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REGULATION OF THE EARLY DEVELOPMENT OF THE NERVOUS SYSTEM BY GROWTH FACTORS

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Abstract—Development of the nervous system, although patterned by intrinsic genetic expression, appears to be dependent on growth factors for many of the differentiation steps that generate the wide variety of neurons and glia found in the both the central and peripheral nervous system. By using *in vitro* assays, including clonal analysis, the precise function of the various growth factors and the differentiation potential of the various neural populations has begun to be described. This review discusses some of the recent findings and examines how neuronal differentiation may result from the interaction of several growth factors.

Keywords—Neural precursors, clonal analysis, fibroblast growth factors, neural crest, multipotentiality, lineage commitment.

CONTENTS

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Abbreviations—aFGF, acidic fibroblast growth factor; BAG, β-galactosidase; BDNF, brain-derived neurotrophic factor; bFGF, basic fibroblast growth factor; CNTF, ciliary neurotrophic factor; DRG, dorsal root ganglia; E, embryonic day; EGF, epidermal growth factor; FGF, fibroblast growth factor; GFAP, glial fibrillary acidic protein; IGF-1, insulin like growth factor-1; LIF, leukemia inhibitory factor; MBP, myelin basic protein; NGF, nerve growth factor; NT-3, neurotrophin-3; PLP, proteolipid protein; PNS, peripheral nervous system; SCG-10, superior cervical ganglion-10; TGF, transforming growth factor; TPA, 12-*O*-tetradecanoyl-phorbol-13-acetate.

1. INTRODUCTION

Underlying early mammalian neural development are complex mechanisms, which include intercellular signalling and differential gene expression. These influences are exerted upon a precursor cell population, which can become committed to either the neuronal or glial lineages in the CNS, or to neurons, glia, melanocytes and numerous other mesenchymal cells in the peripheral nervous system (PNS). The lineage potential of individual precursor cells, however, remains the subject of intensive study, as do the relative influences that intrinsic commitments and epigenetic factors, such as growth factors, might play in both lineage determination and in instructing regionally specific development. Furthermore, whereas conventional wisdom suggests that it is the depletion of precursor cells that accounts for the cessation of neurogenesis in late development, recent studies indicate that neuronal precursor cells may persist in late embryogenesis and even within the adult mammalian brain. This implies that it is a change in the factor regulation of precursors, rather than a depletion of precursor cells *per se*, that is responsible for the failure to generate neurons in the adult brain. This review explores these issues, with particular emphasis on the role growth factors play in converting an apparently homogeneous population of precursor cells into the complex cellular and structurally diverse tissues of the adult mammalian nervous system.

2. EARLY CENTRAL NERVOUS SYSTEM DEVELOPMENT

In the developing embryo, the primordial neural tissue is first recognisable as a pseudostratified, columnar layer of precursor cells known as the neuroepithelium, which forms the neural tube and generates the entire nervous system (Theiler, 1972). Around the time of neural tube closure, the dorsal segment of the tube gives rise to a migrating, cellular population, the neural crest, which generates most of the PNS and a variety of other cell types, including mesenchymal tissue, endocrine and melanocyte derivatives. The neural tube also generates the CNS, which is comprised of two major lineages, neurons and glia, the latter being further subdivided into two principal cellular types, namely astrocytes and oligodendrocytes.

The initial phase of neural development is characterised by cellular proliferation (Cowan, 1979). As cell numbers increase, the epithelium thickens, becoming separated into a ventricular zone and a less well-defined and transient outer region, the subventricular zone, which becomes populated by committed precursor cells. In mammalian development, lineage commitment of CNS neural precursor cells is first apparent at around mid-gestation (Bailey, 1987). For instance, in the rat, the first terminally differentiated (i.e. post-mitotic) neurons develop at about embryonic day 11 (E11) (Nornes and Das, 1974). This, however, does not necessarily imply that uncommitted precursor cells are depleted thereafter from the neural anlage nor that committed precursor cells have necessarily lost the ability to proliferate. In fact, in the rat cerebral cortex, neurogenesis, as determined by the proliferation and subsequent differentiation of committed neuronal precursors, is well documented to continue until around E20 (Angevine and Sidman, 1961; Frederiksen and McKay, 1988). On the other hand, gliogenesis, as determined by the generation of glial fibrillary acidic protein (GFAP)-positive astrocytes, has been reported to commence at E16 in the rat (Abney *et al.*, 1981) and continues well into postnatal development (Miller *et al.*, 1985; Frederiksen and McKay, 1988). However, at least in some species, there may not be a temporal dispersion between the phases of neurogenesis and gliogenesis. For instance, in the monkey, a specialised subset of glial cells, known as radial glia, are generated at the time of neurogenesis (Schmechel and Rakic, 1979), and it has been proposed that mature astrocytes may be derived from these cells (Schmechel and Rakic, 1979; Culican *et al.*, 1990). Alternatively, astrocytes may be derived from morphologically undifferentiated precursor cells, which, nevertheless, are committed to the glial lineage from the earliest phase of neural development.

2.1. The Concept of Multipotentiality

In order to study the interplay between intrinsic and environmental (growth factor) regulation of precursor cells within the developing nervous system, it is important to establish whether individual neuroepithelial cells retain the capacity to differentiate into both neurons and glia; in other words,

to what extent do regulatory processes acting after neural induction exert an instructive effect upon neural cell lineage?

There is evidence to suggest that the morphologically homogeneous population of neuroepithelial cells is heterogeneous. This implies that, amongst the undifferentiated precursors, there are cells that are already restricted in their lineage potential. The concept of a heterogeneous population of precursor cells dates back to the studies of His (1889), in which two morphologically distinct cell types were identified; round cells, or neuroblasts, thought to be neuronal precursors, and columnar cells, or spongioblasts, hypothesised to be glial progenitors. However, it was subsequently shown that these morphologies reflected different phases of the mitotic cycle, rather than specifying lineage restriction (Schaper, 1897). Levitt *et al.* (1981) provided more convincing evidence of preexisting cellular determination by showing that, at midgestation in the foetal monkey, a subset of the morphologically undifferentiated ventricular cells expressed GFAP, suggesting that subpopulations of these precursors already possessed discrete commitment potentials. The operational use of GFAP as a lineage marker was probably justified, as proliferating precursor populations that invariably give rise solely to neurons (e.g. cerebellar granule cells) never expressed detectable levels of GFAP.

Current opinion favours the view that some neuroepithelial cells retain a multipotential capacity, with the ability to differentiate into both neurons and glia. Evidence for this has accumulated from immunohistochemical staining (De Vitry *et al.*, 1980), retroviral infection (Bartlett *et al.*, 1988; Frederiksen *et al.*, 1988) and the single cell cloning (Temple, 1989; Kilpatrick and Bartlett, 1993) of neural precursor cells. One of the earliest studies to claim multipotentiality (De Vitry *et al.*, 1980) demonstrated that murine hypothalamic precursor cells expressed both glial and neuronally restricted antigens, but this conclusion was dependent upon the absolute specificity of these lineage markers. More recently, fluorescent tracers have been injected into precursor cells in the developing frog retina and avian neural crest, and provide strong evidence to support the presence of multipotential precursors in these tissues (Holt *et al.*, 1988; Wetts and Fraser, 1988; Bronner-Fraser and Fraser, 1988). However, the technique is limited to short-term studies and can only be used *in vivo* in readily accessible structures.

Frederiksen *et al.* (1988) immortalised cerebral precursor cells with a temperature-sensitive mutant of the proto-oncogene SV40 large T antigen and investigated the lineage potential of the resultant immortalised cell lines. These cells retained the morphological characteristics of precursor cells at the permissive temperature, but, at elevated temperatures, both GFAP-positive cells and neuron-like cells were generated. The precursor cell lines have also provided evidence to support a role for epigenetic factors in the regulation of lineage commitment. Using v-*myc* immortalised cells, Frederiksen *et al.* (1988) found that cells of both neuronal and glial-like phenotypes could be induced by culture with dibutyryl cAMP and retinoic acid. In addition, Bartlett *et al.* (1988) were able to induce the commitment of c-*myc* immortalised precursor cells, by adding fibroblast growth factor (FGF).

The phenotypic potential of retrovirally infected neural precursor cell lines has been more recently explored by transplantation experiments (Renfranz *et al.*, 1991; Snyder *et al.*, 1992). These studies confirm that these cells have a multipotential capacity and that they, or their differentiated progeny, may have the ability to migrate to specific zones of the postnatal rodent brain, including the dentate gyrus of the hippocampus and the internal granular layer of the cerebellum. However, alteration of the genome induced by retroviral infection could alter the developmental potential of the immortalised cells and, thus, it is unclear if these results reflect the lineage potential of primary cells.

Replication-incompetent retroviruses containing the *Escherichia coli LacZ* gene, the β-galactosidase (BAG) retrovirus, detectable by the chromogenic substrate X-gal, have also been used to assess lineage, both *in vivo* and *in vitro* (Sanes *et al.*, 1986; Price *et al.*, 1987; Price and Thurlow, 1988; Luskin *et al.*, 1988, Williams *et al.*, 1991). When this technique has been applied to the developing chick spinal cord (Leber *et al.*, 1990), tectum (Gray *et al.*, 1988; Galileo *et al.*, 1990) and forebrain (Gray *et al.*, 1990) and to rat retinal development (Turner and Cepko, 1987), the presence of multipotential precursor cells has been established. However, when applied to the study of precursor cells in the mammalian cerebral cortex, the retroviral-labelling technique has produced disparate results. Problems with the technique include the variable expression of BAG by infected cells and, until recently, the full migration potential of clonally related cells may not have been appreciated (Walsh and Cepko, 1992), making interpretation of the reported data difficult. Luskin

et al. (1988) reported that the vast majority of clones derived by the *in vivo* infection of E12–14 murine cortical cells with the BAG retrovirus were homogeneous with respect to cell type; most (81%) of the derived clones were of neuronal morphology, although a smaller percentage contained phenotypic oligodendrocytes (13%) and astrocytes (4%). Price and Thurlow (1988) studied cell lineage in the E16 rat cerebral cortex and reported similar findings. It was of note that in both studies, glial clones were less frequent, but contained more cells, suggesting that neurons and glia might accumulate from precursor cells either at different rates or by different mechanisms (Luskin *et al.*, 1988; Price and Thurlow, 1988). Alternatively, it may be that glial precursors incorporate the BAG retrovirus at a lower efficiency than neuronal precursors. Williams *et al.* (1991) used retroviral infection to study the *in vitro* clonal development of cells derived from embryonic rat cerebral cortex. This study found that most clones derived from cells isolated from the cerebral cortex at E16 were once again restricted to a single lineage; after 7–8 days *in vitro*, 39% contained oligodendrocytes, 18% contained neurons and 3% contained GFAP-positive astrocytes. In addition, 36% of the clones were composed of undifferentiated cells and 5% contained both neurons and oligodendrocytes, but no astrocytes. Although collectively these results suggest significant lineage restriction within the developing rodent cerebral cortex, both the studies of Luskin and Williams identified cells of undifferentiated phenotype in some clones; consequently, the full lineage potential of the precursor cells may not have been apparent, possibly because the appropriate growth conditions for the differentiation of astrocytes were not provided. Clonal boundaries and, thus, lineage now have been more rigorously analysed in this system by infecting the developing brain with a library of genetically distinct viruses and amplifying single viral genomes using the polymerase chain reaction (Walsh and Cepko, 1992). This study has suggested that at least 5% of clones labeled from E15 rat neocortex contained both neurons and astrocytes and, thus, that they were derived from multipotential precursors. However, this result may still fall short of being definitive, given that its interpretation is dependent upon the statistical chance of the same retroviral tag infecting more than one precursor within the cortex (Guthrie, 1992).

Clonal analysis of *Xenopus* and rat retinal cellular development also suggests that a single neuroepithelial cell can generate multiple cell types (Turner and Cepko, 1987; Turner *et al.*, 1990; Holt *et al.*, 1988). These results, together with the observation that clonally related cells migrating radially within the retina encounter different microenvironments, suggest that epigenetic factors may influence lineage (Holt *et al.*, 1988). The molecular nature of this postulated epigenetic influence, however, has not been investigated. An alternative explanation for these results is that a cell-autonomous programme may regulate cell type and that epigenetic factors influence migration and cytodifferentiation, rather than instructing commitment to a particular cell lineage (Raff, 1989; Watanabe and Raff, 1990).

Temple (1989), in an *in vitro* study of E13.5–14.5 rat septal precursor cells, found that there was heterogeneity within the isolated population. By clonal analysis, it was shown that some cells exhibited multipotentiality, whilst others were restricted to a single lineage, a finding interpreted as reflecting some intrinsic commitment. Kilpatrick and Bartlett (1993) undertook clonal analysis of E10 murine neuroepithelial cells, and confirmed that there was heterogeneity within the population, in that two predominant and morphologically distinct types of clone were identified. The first type (Type A) consisted of large, amorphous cells (37% of clones) and the second (Type B) contained cuboidal, epithelial-like cells (54% of clones). In many of the Type B clones, very large numbers ($>5\times10^4$) of precursor cells were produced, suggesting that their differentiation was not due to a 'biological clock' that counts cell divisions. Although the vast majority of the clones contained undifferentiated cells, 24% of Type B clones contained a small number of neurons ($<1\%$ of the component cells). It was also found that 59% of the clones that contained neurons also contained GFAP-positive astrocytes, indicating that many of the Type B clones were derived from bipotential precursors.

At a later stage of development, the lineage potential of glial progenitors has been studied extensively, and the results suggest that one precursor can generate progeny of more than one glial cell type (Raff *et al.*, 1983b; Temple and Raff, 1985). These studies have also suggested that there are two major types of astrocyte (Raff *et al.*, 1983a). Type 1 astrocytes are thought to be important in the maintenance of the blood–brain barrier (Janzer and Raff, 1987) and in the production of neurotrophic factors (Raff, 1989), whilst Type 2 astrocytes may be important in the maintenance of

the nodes of Ranvier (ffrench-Constant and Raff, 1986b). *In vitro* lineage studies of cells isolated from the postnatal rat optic nerve, and, more recently, from the adult brain (ffrench-Constant and Raff, 1986a; Wolswijk and Noble, 1989), have suggested that these two cell types may be derived from different precursors. In particular, it has been thought that Type 2 astrocytes share a common precursor, the oligodendrocyte-Type-2 astrocyte precursor, with oligodendrocytes (Raff *et al.*, 1983b). However, it remains uncertain if these *in vitro* observations are pertinent to *in vivo* development, in particular because it has been difficult to identify Type 2 astrocytes definitively *in vivo* (Lillien and Raff, 1990). Further, the expression of GFAP by these cells *in vitro* is sometimes a transient event and, thus, is not by itself indicative of lineage commitment (Hughes *et al.*, 1988). The recent observations of Williams *et al.* (1991) analysing clones labeled with the *lacZ* reporter gene have failed to identify astrocytes and oligodendrocytes in a single clone. However, these studies did reveal a previously unidentified clonal association, suggesting a common neuronal and oligodendrocyte precursor.

To resolve the apparent discrepancies between the observed restricted nature of clones *in vivo* and the identification of proliferating precursors with multipotential capacity *in vitro*, it is necessary to postulate that cells with a multipotential capacity become progressively dormant *in vivo*, as development proceeds. This would explain why few clones with bipotential capacity are detected by labelling E12–E16 brain with retroviral markers (Luskin *et al.*, 1988; Price and Thurlow, 1988), given that, with this technique, only actively dividing cells are detected. It is also possible that multipotential cells might be present, but remain dormant both at later developmental stages and within the adult brain (see Sections 2.3 and 2.4 for further discussion). This would imply that proliferating progenitor cells and/or their differentiated progeny inhibit the mitotic division of the multipotential precursor, as development proceeds.

2.2. The Role of Growth Factors in the Instruction of the Developmental Fate of Neural Precursor Cells

Epigenetic factors may act upon neuroepithelial cells, either to potentiate their survival, to induce their proliferation or to facilitate their differentiation into mature cell phenotypes. Indeed, it has been found that insulin-like growth factor 1 is a necessary epigenetic requirement for neuroepithelial cell survival (Drago *et al.*, 1991). Growth factors that regulate the *in vitro* proliferation of neuroepithelial cells have also been identified (Gensburger *et al.*, 1987; Murphy *et al.*, 1990; Cattaneo and McKay, 1990; Anchan *et al.*, 1991), and, in particular, FGF has been shown to induce the proliferation of neuroepithelial cells isolated from E10 murine telencephalon and mesencephalon (Murphy *et al.*, 1990). In addition, Cattaneo and McKay (1990) have reported that neuronal precursor cells isolated from E13.5–14.5 rat striatum not only respond to FGF, but that proliferation was further potentiated by nerve growth factor (NGF), which correlates with the finding that embryonic striatal cells express the NGF receptor (Gage *et al.*, 1989). Recent reports by Anchan *et al.* (1991) and Reynolds *et al.* (1992) also suggest that epidermal growth factor (EGF) provides a proliferative stimulus for CNS neuroepithelial cells isolated from E17 rat retina and E14 murine striatum, respectively.

In dissociated cell cultures, at high cell density, FGF also induces the differentiation of neuroepithelial cells into neurons and astrocytes (Murphy *et al.*, 1990). However, this latter response may have resulted from the secondary production of other factors within the cultures. Clonal studies provide a more precise means of analysing these potential influences. Using this analytical method, Kilpatrick and Bartlett (1993) found that the primary response of FGF was to induce the proliferation of the neuroepithelial population, given that the vast majority of cells in the derived clones were of the undifferentiated phenotype. Further, in these studies, the removal of the proliferative signal was inadequate by itself to induce differentiation, a process that required quite discrete and separate epigenetic signals. These findings differed from those of Cattaneo and McKay (1990), who found that, in the rat, the withdrawal of FGF and NGF was sufficient to invoke the differentiation of colonies of precursor cells into neurons. The differences in the findings of the two studies may reflect either ontogenic or phylogenetic differences. Alternatively, in the culture system of Cattaneo and McKay (1990), accessory cells could have produced secondary differentiative factors, whose effects were inhibited by FGF and/or NGF. Indeed, it was apparent in the studies of Kilpatrick and Bartlett (1993) that, when the neuroepithelial precursor cells were exposed to

soluble activities produced by an N-*myc*-immortalised, astrocyte-precursor cell line (Kilpatrick *et al.*, 1993), significant numbers of differentiated neurons were generated. This finding is consistent with the observation that astroglial cells can inhibit precursor proliferation, further confirming that glial cells play a role in the terminal differentiation of CNS progenitor cells (Gao *et al.*, 1991), although in the latter study the effect was dependent upon cell surface membranes. The differentiation of precursor cells into neurons, however, also does appear to require the removal of the proliferative signal, as the effectiveness of the astrocyte-derived factor was diminished by the presence of basic FGF (bFGF) (Kilpatrick and Bartlett, 1993). It is of interest that a recent report by Nurcombe *et al.* (1993) suggests that bFGF and acidic FGF (aFGF) may have different roles in early neural development. The expression patterns of these molecules show that bFGF is expressed at the time of precursor proliferation (E10), but that aFGF is first expressed at E12, corresponding to the time of neurogenesis. However, it remains to be determined if aFGF is specifically involved in neuronal commitment and/or differentiation. Preliminary results, using clonal analysis, suggest that aFGF is not a factor that invokes neuronal differentiation in this system (T. J. Kilpatrick, unpublished observations).

The nature of the regulation that invokes the differentiation of neuroepithelial cells into astrocytes remains largely unknown. Cell–cell interactions may be important in this process, as the generation of GFAP-positive astrocytes amongst clonal populations derived from multipotential precursors occurs predominantly in regions comprised of multiple layers of cells (Kilpatrick and Bartlett, 1993). In optic nerve cultures, the fate of committed glial progenitor cells has been shown to be dependent on epigenetic factors, in that their proliferation is stimulated by either platelet-derived growth factor (Richardson *et al.*, 1988; Noble *et al.*, 1988; Raff *et al.*, 1988), FGF (McKinnon *et al.*, 1990) or a combination of both platelet-derived growth factor and FGF (Bögler *et al.*, 1990), and withdrawal of these factors induces oligodendrocyte differentiation. Alternatively, the cells can be stimulated to differentiate into Type 2 astrocytes by culture with ciliary neurotrophic factor (CNTF) (Hughes *et al.*, 1988) and the extracellular matrix (Lillien *et al.*, 1990), in the presence of serum (Raff *et al.*, 1983b). However, in the assay system established by Murphy *et al.* (1990), CNTF has no identifiable effect upon E10 murine neuroepithelial cells.

Cell-signalling molecules may not only influence cell lineage, but they could also instruct regionally specific development within the mammalian CNS, either by influencing cell fate or by potentiating the survival and/or proliferation of regionally committed progenitors (McMahon *et al.*, 1992). Circumstantial evidence links retinoic acid with the regulation of *Hox* genes, but, as emphasised by McGinnis and Krumlauf (1992), the effects of retinoic acid are pleiotropic, making it difficult to conclude that its influence upon *Hox* gene expression is necessarily direct. In contrast, there is good evidence to suggest that the *Wnt* proto-oncogenes (Nusse and Varmus, 1992) could represent a family of genes encoding signalling molecules that directly influence regional development: firstly, *Wnt* genes have highly restricted patterns of expression within the developing brain (Wilkinson *et al.*, 1987) and secondly, mice homozygous for null alleles of *Wnt*-1 (*int*-1) exhibit loss of the midbrain and cerebellum (McMahon and Bradley, 1990). Of further interest is the finding that these mutant mice also exhibit perturbed transcriptional factor expression within the hindbrain, in particular of the homeobox-containing gene, *En*. In fact, the first abnormality identifiable in these mice is of loss of anterior expression of *En* within the midbrain, suggesting that although *Wnt*-1 is not implicated in the activation of *En*, it may be necessary for the maintenance of *En* expression (McMahon *et al.*, 1992). This interaction may be of great significance as, in *Drosophila*, the orthologue of *En*, *engrailed*, is implicated in the specification of posterior segment identity in response to the polarised expression of the orthologue of *Wnt*-1, *wingless* (Martinez Arias *et al.*, 1988; McMahon *et al.*, 1992). This potential signalling cascade serves to demonstrate how growth factors may interact with homeobox genes to influence pattern formation within the developing embryo.

These findings, however, do not preclude the possibility that a cell-intrinsic developmental programme is important in guiding either regionally specific pattern formation or cell lineage. In terms of regional development, this influence could be mediated by the intrinsic control of *Hox* gene expression. This might be effected by either autoregulatory circuits (McGinnis and Krumlauf, 1992) or by the expression of transcription factors that directly enhance *Hox* gene expression, as exemplified by the regulation of *HoxB2* (2.8) by the zinc finger gene *Krox20* during hindbrain segmentation (Sham *et al.*, 1993). At the single cell level, subsets of committed but morphologically homogeneous and

undifferentiated progenitor cells could be produced by the asymmetrical division of multipotential precursors. If this were so, epigenetic factors may provide the necessary requirements to invoke differentiation, but may not, by themselves, instruct cell type. The best evidence for such a combinational influence is derived from studies of the oligodendrocyte-Type-2 astrocyte progenitor (Raff, 1989) and of rat retinal neuroepithelial cell development (Watanabe and Raff, 1990), in which it has been demonstrated that cell–cell interactions and a cell-autonomous developmental programme combine to instruct both the timing and the nature of precursor cell differentiation.

2.3. Age Dependency: An Ontogenic Hierarchy of Stem Cells?

It has been found that E10 murine neuroepithelial cells proliferate in response to bFGF, but not to EGF (Murphy *et al.*, 1990; Kilpatrick and Bartlett, 1993). In contrast, Reynolds *et al.* (1992) identified EGF and transforming growth factor (TGF)-a as mitogens for multipotential precursors isolated from E14 murine striatum, and Anchan *et al.* (1991) identified EGF as a mitogen for E17 rat retinal neuroepithelial cells. These disparate results suggest that the capacity of a precursor cell to respond to a given growth factor is dependent on developmental stage. Of relevance to this issue are the findings of Lillien and Cepko (1992), which demonstrated that rat retinal neuroepithelial cells change during development in terms of their responsiveness to mitogenic signals: in particular, progenitor cells from younger retinas were more responsive to FGF, whereas those isolated from older retinas were more responsive to TGFa. This issue has been further addressed by cloning E17 murine cerebral cells (T. J. Kilpatrick and P. F. Bartlett, unpublished observations). The results indicate that it is possible to isolate proliferating precursor cells from this population with either bFGF or EGF, although the cloning efficiency is superior with bFGF. However, whereas E17 precursor cells gave rise to astrocytic progeny with either bFGF or EGF, it was only possible to identify proliferating multipotential precursors with bFGF, suggesting that the effects of EGF upon precursor cells are confined to committed glial progenitors. This contrasts with the findings of Reynolds *et al.* (1992), who have suggested that EGF and TGFa induce the proliferation of CNS multipotential progenitors. However, these latter results were obtained from bulk cultures that contained 2500 cells/cm^2, and when single-cell analysis was undertaken, neither the generation of GFAP- nor neurofilament-positive cells was reported. This observation suggests that the differentiative effects of EGF could be indirect, and that neuronal differentiation is reliant upon the production of secondary factors by either committed or post-mitotic cells in the bulk cultures. Furthermore, it is unclear as to whether the proliferating cells in the clonal cultures had multipotential capacity, even though they stained positively for nestin, a putative precursor-cell marker (Frederiksen and McKay, 1988). Indeed, it remains possible that these cells were committed, but GFAP-negative, glial progenitors, an interpretation that would be consistent with our findings (T. J. Kilpatrick and P. F. Bartlett, unpublished observations).

We have also found that, unlike E10 precursors, E17 multipotential precursors cultured with bFGF exhibit the intrinsic capacity to generate large numbers of neurofilament positive cells, even in the continued presence of the proliferative signal. Although the majority of these latter cells remained morphologically undifferentiated, this finding indicates that multipotential precursors have an age-dependent variability in their capacity to exhibit commitment to the neuronal lineage, in response to set epigenetic conditions. Furthermore, the proliferative potential of cells within clones derived from multipotential precursors isolated at E17 is much lower than from those isolated at E10 (T. J. Kilpatrick and P. F. Bartlett, unpublished observations). Lillien and Cepko (1992) also found that the proliferative potential of progenitors isolated from the retina tended to reduce with age. Thus, there may be an ontogenetic hierarchy of multipotential precursor cells within the CNS, analogous to the well-defined hierarchy of precursors within the haematopoietic system (Metcalf and Moore, 1971).

2.4. The Identification of Multipotential Precursors in the Adult Mammalian Brain

In the mouse, the majority of neurons are formed prenatally, although there are well-identified exceptions, including granule neurons in the hippocampus (Altman, 1963; Schlessinger *et al.*, 1975; Altman and Bayer, 1990) and cerebellum (Altman, 1972), and the olfactory neuroepithelium

(Graziadei and Graziadei, 1979). It is already well established that cells within the subventricular zones of the brains of adult fish, amphibians and some birds retain the capacity for proliferation (Alvarez-Buylla *et al.*, 1988). In canaries, these proliferating cells have been shown to maintain the capacity to differentiate into neurons and to populate song-bird nuclei, in response to hormonal stimuli (Nordeen and Nordeen, 1989). Further, it has been shown that proliferating cells can be identified in the subventricular zone of adult mammals, as assessed by tritiated thymidine incorporation studies (Morshead and van der Kooy, 1992). When these cells were marked by *LacZ* infection, it was shown that the resultant clones were virtually never more than two cells in size, whereas some 33% of the subventricular cellular population was shown to be undergoing cell division. This finding suggests that the proliferating cells were dividing asymmetrically, with one progeny maintaining an undifferentiated phenotype, thus contributing to the self-renewal of the precursor, whereas the other daughter cell invariably died. If this were so, it could be that the proliferating cells are precursors, which, in the absence of appropriate epigenetic conditions, lack the ability to differentiate into mature neural cell phenotypes. The elucidation of the epigenetic conditions that induce the neuronal differentiation of neuronal precursors in embryogenesis has provided the means to address this issue.

Two recent reports suggest that neuronal precursor cells exist in the adult mammalian brain (Reynolds and Weiss, 1992; Richards *et al.*, 1992). Reynolds and Weiss (1992) isolated cells from the striatum of the adult mouse brain and induced the *in vitro* proliferation of precursors with EGF in serum-free conditions. These cells were also demonstrated to have the capacity to differentiate into neurons and glia. Richards *et al.* (1992) reported that neuronal induction from murine cerebral precursors was optimal under *in vitro* conditions in which the cells were initially stimulated with bFGF and then with medium conditioned by an N-*myc* immortalised astrocyte-precursor cell line (Kilpatrick *et al.*, 1993). In this study, neuronal induction was not potentiated by initial culture with EGF. The reason for the disparate results of the studies with regard to the efficacy of EGF remains unclear. It may be that under the culture conditions employed by Reynolds and Weiss (1992), there was production of secondary factors, including FGF, within the cultures (see Section 2.2). Alternatively, it is possible that the differences relate to a disparity in the expression of the EGF receptor by different subpopulations of precursor cells.

One major challenge is to determine if the *in vivo* differentiation of these precursor cells can be achieved. In order to investigate this, it will be necessary to determine whether the precursors survive within the brain in a dormant, non-proliferative state or whether they represent an analogous population to the proliferating subependymal cells identified by Morshead and van der Kooy (1992).

2.5. Potential Clinical Applications

The results of adult neural cell culture, together with the results of clonal analysis of embryonic neuroepithelial cells, suggest that the differentiation of precursor cells is invoked by specific epigenetic conditions. These observations raise the possibility of utilising precursor cell populations, present *in situ*, to replace degenerating neurons by the *in vivo* administration of differentiative factors. The ability to generate large clonal populations from multipotential precursors *in vitro* suggests that neuronal repopulation could also be achieved by precursor cell transplantation. Renfranz *et al.* (1991) and Snyder *et al.* (1992) have recently transplanted retrovirally immortalised multipotential cells into postnatal rat brain, and have provided convincing evidence of *in vivo* differentiation of these cells into neuronal and glial elements. The elucidation of epigenetic conditions, which are permissive for the proliferation of multipotential precursors, has provided the methodology to repeat the above transplantation experiments with primary cells. Such experiments would have obvious advantages over those using cell lines. Firstly, lineage could be studied using cells that have not been affected by genetic manipulation and, secondly, if precursor cell transplantation is to be applied to human degenerative disease, primary cells will be the preferred source.

Transplantation experiments could also be performed using primary cells after they have been exposed to either specific growth factors or to antisense oligonucleotides designed to inhibit the effects of specific growth factors or their receptors. Such experiments would also provide further insight into how exogenous and endogenous mechanisms interact to regulate the differentiation of neural cells.

Preliminary results indicate that transplanted neuroepithelial cells can successfully engraft into the

postnatal mammalian brain (T. J. Kilpatrick, S. Cheema, S. Kobler and P. F. Bartlett, unpublished observations). These experiments also suggest that cells injected into the hippocampus selectively migrate to regions of continuing neurogenesis in the host brain. In particular, some of the transplanted cells align within the granular layer of the dentate gyrus, where they elaborate processes that extend toward the CA2 region of the pyramidal layer, indicating that the donor cells can adopt similar morphologies to those of host granule neurons. An additional challenge will be to determine the lineage potential of the injected cells in other regions of the brain. Furthermore, if this technique is ever to be applied to human degenerative disease, it also will be necessary to establish that cells can engraft and differentiate within the brain of adult hosts, especially animals with cerebral lesions.

3. EARLY DEVELOPMENT OF THE PERIPHERAL NERVOUS SYSTEM

The neural crest is a transient structure that arises from the dorso-lateral aspect of the closing neural tube. The cells migrate along several discrete tissue pathways and give rise to the majority of cells of the PNS, 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, the neural crest 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.

3.1. 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 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, 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; 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. Over a period of 10 years or more, this approach 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, that the resultant neural crest derivatives correspond to their original position along a rostral–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 located mainly in the head and neck.

3.2. Commitment Versus Multipotentiality

The fate map described in Section 3.1 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 by 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, which are located exclusively 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 neural crest 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 crest cells migrate into the dorsal mesentery and colonise 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 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 vertebrate neural development may be primarily the result of mesenchymal influence, although the inductive agents are unknown as yet (see Dodd, 1992, for review).

3.3. Neural Crest Cells Display Multipotential and Committed Characteristics When Grown *In Vitro*

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 for 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 are 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 non-neuronal 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.

Thus, these studies 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, but 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 co-segregation 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 support the concept of sequential differentiation from a multipotent 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 analysed after a number of weeks, when there can be thousands of cells in each clone. Under these conditions, the microenvironment of each clone itself might vary; there might be endogenous production of different growth factors that could influence cell phenotype. One labour-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 occurring during crest development.

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 fluorescent dye prior to migration from the neural tube (Bronner-Fraser and Fraser, 1988, 1989; Fraser and Bronner-Fraser, 1991). After 2 days, the clonal progeny of the cells sometimes were 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 (DRG), 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 Fraser, 1988, 1989). Whilst these studies indicate the diversity of cell products, there has been concern as to whether they represent the progeny of a single cell. Confining the injection to just one cell still appears to present considerable technical difficulties to other workers. However, results obtained by following the progeny of neural crest cells infected with *lacZ*-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.

3.4. 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. One approach to studying the mammalian neural crest is to make immortalised 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 own and other laboratories 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).

In similar manner, we immortalised 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 classified broadly 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 NGF and its receptor, which are expressed by cells in the PNS, myelin basic protein (MBP) and the proteolipid protein (PLP) of myelin, which, in the PNS, 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 this group also was 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 committed to a single developmental pathway yet and probably represent stem cells. These stem cells presumably have 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, cells of this line expressed neurofilament, as well as MBP and PLP, showing that they 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 expressed neurofilament. Similarly, multipotent neural cell lines have been isolated from newborn brain (Frederiksen *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, the NC14.4.8 cell line contained cells that differentiated, after 1–2 weeks in culture, into Schwann-like cells. Further, these older cultures expressed mRNA for MBP, PLP, NGF and NGF receptor. 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, which 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 immortalised 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, that displayed bipotential characteristics was generated. 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.

3.5. Growth Factor Regulation of Neural Crest Proliferation

We have shown previously that 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 DRG anlage, there is 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 bFGF, there is significant survival of the neural crest cells for a period of over 30 hr 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 non-neuronal 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 (M. Murphy, K. Reid, J. Furness and P. F. Bartlett, 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 non-neuronal 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 radioimmunoassays, which showed that bFGF was detectable in spinal cords from as early as E3 and 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 NGF-like peptides. In mixed cultures of somites and neural crest cells, NT-3 caused a significant increase in the number of neural crest cells incorporating ^{3}H-thymidine and increased total cell numbers. 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 those committed to a particular lineage.

3.6. Growth Factor Regulation of Neural Crest Differentiation

3.6.1. *Sympathoadrenal Lineage*

Developmentally, the best characterised 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 fluorescent 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 in Section 3.5. Thus, the possibility that the developing sympathetic neuron precursors will find a supply of this factor at the site of ganglia is 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 sympathetic neurons has been demonstrated over the past

40 years using numerous experimental systems (see Levi-Montalcini and Angeletti, 1968). It remains the only molecule shown unequivocally to be critical for neuron survival *in vivo*. The injection of anti-NGF 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. Insulin like growth factor-1(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. Conversely, CNTF 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 been purified recently and is equivalent to leukemia inhibitory factor (LIF) (Yamamori *et al.*, 1989). As discussed in the next section, it is beginning to emerge that LIF has multiple activities within the nervous system, as well as outside it.

3.6.2. *Sensory Lineage*

The processes that regulate the development of sensory neurons from their precursors in the embryonic neural crest have not been well characterised. 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 Section 3.6.1) 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 non-dividing precursors, a characteristic observed previously 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 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 DRG 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 non-neuronal 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 once the factor is taken up by the nerve terminals, it should be retrogradely transported back to the neural perikaryon. The transport of the neurotrophic factor is the signal from the target tissue to the neuron that results in neuronal survival (Hendry *et al.*, 1974). To test the possibility that LIF is transported retrogradely, 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 DRG (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 at sites of peripheral sensory innervation.

The best characterised 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 acts most probably as a target-derived survival factor during the period of natural neuron death, as discussed in Section 3.6.1 for sympathetic neurons. The evidence for the time of action of NGF on sensory neurons comes from expression studies: mRNA for NGF is first observed in the target tissue at the time of innervation of the newly formed neurons, which is concomitant with appearance of NGF receptors on nerve fibres as they innervate these target tissues (Bandtlow *et al.*, 1987; Davies *et al.*, 1987). In addition, the role of NGF *in vivo* has been established by Johnson and coworkers, who immunised female guinea pigs with NGF and showed that their offspring, which were exposed to NGF antibodies during the period of sensory development, lost up to 80% of their sensory neurons (see Johnson *et al.*, 1986 for review).

Other factors, such as the other neurotrophins, also act 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. 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.

3.6.3. *Parasympathetic Lineage*

While the identity of the factor(s) is unknown as yet, 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 recognise 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-migrating 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 behaviour under a variety of different culture conditions. The cells proliferate in the presence of 15% foetal bovine serum and high concentrations of chick embryo extract, but do not differentiate. However, in chick serum, elevated K^+ or heart-, iris- or lung-conditioned medium, the cells stopped proliferating and all of the cells became neuron-like within 10 days (Barald, 1989). These cells also stained positively for choline acetyl transferase.

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 choline acetyl transferase-positive neuron-like cells suggests that they, indeed, are 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 represents ciliary neuron precursors (Barald, 1989).

3.6.4. *Melanocyte Lineage*

The melanocyte lineage is apparently determined early in development in the mouse, and whilst studies in chimeras suggest that 34 primordial melanocytes are lined up in pairs longitudinally during neural crest formation (Mintz, 1967), this observation would appear to be due to the segregation of cohorts of like cells in metameric units along the spinal cord, as described in zebra fish, and not due to 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 to the involvement of a newly characterised growth factor in this process. These are the *White dominant-spotting* (*W*) and *Steel* (*Sl*) mice. Mice homozygous at either of these alleles are black-eyed, white, anaemic and sterile; some of the mutations result in lethality (reviewed in Russel, 1979; Geissler *et al.*, 1981).

An analysis of the mutations in these mice has revealed a complementary molecular relationship between the two alleles. Firstly, it was found that the *W* allele coded for a growth factor receptor-like tyrosine kinase, which 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 and the c-*kit* receptor are strongly implicated in melanogenesis, as well as germ-cell production and in haemopoiesis. Because of this range of involvement, the Sl factor variously has been called mast-cell growth factor, stem cell factor and the c-*kit* ligand.

We first tested whether Sl factor 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 Sl factor 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 Sl factor alone is not sufficient to stimulate the differentiation of melanocytes from their precursors in the neural crest.

In other studies, the phorbol ester drug, 12-*O*-tetradecanoyl-phorbol-13-acetate (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). Therefore, 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 appearance of melanocytes in the neural crest cultures after a period of 2 weeks. 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 Sl factor. We added Sl factor and TPA to the neural crest cultures to test this and found an approximate 10-fold increase in melanocyte numbers compared with cultures with TPA alone (Murphy *et al.*, 1992). Thus, Sl factor is acting with TPA to induce melanocyte differentiation.

The interaction between TPA and Sl factor in the production of melanocytes may involve direct synergistic effect of the two factors acting on the same cell to produce melanocytes. Alternatively, Sl factor may act on the melanocyte precursors to stimulate division and/or survival, but not act as a differentiating agent. These possibilities could be tested partially by pulsing the cultures first with Sl factor, then washing it out and adding TPA to separate temporally the activities of Sl factor and TPA. The results of these experiments indicate Sl factor is mainly acting on the melanocyte precursors, but not as a differentiation agent (Murphy *et al.*, 1992). That there is a requirement for added Sl factor early in the culture period in these pulsing experiments indicates that Sl factor 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 Sl factor in the cultures.

This is quite possible, as there is *in vivo* expression of Sl factor in the neural tube during this time (Matsui *et al.*, 1990).

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

Another molecule that might be involved in the differentiation of melanocytes from their precursors is FGF, which enhances the development of pigment in DRG cultures and peripheral nerve (Stocker *et al.*, 1991). In contrast, TGF-β1 inhibits the formation of melanocytes in these cultures and, thus, may act as a negative modulator in pigment development (Stocker *et al.*, 1991). As stated in Section 3.3, it would be of interest to determine if this represents a separate lineage to that which migrates dorso-laterally. Recent results in our laboratory indicate that FGF overrides the melanogenic capacity of TPA, further suggesting separate identities (M. Murphy, K. Reid and P. F. Bartlett, unpublished observations).

4. INTERACTION BETWEEN GROWTH FACTORS AND TRANSCRIPTIONAL REGULATORS

The reductionist approach of attempting to identify discrete signals for differentiation, by necessity, is 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 shown recently that the formation of rhombomeres in the developing hindbrain is not related to the clonal origin of such cells, but reflects the position in which 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. Whilst 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 identities of the factors that might influence these processes clearly have not been determined. 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 correlated partially 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).

We propose that this two-way interaction between transcription factors, particularly homeobox genes, and growth factors may explain firstly, the process of lineage commitment of nervous system precursor cells and secondly, how morphogenesis of the nervous system occurs. The demonstration of this awaits studies in which the influence of some of the various growth factors described on individual neural crest cells can be followed during differentiation. Of course this model is not limited to growth factors, but could be applied to other cell–cell interactive molecules, such as neural-cell adhesion molecule, and, indeed, recent findings (Jones *et al.*, 1992) have shown that *Hox* gene products can either enhance or inhibit neural-cell adhesion molecule production *in vitro*.

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