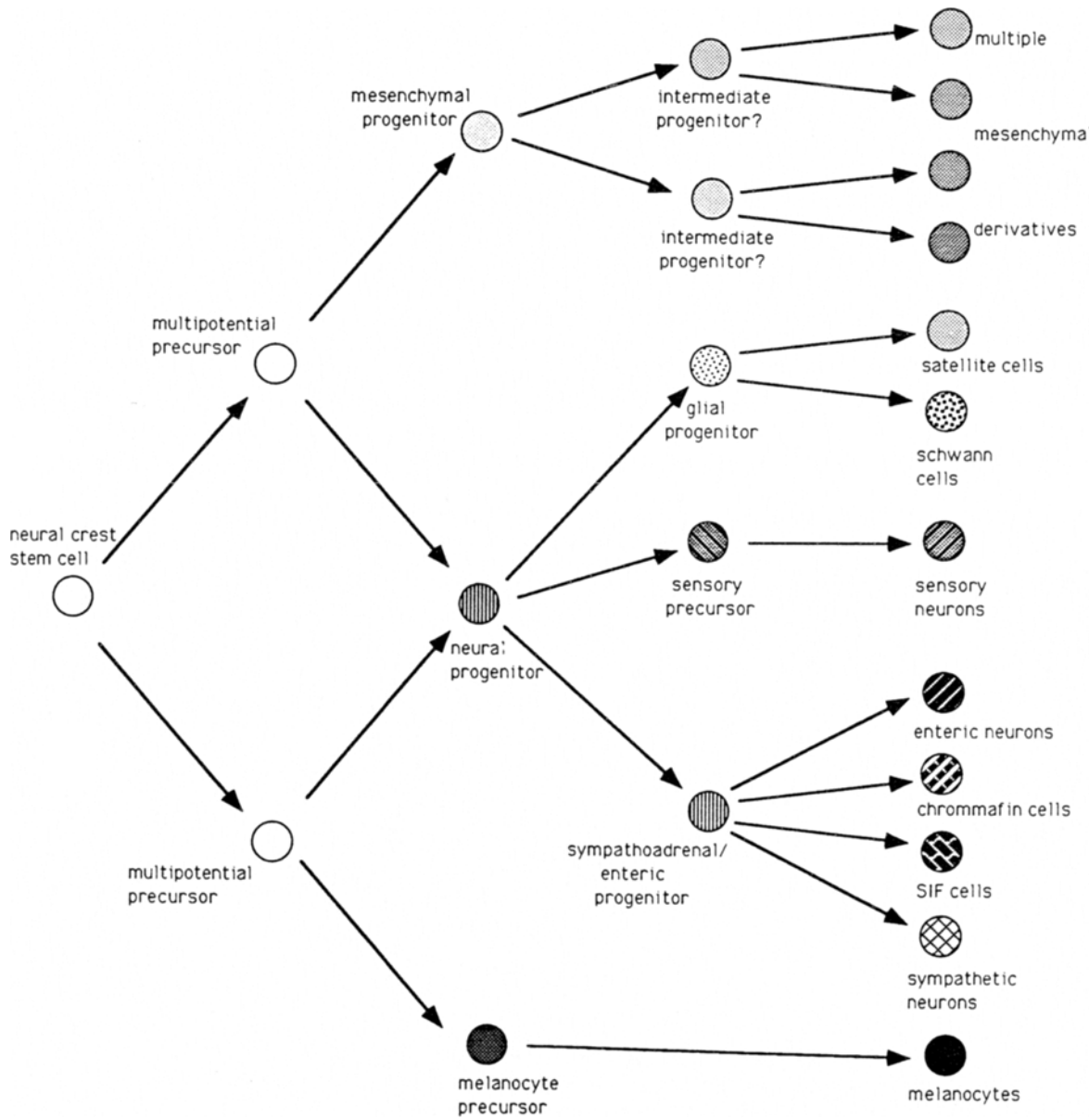

Fig. 1. Cell lineage map of the neural crest. The construction of this map is based on studies of single-cell cloning experiments and characterization of antigen expression patterns as outlined in the text. The full complement of neural crest derivatives is normally only seen in the rostral regions of the neural crest.

Fraser, 1988,1989). Although these studies indicate the diversity of cell products there has been concern as to whether they represent the progeny of a single cell. Difficulties with confining the injection to just one cell appears to still present considerable technical difficulties to other workers. However, results obtained by following the progeny of neural crest cells infected with lac-Z containing retrovirus in vivo in the dorsal root ganglia (Frank and Sanes, 1991) do tend to support the multipotential concept. However, here again, problems with infecting a small cohort of dividing cells rather than a single cell, makes this interpretation somewhat equivocal.

Mammalian Neural Crest Cell Lines Display a Variety of Differentiated Characteristics

In mammals, far less is known about cell lineage and commitment of the neural crest than in avian species. So far, no clonal analyses have been reported for mammalian neural crest and transplantation experiments in mammals are much more difficult to undertake. One approach to studying the mammalian neural crest is to make immortalized cell lines. If clonal cell lines can be obtained that represent neural crest cells or their derivatives, they may be useful in inferring cell lineage relationships in an analogous way to that described for clonal analysis of avian neural crest cells. Previous work from our laboratory and others has shown that retrovirus mediated proto-oncogene transduction of the neural precursor cells from mouse neuroepithelium results in the production of stable neuroepithelial and neural cell lines (Bartlett et al., 1988; Bernard et al., 1989; reviewed in Cepko, 1988, 1989).

In a similar manner, we immortalized mouse neural crest cultures using retroviruses bearing the c-*myc* or the N-*myc* proto-oncogenes (Murphy et al., 1991a). The different lines could be broadly classified into three subgroups. Group 1 contained flat adherent cells, which looked like primary neural crest cells. Group 2 contained flat cells at low density, a proportion of which at higher density and longer time in culture tended to become stellate or dendritic. Group 3 cells grew initially as flat cells but after a relatively short time in culture, most of the cells elongated and put out processes.

These cell lines were examined for the expression of lineage-specific or lineage related antigenic markers and for the expression of neural specific mRNAs. We examined the expression of nerve growth factor [NGF] and its receptor [NGF-R], that are expressed by cells in the peripheral nervous system, myelin basic protein [MBP], and the proteolipid protein [PLP] of myelin that, in the peripheral nervous system, are specific to Schwann cells. A neuron specific gene SCG-10 (superior cervical ganglion, *see* Anderson and Axel, 1985) was also used in the analysis.

Group 1 cell lines not only morphologically resembled migrating neural crest cells, but also were largely devoid of phenotypic markers, both antigenic and mRNA, expressed by mature neural cells. These observations are consistent with the idea that some of the migrating neural crest cells are not yet committed to a single developmental pathway and probably represent stem-cells. These stem cells have presumably been arrested at this stage by the immortalization process.

Some of the cell lines also displayed a plastic or at least a bipotential nature, especially those in Group 2. Particular cell lines expressed their bipotentiality in the expression of markers associated with two lineages. In one case a cell line, NC14.9.1, appeared to be bipotential since in a cloned population these cells expressed neurofilament as well as MBP and PLP, showing that it had characteristics of both neurons and Schwann cells. Likewise another cell line, NC14.4.9D, expressed both PLP mRNA and SCG-10 mRNA and all the cells express neurofilament. Similarly, multipotent neural cell lines have been isolated from newborn brain (Fredericksen et al., 1988; Ryder et al., 1990). These cell lines also share some other characteristics of our cell lines in that some of the antigenic markers examined were expressed on a small proportion of cells in particular cell lines. Cell lines from Group 2 also had the properties of progenitor cells (Murphy et al., 1991a). For example, NC14.4.8 cells contained cells that differentiated after 1–2 wk in culture, into Schwann-like cells. Further, these older cultures expressed mRNA for MBP, PLP, NGF, and NGF-R. All these observations are consistent with this cell line comprising Schwann cell progenitors.

Finally, one of the cell lines (in Group 3) appears to represent differentiated neuronal cells. These cells (NC14.4.6E cells) have fine processes that contain neurofilament. In addition, these cells express mRNA for the neuronal protein SCG-10, as well as for NGF.

The multipotential nature of the neural crest cells that were originally infected with either c-*myc* or N-*myc* containing viruses was also demonstrated by the observation that cell lines that have the same *myc* integration pattern, and thus

must have originated from the same cell, can have quite different phenotypes. It is possible that an immortalized multipotential cell divided a number of times before differentiation of the progeny cells into the different phenotypes took place. Thus, a single crest cell can give rise to an immature neural crest-like line, a Schwann cell progenitor, and a bipotential cell line.

Mammalian cell lines have also been derived from rat primary neural crest cultures (Lo et al., 1991). One cell line, NCM-1, was generated that displayed bipotential characteristics. NCM-1 has the characteristics of a glial progenitor and resembles Schwann cells in serum-free medium. In addition, some of these cells acquire sympathoadrenal characteristics in response to FGF and dexamethasone. Thus, this cell line contains cells with the potential to generate precursors in at least two neural crest sublineages.

The Role of Homeobox-Containing Genes in the Commitment and Development of the Neural Crest

Although evidence has begun to emerge only recently that homeobox-containing genes may play a role in the control of development of the neural crest, the concept has already assumed major importance. These genes were originally characterized in the fruit fly, *Drosophila melanogaster*, as homeotic genes (*see* Akam, 1987; Gehring, 1987; Ingham, 1988). Mutations in these genes lead to the altered specification of whole segments in the fly, which implies that these genes are involved in the determination of body plan. This idea is supported by the finding that expression of different homeotic genes is localized within particular segments in the developing *Drosophila* embryo. A molecular mechanism for the action of these genes is suggested by the presence in all the homeotic genes of a sequence encoding a 60-amino-acid domain, the homeodomain, which encodes a helix-turn-helix motif. The presence of this motif suggests that these genes encode transcription factors, which has been confirmed by many experiments in recent years. Thus, these genes control the expression of other genes which somehow results in the determination of the morphology of whole segments in *Drosophila*.

Hox Genes

By crosshybridization experiments using *Drosophila* probes, homeobox containing genes have been found in all vertebrates so far examined (*see* Holland and Hogan, 1988; Duboule and Dolle, 1989; Graham et al., 1989; Kessel and Gruss, 1990). The single largest class of homeobox genes in mice and humans, *Hox-C*, is most closely related to the *Antennapedia* (*Antp*) Group in *Drosophila* and shows some striking similarities to this group of genes (*see* Fig. 2 for summary). First, *Hox* genes are arranged in four clusters of genes on different chromosomes and each cluster appears to have arisen from duplication of the original *Antp* group, because the genes that show the most homology with the *Antp* group are arranged in the same chromosomal order within each group (Acampora et al., 1989 and Fig. 2). Second, the anterior-posterior expression pattern of all the *Hox* genes and the *Antp* group correlates with their position in the gene cluster in a 3' to 5' direction; the most 3' genes in the cluster having the most anterior expression patterns in the embryo. In the mouse this is most clearly seen at neurulation.

Most importantly, all the *Hox* genes are expressed, albeit not exclusively, in the neural tube during neurulation. The partially restricted patterns of expression of the *Hox* genes along the anterior-posterior neural axis suggests that these genes may play a similar role in determining regional neural development in the vertebrate. What is missing is vertebrate homeotic mutants to give direct functional support to these suggestions. However, two approaches have been used to overcome the lack of homeotic mutants in mice: making transgenic mice disregulated for the expression of particular *Hox* genes; and making deletions in *Hox* genes by homologous recombination in embryonic stem cells and subsequently incorporating these genes into the mouse germ line.

Both of these approaches have produced results that strongly implicate a role for some of the *Hox* genes in neural crest development. In transgenic mice overexpressing *Hox-1.4* under control of its putative endogenous promotor,

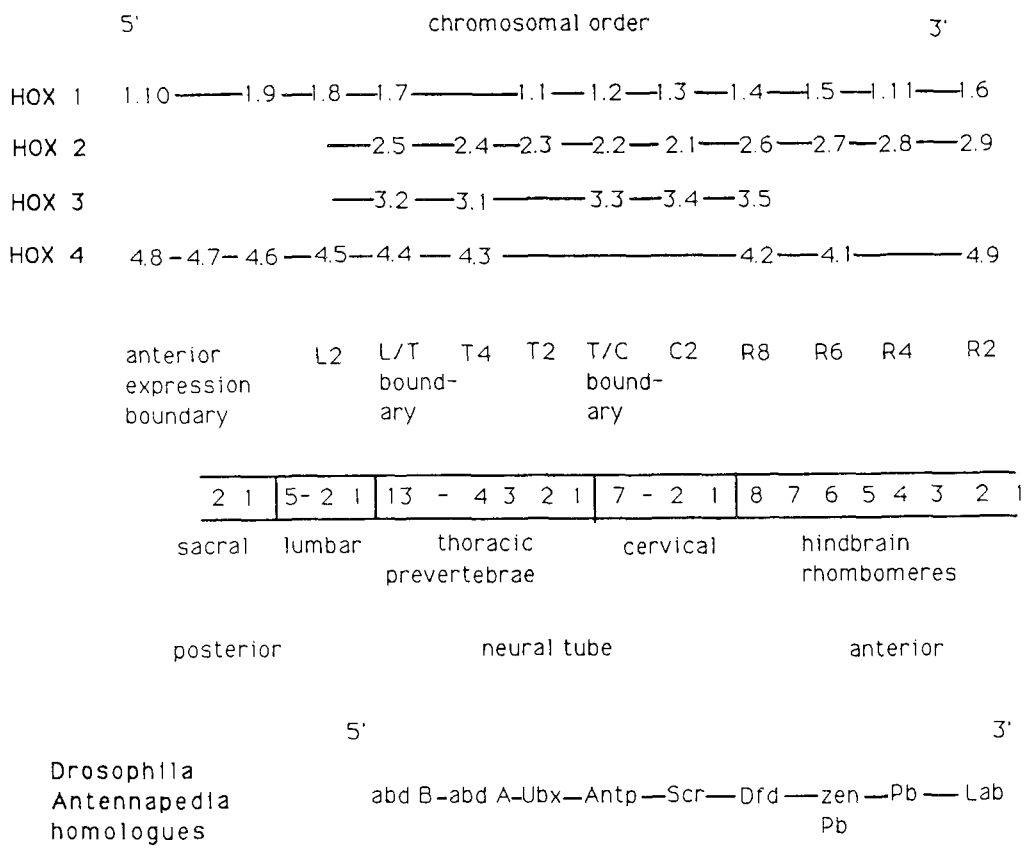

Fig. 2. Expression of *Hox* genes in the mouse at neurulation. The derivation of this map is essentially from Duboule and Dolle (1989) and Hunt et al. (1991) and references contained therein (*see also* McGinnis and Krumlauf, 1992). The upper lines show the chromosomal order of the four different clusters of *Hox* genes of the mouse. *Hox* paralogs are aligned vertically. The anterior expression boundary within the neural tube of each group of paralogs is shown under each group (L, lumber; T, thoracic; C, cervical; R, rhombomere) as well as a schematic view of the neural tube with the positions of the prevertebrae and rhombomeres. The *Drosophila antennapedia* gene cluster is shown at the bottom with the most closely related genes aligned vertically with the *Hox* paralogs (in addition, Abd-B is similarly homologous to all *Hox* genes 5' to its aligned paralogs).

high levels of *Hox-1.4* were found in the gut mesenchyme that caused the mice to develop a fatal condition called congenital megacolon (Wolgemuth et al., 1989). This syndrome has been associated with a deficiency of the neural crest derived myenteric ganglia and these results imply that the positional cues in the gut mesenchyme have been disrupted by the overexpression of *Hox-1.4*. Neural crest cells may have been a direct target in transgenic mice overexpressing *Hox-1.1*. In these mice, the ubiquitous overexpression of *Hox-1.1* leads to abnormalities in neural crest-derived structures, including cleft palate and nonfused pinnae (Balling et al., 1989), as well as somite-derived vertebrae (Kessel et al., 1990).

The more definitive experiments involve incorporating deleted *Hox* genes into the germ line of mice by homologous recombination. So far, mice lines deleted for two *Hox* genes have been constructed and characterized (Chisaka and Capecchi, 1991; Lufkin et al., 1991; Chisaka et al., 1992). Both of these mice show defects in neural crest-derived structures.

In the *Hox-1.5* deletion (Chisaka and Capecchi, 1991), the homozygotes die shortly after birth and have a range of defects, including being athymic, having no parathyroid tissue, little thyroid tissue, cardiovascular problems, skeletal defects, and defects in the muscles of the throat. All of these structures are derived from branchial arch mesenchyme, which is derived from both mesoderm and from the cephalic neural crest. Other studies show that ablation of the cephalic neural crest results in a similar phenotype to that seen in the *Hox-1.5*-homozygotes (Kirby et al., 1983). Thus, it seems likely that a major defect in these mice originates in the cephalic neural crest cells. Correspondingly, *Hox-1.5* expression has a discrete anterior boundary of expression during neurulation at the level of the second branchial arch and is also most strongly expressed in this region (Gaunt, 1987 and Fig. 2). These findings suggest, in their simplest interpretation, that *Hox-1.5* is essential for the development of neural crest mesectoderm and may help to explain the above noted restriction of neural crest mesectoderm to the cephalic regions.

In contrast to *Hox-1.5* mutants, mice deleted for *Hox-1.6* (Lufkin et al., 1991; Chisaka et al., 1992) have defects that affect derivatives from rhombomeres 4–7 and could be ascribed to having origins in the neuroectoderm, including all the neural crest derived cranial ganglia in this region, but also inner ear and skull bones. These defects are confined to tissue originating near the anterior expression boundary of *Hox-1.6*, but interestingly, the expression of *Hox-1.6* in this region is over by the time the neural crest cells migrate. It is therefore possible that *Hox-1.6* expression in the neuroectoderm during neurulation confers commitment to a subset of cells that later migrate out as cephalic neural crest cells.

Hox-1.6-mice show no defects in any mesenchymal structures, whereas the *Hox-1.5*-mice show defects that are exclusively mesenchymal. Thus, these two *Hox* genes appear to be involved in the determination of two separate lineage groups arising from the same region of the developing embryo and involving the major neural crest derivatives in this region.

The interpretation of these experiments is clearly more intricate than outlined above. For instance, as *Hox* genes are also expressed in mesenchymal tissue through which neural crest cells pass, the resultant phenotypic changes could be ascribed to environmental influences. Changes in growth factors *(see below)* or changes in cell interactive molecules, such as N-CAM, have recently been shown to be influenced by *Hox* genes (Jones et al. 1992). Another possible mediator of the phenotypic change is potential alteration in the expression of paralogs in the 2 and 4 *Hox* gene clusters (*see* Fig. 2), that may lead to some type of homeotic change. An essential piece of information that is required before further inferences can be made as to the influence of *Hox* genes on neural development is the identification of the *Hox* repertoire of individual neural crest cells at the time of migration. This coupled with an ability to trace the ultimate phenotype of the progeny should enable a more cogent assessment of *Hox* gene regulation of cell lineage.

Pax Genes

The mouse *Pax* gene family is comprised of eight genes sharing with a group of *Drosophila* genes a highly conserved domain, the paired domain, which encodes a 128-amino-acid sequence and contains a helix-turn-helix motif (Kessel and Gruss, 1990). Three of the *Pax* genes and three of the *Drosophila* genes also contain a paired homeodomain. The expression patterns of these genes makes it also likely that they, along with the *Hox* genes, play key roles in embryonic development. One current advantage in the study of the function of *Pax* genes is that there are a number of mouse mutants that have mutations in the coding regions of the *Pax* genes themselves (Balling et al., 1988; Epstein et al., 1991; Hill et al., 1991).

In terms of neural crest development, *Pax-3* is currently the most interesting gene in this family. *Pax-3* is expressed just prior to neural tube closure in the dorsal aspect of the neuroectoderm extending along almost the entire length of the neural tube (Goulding et al., 1991). It is also expressed in neural crest derived spinal ganglia, dorsal root ganglia, somitic mesoderm, and some

cranio-facial neural crest derivatives. The mouse mutant, *splotch*, has recently been found to be mutated in *Pax-3* (Epstein et al., 1991). *Splotch* homozygote embryos survive until approx day 16 of gestation and exhibit exencephaly, meningocele, spina bifida (Auerbach, 1954; Beechey and Searle, 1986; Franz, 1989), as well as dysgenesis of the neural-crest derived spinal ganglia (Auerbach, 1954), Schwann cells (Franz, 1990), and structures of the heart (Franz, 1989). The heterozygotes are characterized by white spotting of the abdomen, tail, and feet, possibly caused by defects in melanocyte production (Auerbach, 1954).

These results show *Pax-3* is functionally very important in the development of the neural crest and tube, apparently along the entire embryonic axis. One possibility is that *Pax-3* regulates the expression of proteins essential for the migration of neural crest cells, such as extracellular matrix molecules, and cell adhesion molecules, such as N-CAM (Moase and Trasler, 1990; Epstein et al., 1991). Some support for this contention comes from the finding that N-CAM expression profiles are altered in *splotch* homozygotes compared with +/+ littermates (Moase and Trasler, 1991).

Clearly these studies are but the first of a great number of experiments required to elucidate the role this group of genes plays in the control of the development of the neural crest. For example, Le Douarin's laboratory is beginning to address specific questions in this area, such as the role of homeobox genes in bone differentiation from neural crest mesenchyme (Takahashi et al., 1991), and work from Anderson's laboratory is addressing the role of another class of transcription factors, the basic helix-loop-helix proteins, in neural crest development (Johnson et al., 1990).

When Does Commitment Occur in the Neural Crest?

Superficially, some of these findings discussed above appear to be contradictory; for example, how can neural crest cells be multipotential when they already express discrete combinations of *Hox* genes associated with a particular region? A view of the developmental process that can accomodate these different findings can be proposed and is presented diagramatically in Fig. 3. In this scheme we propose that commitment of the neural crest might be regarded as a continuous or multistep process whereby as soon as the neural crest begins to form, early in neurulation, the commitment process begins. The expression of *Pax-3* in the dorsal aspect of the neural tube may be one of the initial steps in the process of commitment of those cells destined for migration from the tube. The differential *Hox* expression patterns along the anterior-posterior axis (Figs. 2 and 3) may impose a spatially regulated commitment of neural crest cell populations, either directly or indirectly as opposed to the strictly temporal pattern of lineage restriction shown in Fig. 1. At this early stage, the steps in the commitment process may be reversible so that transplantation of the neural primordium from one region to another may change the commitment process and redirect the neural crest cells to another fate. Support for this concept of plasticity is the finding that the expression of two of the segment specific genes, *Hox-2.9* and *Krox-20*, can be altered during neurulation by the administration of retinoic acid (Morris-Kay et al., 1991); this has pronounced effects on neural crest and central nervous system development.

At the time the neural crest cells migrate, some of the early commitment steps may have already occurred, such as commitment to a neural or a mesenchymal fate, but further steps down particular sublineages occur postmigration. The heterochronic grafting experiments generally support this idea as well as the in vitro cloning experiments. Cells labeled with fluoresceinated dextran as they emigrate from the neural tube *in situ* (Bronner-Fraser and Fraser, 1989) also provide supportive evidence for this concept. Clearly then, the embryonic environment that the cells find themselves in during and postmigration must directly influence the phenotype of these cells.

Another influence on the development of the neural crest can come from a selective survival of precommitted cells, as exemplified in the dual cell line segregation model for autonomic and sensory precursors proposed by Le Douarin (*see* Le Douarin, 1986). This model proposes that in

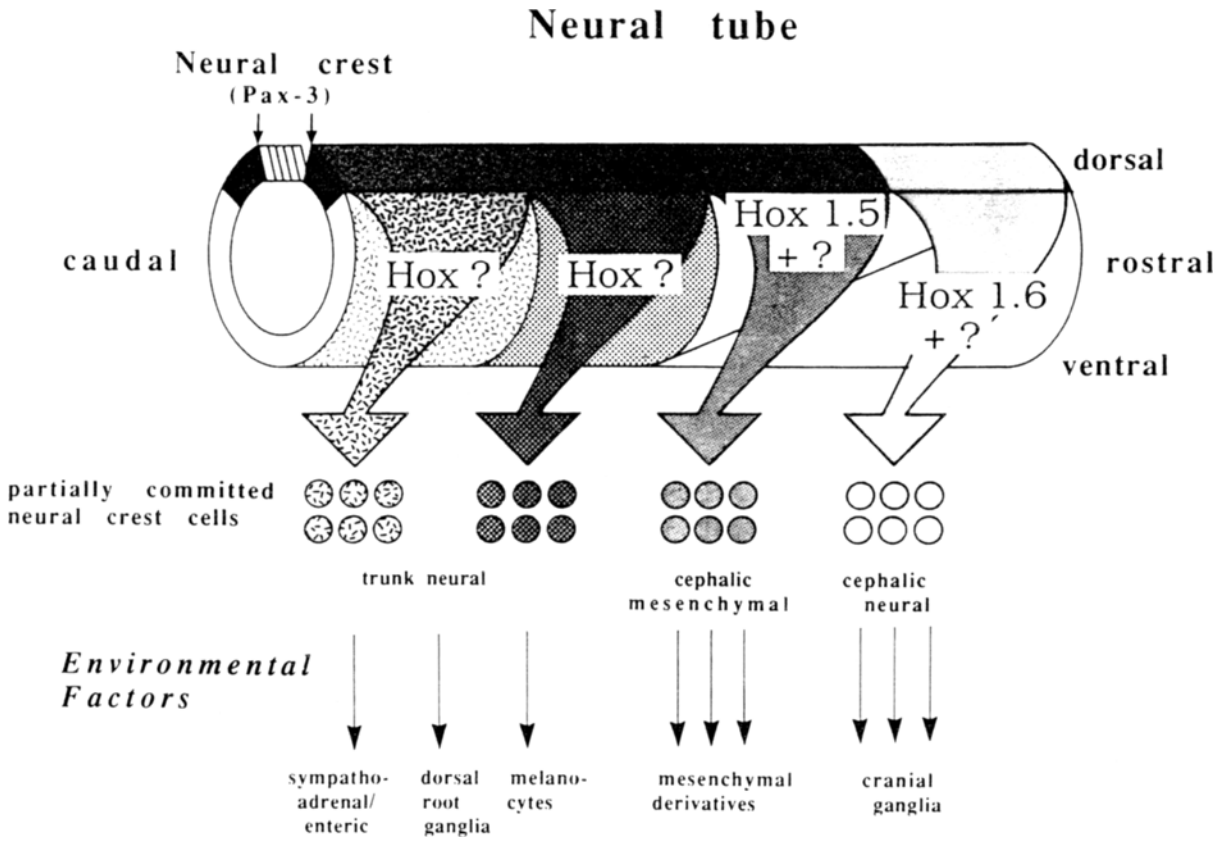

Fig. 3. A scheme of neural crest commitment and development. *Pax-3* expression is restricted to the dorsal neural tube, including the premigratory neural crest cells, but extends along the entire rostro-caudal axis. There is differential expression of the *Hox* genes along the rostro-caudal axis and the particular expression pattern of *Hox* genes at any point on this axis may be involved in the specification of neural crest cell phenotype. In the case of *Hox 1.5* and *Hox 1.6* there are overlapping domains of expression along the rostral-caudal axis. Partially committed neural crest cells migrate away from the neural crest cells (large arrows of migration show different patterns of expression present in the cells as they migrate). The partially committed neural crest cells are then acted on by different environmental factors they encounter in the embryo to differentiate into all the neural crest derivatives.

very early ganglia, the developmental potentials of the two precursors have already been determined, presumably according to the interactions described above. By doing back transplantation experiments of either sensory or autonomic ganglia, these precursors could be distinguished on the basis of proximity to the spinal cord. Sensory neurons only arose from proximal grafts, whereas autonomic neurons arose from all types of grafted peripheral ganglia. These findings were interpreted to indicate that sensory and autonomic precursors have different survival requirements, where sensory precursors require CNS derived survival factors, and autonomic precursors do not.

In summary, neural crest commitment may be regarded as being determined in both spatial and temporal contexts. Within the neural tube the temporal specific expression of *Pax-3* and the spatial and temporal specific expression of the *Hox* genes may become markers of the early steps in the commitment process. During and post migration, the cells become spatially defined and thus under the influence of spatially distinct

environmental factors that both directly determine phenotype and may further select for particular cell types by selective survival. The nature of the environmental factors involved in this process will be described below. The argument between commitment and multipotentiality can be seen as a "straw-man," whose resolution appears superfluous to the important questions of what mechanisms regulate phenotype and morphogenesis.

Growth Factor Regulation of Neural Crest Development

All the steps in the development of the neural crest are determined by cell intrinsic signals, such as the transcription factors described above, in coordination with environmental factors. The early commitment steps that occur within the neural tube, such as establishment of differential expression patterns of the *Hox* and *Pax* genes, are presumably themselves regulated by environmental factors. Retinoic acid is one factor implicated at this stage in the establishment of the domains of expression of the *Hox* genes along the anterior-posterior axis (*see* Kessel and Gruss, 1991, and papers contained therein). Clearly other factors, as yet undescribed, must also be involved in this process (*see also* last section). It is also clear from the above discussion that environmental factors must influence the phenotype of neural crest cells after, and probably during, migration. In the discussion below we focus on the few characterized growth factors that have been implicated in this process. A summary of these growth factors and their proposed involvement in neural crest development is presented in Table 1.

Growth Factors that Stimulate Neural Crest Proliferation

We have previously shown that fibroblast growth factor (FGF) stimulates the proliferation of freshly isolated neuroepithelial cells (Murphy et al., 1990). Given that the neural crest is initially contiguous with the neuroepithelium, FGF appears to be a good candidate for involvement in neural crest proliferation. There are a number of indications that FGF plays a role at this early stage of neural crest migration. FGF has been reported to have a survival role for neural crest cells (Kalcheim, 1989). If silastic membranes are inserted between the neural tube and the neural crest cells of the dorsal root ganglion anlage, there is a selective death of the neural crest cells that are distally located with respect to the silastic implants. If these silastic membranes are implanted with laminin and basic FGF (bFGF) there is significant survival of the neural crest cells for a period of over 30 h after grafting (Kalcheim, 1989).

In addition to these in vivo studies, the effects of bFGF were examined in mixed cultures of trunk neural crest cells and somite cells or in pure cultures of neural crest cells. Under the conditions of the assay, that is, in a serum-free defined medium, bFGF was found to act as a survival agent for nonneuronal cells of neural crest origin (Kalcheim, 1989). We have found that most of our mouse neural crest cell lines respond to FGF by proliferating and by changes in morphology (Murphy et al., 1991a). In addition, recent studies from our laboratory show that FGF can act as a proliferation factor for the majority of primary neural crest cells (unpublished observations).

Studies have shown the presence of FGF in culture and *in situ* at the time of neurogenesis and neural crest migration (Kalcheim and Neufeld, 1990). bFGF was found by immunocytochemistry in quail neural tube cells, sensory neurons, and in some nonneuronal cells in neural crest cultures. Spinal cord and ganglionic neurons expressed bFGF *in situ* from E6 until E10. In addition, bFGF was detected in mesodermal tissues dorsal to the neural tube as well as in other mesoderm-derived structures. These *in situ* immunohistochemical observations were supported by radio-immunoassays that showed levels of bFGF in spinal cords from as early E3 and that increased to a maximum at E10.

Another factor implicated in the proliferation of neural crest cells is neurotrophin-3 (NT-3; Kalcheim et al., 1992), a member of a family of nerve growth factor-like peptides. In mixed cultures of somites and neural crest cells, NT-3 stimulated a significant increase in the number

Table 1
Growth Factors Implicated in Neural Crest Development

Neural crest lineage and cell type	Factor	Activity
Primary neural crest cell	FGF	Survival and proliferation
	NT-3	Proliferation
Sympathoadrenal lineage		
Progenitor cell	FGF	Proliferation and commitment
Presympathetic neuron	CNTF	Inhibition of proliferation, commitment?
	IGF-I	Proliferation
	NGF	Differentiation, survival
Sympathetic neuron	LIF	Cholinergic switching
Prechromaffin cell	Glucocorticoids	Differentiation
Sensory lineage		
Precursor cell	LIF	Differentiation, survival
Immature neuron		
(Nonfactor dependent)	BDNF	Differentiation, maturation
	NT-3	Maturation
Immature neuron		
(Factor dependent)	NGF	Survival, maturation
Melanocyte lineage		
Precursor	SLF	Maintenance
	TPA	Differentiation
	FGF	Differentiation
	TGF-beta 1	Differentiation inhibition

See text for details.

of neural crest cells incorporating ^{3}H-thymidine and an increase in cell number. In homogeneous cultures of neural crest cells, NT-3 caused a smaller but still significant increase in the proliferation of a subpopulation of neural crest cells. Thus, NT-3 may act to stimulate the proliferation of a subset of neural crest cells, perhaps committed to a particular lineage.

Growth Factors that Influence Differentiation of Neural Crest Cells

Sympathoadrenal Lineage

Developmentally, the best characterized cell lineage within the neural crest is probably the sympathoadrenal lineage. There are three cell types in this lineage, the sympathetic neuron, the adrenal chromaffin cell, and a third cell of an intermediate phenotype, the so called small, intensely flourescent cell (SIF cell) (*see* Patterson, 1990; Anderson, 1989). Although progenitors of this lineage have not been isolated from neural crest cultures, they have have been isolated from embryonic adrenal medulla as well as from both embryonic and neonatal sympathetic ganglia.

These progenitors will differentiate into either chromaffin cells or sympathetic neurons depending on culture conditions (Doupe et al., 1985a,b; Anderson and Axel, 1986). FGF will initiate neuronal differentiation as well as a dependency of the cells on NGF for their survival. Glucocorticoids will stimulate the cells to differentiate into mature chromaffin cells. The evidence for the presence of FGF in the embryo around the neural tube has been presented above. The possibility that the developing sympathetic neuron precursors will find a supply of this factor at the site of ganglia is thus quite reasonable. In the adrenal medulla, on the other hand, when the precursors migrate into the adrenal gland they may be subject to a high concentration of steroids produced in the adrenal cortex.

The role of NGF as a survival factor for the sympathetic neurons has been demonstrated over the past 40 yr using numerous experimental systems (*see* Levi-Montalcini and Angeletti, 1968). It remains the only molecule to be unequivocally shown to be critical for neuron survival in vivo. The injection of antiNGF antibodies into newborn mice results in the destruction of the sympathetic nervous system. Studies of the mechanism of action of NGF have resulted in it becoming the prototype of target derived neurotrophic factors. In this model, the newly differentiated neurons sprout axons to their target fields, where there is a limited supply of a target derived survival factor. It is postulated that only those neurons that have made the appropriate connections will obtain this factor and survive. Thus, this model provides a part of a mechanism for the control of the development of the nervous system into a three dimensional network.

A number of other factors have been implicated in the development of the sympathoadrenal lineage and in particular the development of sympathetic neurons. IGF-1 stimulates proliferation in cultures of rat sympathetic ganglia (DiCicco-Bloom et al., 1990). Whether this is a direct effect of IGF-1 on the proliferation of the neuronal precursor cells or whether the IGF-1 is acting principally as a survival agent and there are endogenous proliferative factors in these cultures, as we have observed in cultures of neuroepithelial cells (Drago et al., 1991), is unclear at present. Ciliary neurotrophic factor (CNTF), conversely, inhibits the proliferation of the neuroblasts and may provide a signal to initiate the differentiation of the cells (Ernsberger et al., 1989).

Other factors have been described that influence the transmitter phenotype of the sympathetic neurons. Most of the sympathetic neurons are adrenergic, except for those that innervate the sweat glands, which are cholinergic. One of the factors that may influence the switching of phenotype of these neurons to cholinergic has recently been purified and is equivalent to leukemia inhibitory factor (LIF, Yamamori et al., 1989). As discussed below, it is beginning to emerge that LIF has multiple activities within the nervous system as well as outside it.

Sensory Lineage

The processes that regulate the development of sensory neurons from their precursors in the embryonic neural crest have not been well characterized. We recently reported that LIF, a protein with multiple activities (Gearing et al., 1987; Abe et al., 1986; Yamamori et al., 1989; Williams et al., 1988; Baumann and Wong, 1989, *see above)* stimulated the generation of neurons in cultures of mouse neural crest (Murphy et al., 1991b). These neurons have the morphology of sensory neurons and contain neuropeptides, such as calcitonin gene-related peptide, found in mammalian sensory neurons. Consistent with these neurons being of the sensory lineage was the finding that they arose from nondividing precursors, a characteristic previously observed for early arising sensory precursors in neural crest cultures (*see* Weston, 1991). In addition, LIF supported the generation of sensory neurons in cultures of cells obtained from embryonic dorsal root ganglia (DRG). The full differentiation of sensory neurons in these cultures is dependent on the presence of NGF (Murphy et al., 1993). Thus, the role of LIF early in the differentiation of sensory neurons appears to be primarily at the step of differentiation of neuronal precursor cell to newly differentiated neuron (Murphy et al., 1993).

At later stages in sensory development, LIF can act as a survival factor. In cultures of dorsal root ganglia isolated at postnatal day 2, a high proportion of neurons survived in the presence of LIF (Murphy et al., 1991b). Thus, LIF may also be a neurotrophic factor, like NGF. Binding studies on the DRG cultures from P2 mice showed that greater than 60% of the neurons bound significant amounts of ^{125}I-LIF, which was completely inhibited by the addition of cold LIF (Hendry et al., 1992). Furthermore, there was negligible specific binding of ^{125}I-LIF to nonneuronal cells in the culture. Thus, at this age, the only cells capable of responding to LIF in the DRG are the sensory neurons.

One of the essential criteria to be fulfilled by a neurotrophic factor is that there appears to be a requirement for factors taken up by the nerve terminals to be retrogradely transported back to the

neural perikarion. The transport of the neurotrophic factor is the signal from the target tissue to the neuron that results in neuronal survival (Hendry, 1974). To test the possibility that LIF is retrogradely transported, mice were injected in the skin or muscle with ^{125}I-LIF, and in those animals injected in the skin of the foot, there was a significant accumulation of radioactivity in the dorsal root ganglia (Hendry et al., 1992). The retrograde transport of LIF into the DRG was confirmed by autoradiographic examination of histological sections of ganglia from these animals, which revealed radioactive material only within the cell bodies of the sensory neurons. Thus, LIF may have a dual role in the sensory nervous system, first as a differentiation stimulus for the sensory precursors and second as a neurotrophic factor for mature sensory neurons.

Further supportive evidence that LIF has a role in sensory development in vivo comes from the finding of LIF mRNA in developing DRG from as early as E13 and possibly earlier (Murphy et al., 1993). In addition, LIF mRNA is present in the spinal cord region from E12, as well as sites of peripheral sensory innervation.

The best characterized factor shown to play a role in the development of sensory neurons is NGF (Levi-Montalcini and Angeletti, 1968; Levi-Montalcini, 1982; Thoenen and Barde, 1980). NGF most probably acts as a target-derived survival factor during the period of natural neuron death as discussed above for sympathetic neurons. The evidence for the time of action of NGF on sensory neurons comes from expression studies: The message for NGF is first observed in the target tissue at the time of innervation of the newly formed neurons, concomitantly with appearance of NGF receptors on the innervating nerve fibers of these neurons (Bandtlow et al., 1987; Davies et al., 1987). In addition, the role of NGF in vivo has been established by Johnson and coworkers, who immunized female guinea pigs with NGF and their offspring, which are exposed to NGF antibodies during the period of sensory development and lose up to 80% of their sensory neurons (*see* Johnson et al., 1986 for review).

Other factors, such as the other neurotrophins, also have activities on developing sensory neurons. Brain derived neurotrophic factor (BDNF) in particular has been implicated in sensory neuron development and at similar stages to those described here (Kalcheim and Gandreau, 1988; Sieber-Blum, 1991). In recent studies from Davies laboratory (Wright et al., 1992), it has been proposed that BDNF or NT-3 acts at the stage after neuronal differentiation, but before the neurons become dependent on NGF. BDNF or NT-3 accelerate the maturation of neurons before they become dependent on neurotrophic factors for survival, but the maturation process can still occur in the absence of these factors.

If these findings are taken together then a sequence of steps from neural crest precursor cell to mature sensory neuron can be proposed, each driven by a different factor. A schematic view of these developmental steps is presented in Fig. 4. The first step, from precursor cell to immature neuron, requires LIF. The second step, from immature neuron to factor-dependent immature neuron, requires (or is stimulated by) BDNF or NT-3; the third step, the survival of the factor-dependent neuron during target innervation and further maturation, requires NGF.

Parasympathetic Lineage

Although the identity of the factor(s) is as yet unknown, there is now evidence that a soluble factor can direct the differentiation of parasympathetic neurons from precursor cells in the neural crest. By the use of monoclonal antibodies to cell surface antigens, Barald and coworkers have identified a subpopulation of cephalic neural crest cells that are committed to a cholinergic neurogenic fate (Barald, 1988a,b). The monoclonal antibodies recognize an antigen on the cell surface that is concerned with the high affinity choline uptake. These antibodies label all the neurons in the chick and quail ciliary ganglion in vivo and in vitro. In addition, the antibodies label a subpopulation of early migratory cephalic neural crest cells.

By the use of no-flow cytometry, Barald (1989) has isolated this subpopulation of cells from neural crest cultures and studied its behavior under a variety of different culture conditions. The cells proliferate in the presence of 15% fetal bovine serum and high concentrations of chick

embryo extract, but do not differentiate. However, in chick serum and elevated K^+ and in heart, iris-, or lung-conditioned medium, the cells stopped proliferating and all of the cells became neuron-like within 10 d (Barald, 1989). These cells also stained positively for choline acetyl transferase (ChAT).

These experiments were the first to demonstrate that the development of a presumably committed population of neural crest cells can be directly manipulated by culture conditions. The continued proliferation of the cells under one set of conditions indicates that the precursors can still divide, and the observation that they will all differentiate into $ChAT^+$ neuron-like cells suggests that they are indeed neuronal precursor cells. The conditions used to stimulate the differentiation of the cells are the same that promote the survival and/or cholinergic development of ciliary ganglion neurons. This reinforces the idea that the subpopulation of neural crest cells used in this study are ciliary neuron precursors (Barald, 1989).

Melanocyte Lineage

The melanocyte lineage is apparently determined early in development in the mouse and whereas studies in chimeras suggested that 34 primordial melanocytes are lined up in pairs longitudinally during neural crest formation (Mintz, 1967), this observation would appear to be because of the segregation of cohorts of like cells in metameric units along the spinal cord as described in zebra fish (*see* Kimmel et al. 1992), and not because of clonal expansion of a single primordial melanocyte. From related studies in the chick, the melanoblasts then undergo rapid proliferation and migrate laterally to the skin (Rawles, 1944; Weston, 1963) where they differentiate into mature melanocytes. The processes that control the proliferation, migration, and differentiation of these melanocyte precursors are not clearly understood, however two classes of mouse mutants point the way for the involvement of a newly characterized growth factor in this process. These are the *White dominant-spotting (W)* and *Steel (Sl)* mice. Mice homozygous at either of these alleles are blacked-eyed white,

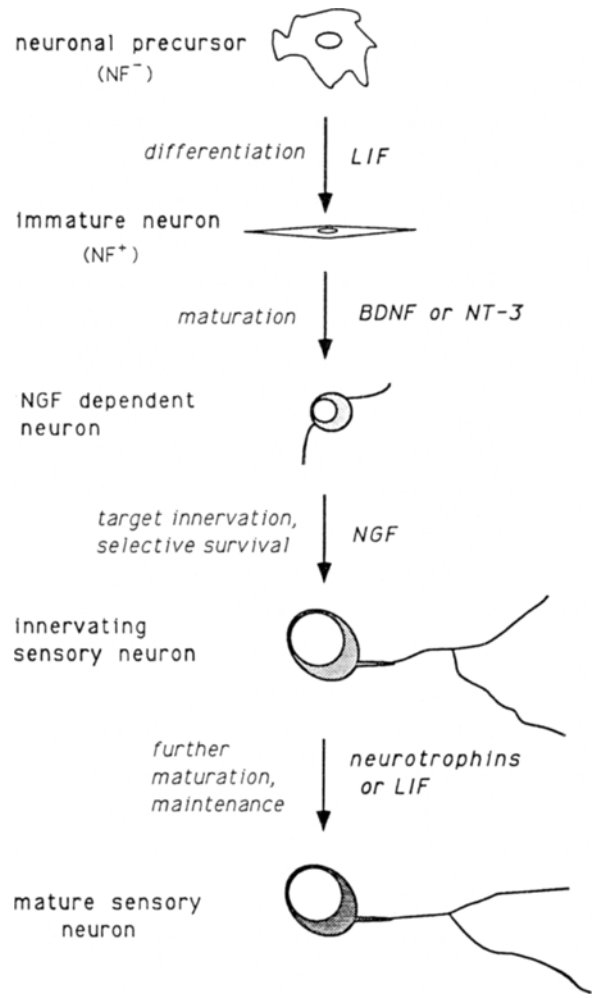

Fig. 4. A scheme for the development of sensory neurons from the neural crest. In this scheme, the development of sensory neurons is seen as a series of steps, each of which is controlled or promoted by a particular factor or factors. All steps presumably occur after the neural crest cells have migrated to the primordial sensory ganglia. *See* text for details.

anemic, and sterile; some of the mutations result in lethality (reviewed in Silvers, 1979; Russel, 1979; Geissler et al., 1981).

An analysis of the mutations in these mice has revealed a complementary molecular relationship between the two alleles. First, it was found that *W* allele coded for a growth factor receptor-like tyrosine kinase and was identical to the proto-oncogene *c-kit* (Geissler et al., 1988; Chabot et al., 1988). Subsequently, the ligand for

c-kit was purified and cloned and was found to be encoded by the *Sl* locus (Anderson et al., 1990; Williams et al., 1990; Martin et al., 1990; Copeland et al., 1990; Zsebo et al., 1990a,b; Huang et al., 1990). Thus, this *Sl* factor (SLF) and the *c-kit* receptor are strongly implicated in melanogenesis as well as germ cell production and in hemopoiesis. Because of this range of involvements the Sl factor has been variously called mast cell growth factor, stem cell factor, and the *c-kit* ligand.

We first tested whether SLF could stimulate the production of melanocytes in our neural crest cultures by adding it at the time of plating of the neural tubes. However the presence of SLF had no observable effect on the cultures and in particular no melanocytes arose in these cultures (Murphy et al., 1992). Thus, it must be concluded that SLF alone is not sufficient to stimulate the differentiation of melanocytes from their precursors in the neural crest.

In other studies, the phorbol ester drug, TPA, has been shown to influence the development of melanocytes. Human melanocytes will grow for long periods when stimulated with TPA (Eisenger and Marko, 1982; Halaban et al., 1983). Further, TPA appears to stimulate the development of melanocytes in cultures of avian dorsal root ganglia (Ciment et al., 1986). Thus, we investigated the effects of TPA on the development of melanocytes in mouse neural crest cultures. The addition of TPA to the cultures resulted in the arisal of melanocytes in the neural crest cultures after a period of 2 wk. Invariably, the melanocytes appeared on the neuroepithelial sheet that grew out from the neural tube.

Given that TPA stimulates melanocyte differentiation in the neural crest cultures, it was possible that this differentiation could be influenced by SLF. We added SLF and TPA to the neural crest cultures to test this and found an approx 10-fold increase in melanocyte numbers compared to cultures with TPA alone (Murphy et al., 1992). Thus SLF is acting with TPA to induce melanocyte differentiation.

The synergy between TPA and SLF in the production of melanocytes may be a direct synergistic effect of the two factors acting on the same cell to produce melanocytes. Alternatively, SLF may act on the melanocyte precursors to stimulate division and/or survival but not act as a differentiating agent. These possibilities could partially be tested by pulsing the cultures first with SLF, then washing it out and adding TPA to separate temporally the activities of SLF and TPA. The results of these experiments indicate SLF is mainly acting on the melanocyte precursors but not as a differentiation agent (Murphy et al., 1992). That there is a requirement for added SLF early in the culture period in these pulsing experiments indicates that SLF is acting as a survival agent for the melanoblasts. The reason that melanocytes arise in cultures containing TPA from the start might be that there is a limited amount of endogenous SLF in the cultures. This is quite possible as there is expression of SLF in the neural tube during this time in vivo (Matsui et al., 1990).

Presumably, TPA is mimicking a function normally found in vivo at the time and place of melanocyte differentiation, which is postnatally in the skin. One possible hormone implicated in melanocyte differentiation is melanocyte stimulating hormone (Ito and Takeuchi, 1984). However, we have found no activity of MSH in the neural crest cultures either in the presence or absence of SLF.

Another molecule that might be involved in the differentiation of melanocytes from their precursors is FGF, which enhances the development of pigment in cultures of dorsal root ganglia and peripheral nerve (Stocker et al., 1991). In contrast, transforming growth factor-beta 1 inhibits the formation of melanocytes in these cultures (Stocker et al., 1991) as well as in primary neural crest cultures (Rogers et al., 1992), and thus may act as a negative modulator in pigment development. As stated earlier, it would be of interest to determine if the melanocytes arising in the peripheral nerve cultures represent a separate lineage to that which migrates dorso-laterally. Recent results in our laboratory indicate that FGF overides the melanogenic capacity of TPA, further suggesting separate indentities (unpublished observations).

How Many Growth Factors Are Required for Neural Crest Differentiation?

Clearly, there must be many factors involved in the neural crest differentiation process. The factors described herein are only a few of all the factors that are presumably required to determine this specifity. What factors influence the enteric nervous system, the parasympathetic nervous system, the Schwann cells, and other glial cells in the PNS and the myriad mesenchymal derivatives? Some factors, such as glial growth factor (Raff et al., 1978) or Schwannoma derived growth factor (Kimura et al., 1990) may be involved in glial development, but the activities of these factors have not been tested in neural crest cultures. In addition, cell surface molecules, extracellular matrix molecules, and other steroids presumably contribute to this process.

Interaction Between Growth Factors and Transcriptional Regulators

The reductionist approach of attempting to identify discrete signals for differentiation is, of course, by necessity, an oversimplification of the regulation of the differentiation process. There is much evidence to suggest that neural differentiation is the result of a complex interplay between environmental signals and genetic predisposition. For example, it has been recently shown that the formation of rhombomeres in the developing hindbrain is not related to the clonal origin of such cells but reflects the position that a cohort of cells, whose members may have originated at various locations, find themselves (Fraser et al., 1990). Boundaries between rhombomeres coincide with boundaries of expression of particular *Hox* genes (*see* Fig. 2). Although it may be that a certain number of the cells are precommitted to express these homeobox genes it would appear that a process of recruitment is essential. Recruitment of this type requires some epigenetic signalling to take place between cells, and secreted growth factors, such as those mentioned here, are prime candidates for this role.

The identity of the factors that might influence these processes have clearly not been identified, however there are some examples of growth factors that do influence the expression of particular homeobox genes. A case in vertebrate neurogenesis is the interaction of *wnt-1* and *engrailed (en)*. *Wnt-1* has characteristics of a growth factor and is expressed at early times in the neural tube (Davis and Joyner, 1988). In mice containing deleted *wnt-1* genes, major defects are observed in the midbrain and cerebellum and these defects have been partially correlated with a loss of expression of the *en* homeobox gene (McMahon et al., 1992), which is also implicated in cerebellum development (Joyner et al., 1991). Thus, *wnt-1* probably regulates the expression of *en*, as previously shown for its homologue in *Drosophila* (van der Heuval et al., 1989).

Feedback regulation between growth factors and homeobox genes also occurs. A member of the transforming growth factor-β gene family, DPP, has been shown to influence the expression of homeotic genes, including the homeotic selector gene, *Ultrabithorax* (*Ubx*, Panganiban et al., 1990). The reverse regulation is also possible as the DPP growth factor gene itself has been shown to be a target of *Ubx*. (Immergluck et al., 1990; Reuter et al., 1990). The *Ubx* protein has been shown to bind to multiple identical binding sites within the Dpp gene (Ekker et al., 1991).

We propose that this two-way interaction between transcription factors, particularly homeobox genes, and growth factors may explain first, the process of lineage committment of neural crest cells, and second, how morphogenesis of crest related structures occurs. The demonstration of this awaits studies in which individual neural crest cells can be followed during differentiation under the influence of some of the various growth factors described above. Of course, this model is not limited to growth factors but could be applied to other cell-cell interactive molecules, such as N-CAM; and indeed recent findings (Jones et al., 1992) have shown that *Hox* genes products can either enhance or inhibit its production in vitro.

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