

## Involvement of leukemia inhibitory factor and nerve growth factor in the development of dorsal root ganglion neurons

Mark Murphy, Kate Reid, Melissa A. Brown and Perry F. Bartlett

The Walter and Eliza Hall Institute of Medical Research, Post Office, Royal Melbourne Hospital, Parkville, Victoria 3050, Australia

### SUMMARY

**Leukemia inhibitory factor (LIF) was recently shown to stimulate the generation of sensory neurons from the murine neural crest in vitro. Here, we examine the respective activities of LIF and nerve growth factor (NGF) throughout the embryonic development of sensory neurons in dorsal root ganglia (DRG) and neural crest. In cultures of embryonic day 12 (E12) DRG, which contain sensory neuron precursor cells, a combination of both LIF and NGF are required for the differentiation of mature sensory neurons from their neurofilament negative (NF<sup>-</sup>) precursors. The primary differentiation step from NF<sup>-</sup> cell to NF<sup>+</sup> immature neuron is promoted by LIF, whereas the survival and further maturation of the newly differentiated neurons depends on NGF. In cultures of sensory neurons isolated**

**at the time of target innervation (E14 and E15 DRG), the survival of the majority of the neurons is dependent on NGF. However, LIF acts as a survival agent for a discrete population of NGF non-responsive neurons. From E16, the number of neurons maintained by LIF increases to >90% by birth. Consistent with the in vitro observations, LIF mRNA could be detected at early developmental stages (E12-E13), within the spinal column and DRG as well as the limbs and, later (after E15), in areas of sensory innervation (skin, limbs, feet and gut). This supports the idea that LIF, as well as NGF, may regulate sensory development in vivo.**

Key words: leukemia inhibitory factor, nerve growth factor, sensory neuron development

### INTRODUCTION

The sensory nervous system in vertebrates is derived from the embryonic neural crest and the ectodermal placodes (see Le Douarin, 1986; Le Douarin and Smith, 1988 for review). The sensory precursors from the neural crest separate from the neural tube just prior to its closure and migrate to the sites of ganglia formation adjacent to the somites. There, these cells differentiate into sensory neurons, Schwann cells and accessory cells of the developing sensory ganglia. The immature sensory neurons then begin to innervate the developing skin, muscle and other sites of sensory innervation. At, or directly after, the period of innervation, many of the sensory neurons die, a phenomenon that has been attributed to the elimination of those neurons that have made no or an inappropriate connection with the target tissue and thus are redundant (see Purves, 1988). After innervation and the period of natural neuron death is complete, the sensory ganglia continue to mature, the neurons increase in size, become myelinated and begin to synthesize a variety of neuropeptides.

The control of these developmental stages presumably resides in both cell autonomous and cell extrinsic factors. The most well-characterized factor shown to play a role in the process is nerve growth factor (NGF, Levi-Montalcini, 1982; Thoenen and Barde, 1980). NGF most probably acts

as a target-derived survival factor during the period of natural neuron death, whereby its limited availability results in the selection of only appropriately innervated neurons. The evidence for the time of action of NGF 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 fibres of these neurons (Bantlow et al., 1987; Davies et al., 1987). In addition, NGF is one of the few molecules to have its function in sensory development established beyond reasonable doubt in vivo. Johnson and coworkers have immunized female guinea pigs with NGF and their offspring, which are exposed to NGF antibodies during the period of sensory development, lose up to 80% of their sensory neurons (see Johnson et al., 1986 for review).

A number of other factors act as survival factors for sensory neurons. Two of these, brain-derived neurotrophic factor (BDNF; Barde et al., 1982; Leibrock et al., 1989) and neurotrophin 3 (NT-3; Hohn et al., 1990) are structurally related to NGF. These factors support the survival of overlapping but not identical subpopulations of sensory neurons in the chick. However, the precise role or the time of action of these molecules during development has not been defined, that is whether these factors act before, during or after target innervation and if they have overlapping roles with NGF.

Leukemia inhibitory factor (LIF), a cytokine displaying a diverse range of activities both in and out of the nervous system (see Hilton and Gough, 1991 for review), has also been implicated in sensory development (Murphy et al., 1991). LIF stimulates the development of sensory neurons in cultures of mouse neural crest as well as in developing dorsal root ganglia (DRG). In mature DRG cultures, where the neurons have already differentiated, LIF acts as a survival factor for the neurons, in a similar fashion to NGF. Thus LIF appears to have both separate and similar roles to NGF.

In this paper, we have looked at the relative roles and actions of LIF and NGF throughout the embryonic development of sensory neurons in the DRG.

## MATERIALS AND METHODS

### Preparation of DRG cells

DRG from CBA mice of specified age (according to the criteria described in Theiler, 1989) were dissected free of surrounding spinal tissue using 27-gauge syringe needles and then incubated in HEM, 0.025% trypsin, 0.001% DNAase at 37°C. P2 DRGs were chopped with a scalpel blade prior to trypsinization. The incubation times for the respective ages were as follows: 12 minutes for E12-E14, 20 minutes for E15-E18 and 30 minutes for E19 and P2. Foetal bovine serum (FBS) was added to 20% and the cells were centrifuged at 300 g for 5 minutes, then washed in Hepes-buffered Eagles medium (HEM), 0.01% DNAase. E19 and P2 DRGs required trituration through 18- to 24-gauge needles to obtain a single cell suspension. The cells were then washed again in HEM, 0.01% DNAase and resuspended in Monomed medium (Commonwealth Serum Laboratories, Melbourne, Australia), 10% FBS.

### Preparation of neural crest cells

Neural tubes were dissected from mouse embryos at E9 and plated onto fibronectin-coated wells in the presence of the appropriate growth factors as previously described (Murphy et al., 1991). After 24 hours, the neural crest cells had migrated away from the neural tube explant and the volume of each culture was adjusted to 0.5 ml. In cultures where anti-NGF antibodies (see below) were present, fresh antibody was added to the culture every day for the duration of the experiment.

### Assays

DRG cells were plated at the indicated cell number onto wells of HL-A plates (Nunc, Naperville, IL), which had previously been coated with 50 µg/ml fibronectin (Boehringer-Mannheim, FRG) for 1 hour. The cells were plated in Monomed medium, 10% FBS, growth factors and anti-NGF antibodies where specified. LIF (*E. coli*-derived murine recombinant, specific activity=10<sup>8</sup> units/ml) was used at 10<sup>3</sup> units/ml and was a gift from N. Nicola; mouse 2.5S NGF (Boehringer-Mannheim) was used at 25 ng/ml and a monoclonal anti-2.5S mouse NGF (Korsching and Thoenen, 1983; Boehringer-Mannheim) at 500 ng/ml, which inhibits endogenous plus added NGF (at least 10ng/ml) in cultures of DRG cells plated at 1000 cells/well; this antibody has also been shown to inhibit NGF activity in a variety of applications in vitro and in vivo (Neet et al., 1987; Rohrer et al., 1988).

Since plating efficiencies for preparations at different embryonic ages varied (see Results), the initial cell numbers plated (the total cell number applied to the well including neurons and non-neurons) were as follows: E12-E16, 1000 or 300 cells; E17-E19, 200 cells; P2, 100 cells. After 2 hours, all the cells in the wells

had settled and the cultures were examined for the presence of morphologically defined neurons, which were counted. Neurons were identified in cultures at different ages as follows: in cultures of E12 and E13 DRG, neurons were identified by morphological appearance after several days in culture and/or by neurofilament (NF) staining as discussed in the results. In older cultures (E14 and older), the neurons are large, phase-bright, spherical cells, 2 hours after plating. In cultures of E14 and E15 DRG, it was not always possible to distinguish clearly neurons from other cells in the culture on size difference only after 2 hours, so the cultures were recounted after another 10 hours and compared with the initial counts. By this time, the neurons were clearly distinguishable from the other cells, which had adhered to the substratum and flattened. In these cultures, the number of neurons in the NGF, and LIF plus NGF were either the same or slightly increased over the 2 hour counts and were taken as 100%. Some cultures were also independently stained for the presence of NF to confirm neuronal identity.

To determine accurately the degree of survival in the E14-P2 DRG cultures, the number of neurons in each well were determined initially 2 hours after plating and then every 24 hours on the next 2-3 days. The percentage survival, where 100% is the number of neurons after 2 hours (or after 12 hours for E14-15; see above), could thus be determined for each well. The numbers shown are the mean±s.d. of the percent survival for 6 wells. All experiments were performed at least twice.

### Immunohistochemistry

Cultures were fixed in methanol at -20°C for 30 minutes, washed in PBS and stained for the presence of p150 NF, with detection using immunofluorescence as previously described (Murphy et al., 1991) or using peroxidase as follows: the fixed cultures were washed 3 times in phosphate-buffered saline (PBS), incubated successively in Hepes-buffered Eagles medium (HEM) containing 2% FBS for at least 1 hour then with p150 NF antibody (Chemicon, Temecula, Cal) diluted in HEM, 1% FBS. After washing 3 times in PBS, the cultures were incubated in a biotinylated anti-rabbit immunoglobulin (diluted 1:200 in HEM, 2% FBS, obtained from Vector, Burlingame, Cal) for 45 minutes, washed 3 times and incubated for 45 minutes with an avidin-biotin-peroxidase complex (Vector). The cultures were then incubated for 6-10 minutes in diaminobenzidine tetrahydrochloride (1 mg/ml) and hydrogen peroxide (0.3% of a 30% solution), washed 3 times in PBS and mounted in Aquamount (BDH, England).

### Analysis of LIF expression

A semiquantitative PCR technique was used to measure the low levels of LIF mRNA in embryonic tissues (M. A. Brown, D. Metcalf and N. M. Gough, unpublished data). Tissues were dissected for RNA extraction into a Petri dish containing PBS, from E11-E19 mice using 27-gauge syringe needles and from postnatal mice using small vannas scissors. Tissues dissected were feet (fore and hind), remainder of limb, tail, skin, gut, fore and midbrain, hind-brain, spinal cord (including meninges), DRG and whole spinal column (including spinal cord, meninges, DRG, vertebrae and/or somites). Total RNA was extracted from tissues dissected from E12 to E15 mouse embryos using a guanidinium extraction procedure (Chomczynski and Sacchi, 1987). Poly(A)<sup>+</sup> RNA was extracted from tissues dissected from E19 embryos and P2 mice using a proteinase K/SDS/oligo(dT) procedure (Gonda et al., 1982). First-strand cDNA was synthesized as previously described (Allan et al., 1990) from at least 100 ng of RNA and used as a template for PCR. PCR reactions contained 200 µM dNTP, 1 µM primers, standard PCR buffer (Cetus) with Mg<sup>2+</sup> at 2.5 mM, and 1.25 units of Taq polymerase (Cetus). Cycle conditions were 94°C for 1.5 minutes, 60°C for 2 minutes and 72°C for 3 minutes

through 25 to 30 cycles depending on the amount of RNA. Primers for LIF and the glycolytic enzyme GAP-DH, which was used as a control for RNA quality and quantity, were as previously described (Allan et al., 1990). PCR reaction products were analysed by electrophoresis through 1.2% agarose gels and then transfer to Zeta Probe membrane (Biorad) in 0.4 M NaOH (Reed and Mann, 1985) for 2 to 6 hours for Southern analysis. Filters were prehybridized and hybridized in 50% formamide, 5× SSC, 5% SDS, 10 mM sodium phosphate, 1% skim milk powder at 42°C. DNA fragments (Allan et al., 1990) were radiolabelled with  $^{32}\text{P}$ -a-dATP (Bresatec, Australia) by random priming to a specific activity of approx.  $2 \times 10^9$  cts/minute per  $\mu\text{g}$  and used in hybridizations at a concentration of  $2 \times 10^7$  cts/minute per ml. Following overnight hybridization, filters were washed to a final stringency of  $0.2 \times \text{SSC}$  at 65°C and autoradiographed (Kodak XAR5 film) with two intensifying screens at  $-70^\circ\text{C}$ .

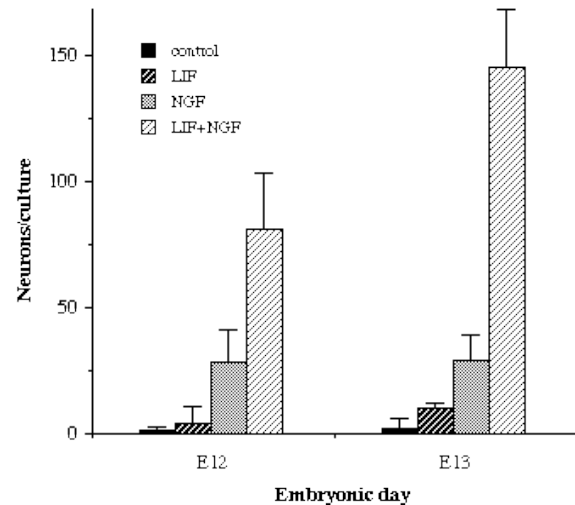
## RESULTS

### Interaction between LIF and NGF in the early stages of sensory neuron differentiation

We initially reported that LIF stimulates the generation of sensory neurons from non-dividing precursors in cultures of mouse neural crest and immature (E12) DRG cultures (Murphy et al., 1991). These cultures were at high cell density: in the case of the neural crest cultures, the region in which the neurons arose was always over a confluent monolayer of neural crest cells and, in the case of the E12 DRG cultures, the development of neurons was dependent on the cell number initially plated. Very few morphologically identifiable neurons (neurons with a large cell body and clearly defined processes) arose in cultures that had been plated at less than 3500 cells/well of an HL-A plate. Thus, it was possible that there was an endogenous activity required for the differentiation of the precursor cells into mature neurons.

As NGF has been so strongly implicated in sensory neuron development (Levi-Montalcini, 1982; Thoenen and Barde, 1980; Johnson et al., 1986), this factor was added to the DRG cultures alone and in the presence of LIF to look for any additive effect on the number of neurons. Whereas no effect was evident at the high cell densities, a marked effect was observed by reducing the initial plating density in the early embryonic DRG cultures (E12 and E13; Fig. 1). At an initial plating density of 1000 cells/well, NGF alone increased the number of morphologically defined neurons that appeared in the cultures; LIF alone had a much smaller effect, but in the presence of both LIF plus NGF, there was a synergistic response with the number of neurons being 3 to 4 times that of the total generated by LIF and NGF separately.

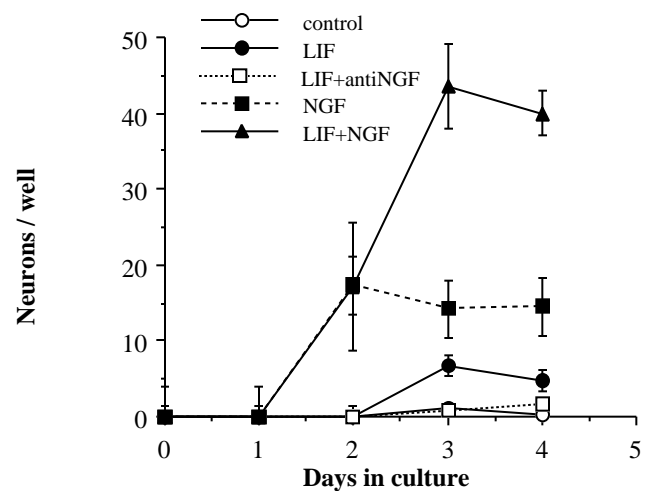
The observed synergy between LIF and NGF was further investigated by following the appearance of neurons over time in cultures of E12 DRG cells plated at 300 cells/well (Fig. 2). In cultures where only exogenous NGF was added, morphologically defined neurons first appeared after 1-2 days and there was no increase in neuron number after this time (Fig. 2). Fewer neurons appeared in LIF-treated cultures; however, this activity was negated by the addition of anti-NGF antibodies to the cultures (Fig. 2). This latter finding indicates that the effects of LIF on the



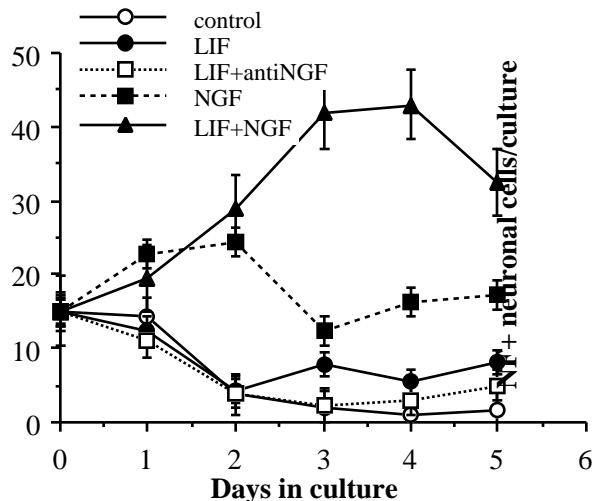
**Fig. 1.** Effects of LIF and NGF on the appearance of neurons in early DRG cultures. DRG cells were plated at 1000 cells/well as described in Materials and methods in the presence of LIF and/or NGF and the number of morphologically mature neurons were determined after 4 days.

generation of morphologically defined neurons were dependent on the presence of NGF. Once again, the greatest effects were seen in cultures treated with LIF plus NGF, where the neuron number increased until 3 days to a level 3 times that of NGF alone cultures (Fig. 2).

At E12, the DRG contain both undifferentiated neuronal precursors as well as immature neurons. Given that NGF's previously described effects have been restricted to differentiated neurons and that there is no evidence that it acts on sensory neuron precursors, it was possible that NGF was acting only on neurons in the E12 cultures, albeit that they were immature and not fully differentiated. To mark morphologically immature neurons, the cultures were stained



**Fig. 2.** Time course of the development of morphologically mature neurons in E12 DRG cultures. DRG cells from E12 embryos were plated at 300 cells/well in the presence of different factors or NGF antibodies as described in Materials and methods. The number of morphologically mature neurons were determined 2 hours and then every 24 hours after plating.

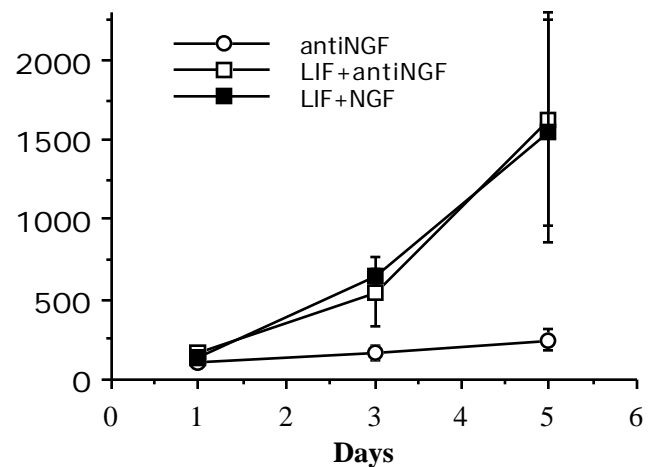


**Fig. 3.** Time course of the appearance of NF<sup>+</sup> cells in E12 DRG cultures. E12 DRG cells were plated as above and after 2 hours, and then every 24 hours, cultures were fixed and stained for the presence of NF, with detection using immunofluorescence as described in Materials and methods. Culture conditions were as described in Fig. 2.

for the presence of p150 neurofilament (NF), which has previously been shown to be expressed early in sensory neuron development (Cochard and Paulin, 1984). It was found that, 2 hours after plating 300 E12 DRG cells into wells of HL-A plates, an average of 15 cells stained positively for p150 NF (NF<sup>+</sup>) and thus were probably immature sensory neurons (Fig. 3). In cultures treated with NGF, the number of NF<sup>+</sup> cells essentially remained unchanged over the culture period (Fig. 3), although the cells matured into well-differentiated neurons. In contrast, cultures treated with LIF alone showed a decrease in the number of NF<sup>+</sup> cells throughout the culture period, to a similar extent to control cultures. Only in the presence of both LIF and NGF was there any significant increase in NF<sup>+</sup> cell number (Fig. 3).

These results indicate that NGF is acting as a survival factor for the immature and mature neurons whereas LIF has no survival-promoting activity for these neurons at this stage. The requirement for both LIF and NGF for full differentiation of new neurons from their NF<sup>-</sup> precursors can be interpreted as either both factors being required simultaneously for differentiation to occur, or that LIF acts during the primary differentiation step and NGF is required for the survival of these newly differentiated neurons.

To distinguish between these possibilities, it was necessary to examine a population of cells at very early developmental times, so that the appearance of NF<sup>+</sup> neuronal cells could be attributed to differentiation of new neurons from their precursors and not to survival of already differentiated neurons. It was also necessary that the NF<sup>+</sup> cells induced could remain viable without the presence of NGF for sufficient time to assess the influence of LIF and NGF on the differentiation step. As this population is not obtainable at an early enough phase in DRG cells, we re-examined this question using primary neural crest cells. Neural tubes were dissected from lumbar and thoracic regions of



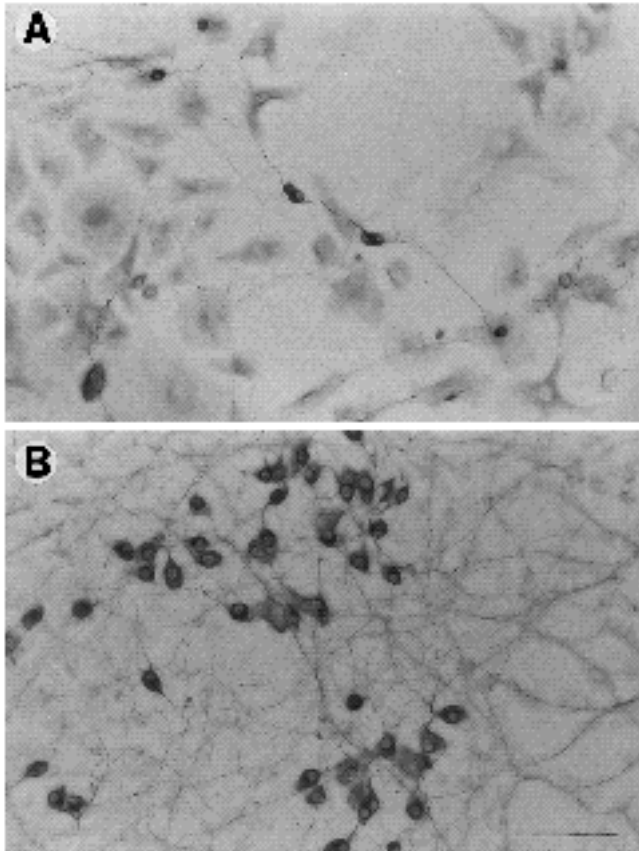
**Fig. 4.** Effect of LIF, NGF and anti-NGF on the generation of neurons in neural crest cultures. Neural crest cultures were incubated in the presence of particular growth factors and antibodies as shown and stained for the presence of p150 NF at the indicated times. The total number of p150 NF<sup>+</sup> neuronal-like cells was determined for each culture and numbers represent mean  $\pm$  s.d. of four determinations.

E9 mice and explanted onto a fibronectin substratum to promote the migration of neural crest cells from the explant as previously described (Murphy et al., 1991). These cultures were incubated in the presence of combinations of LIF, anti-NGF and NGF and the number of NF<sup>+</sup> neuronal cells present within the migrated crest cell population was followed over a period of 5 days. As shown in Fig. 4, significant numbers of new NF<sup>+</sup> neurons only arose in cultures treated with LIF and this was not influenced by the addition of exogenous NGF or by the removal of any endogenous NGF with antibody. These observations support the concept that LIF acts independently to promote the differentiation of NF<sup>-</sup> precursors into NF<sup>+</sup> immature neurons.

Although this indicates that the early steps in the development of sensory neurons are independent of the presence of NGF, their continued maturation, as well as their survival, appeared NGF dependent. In cultures of DRG neurons, in the complete absence of NGF (all cultures with anti-NGF and no added NGF), the NF<sup>+</sup> neurons remained as small spherical cells with very fine processes (Fig. 5A). Large NF<sup>+</sup> neurons with extensive cytoplasm only appeared in the presence of NGF (Fig. 5B) and these were the dominant neuronal type under these conditions.

#### Effects of LIF and NGF on sensory neurons, postdifferentiation and at the time of innervation and natural neuron death.

By analogy with studies in the rat (Lawson et al., 1974), most mouse DRG neurons are probably formed by E14. The newly formed neurons innervate their target tissue and go through a period of natural cell death, the control of which is presumed to be mediated through the availability of NGF. To look for LIF/NGF interactions at this stage, DRG were isolated at E14 and E15. As expected, at E14 and E15, NGF rescued between 65 and 80% of the plated neurons (Fig. 6A,B). In the presence of LIF, a smaller pro-



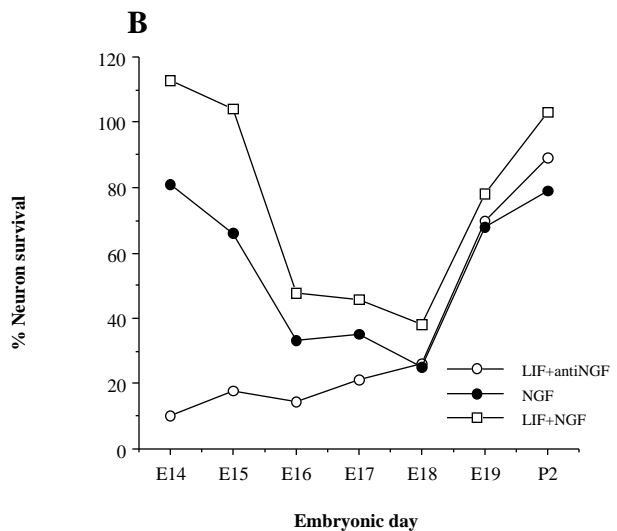
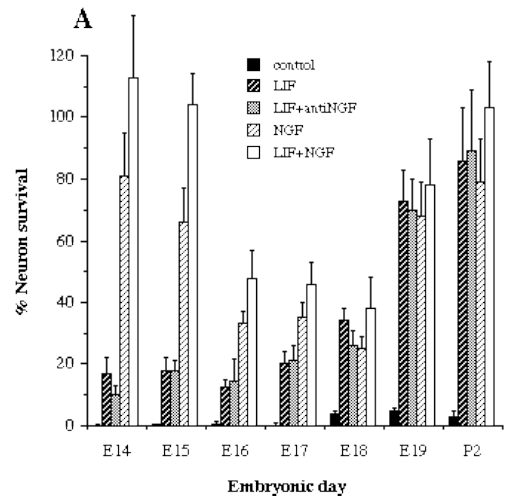
**Fig. 5.** Appearance of neurons cultured in the presence of LIF or NGF. Photomicrographs show morphologies of neurons that have developed from E12 DRG cultures (1000 cells plated) incubated for 4 days in the presence of LIF plus anti-NGF antibodies (A) or LIF plus NGF (B). After culturing, the cells were fixed and stained for the presence of  $\beta$ 150 neurofilament with detection using immunoperoxidase as described in Materials and methods. Bar, 100  $\mu$ m.

portion of neurons survived and this was only slightly reduced in the presence of NGF antibodies. At E14-E15, the effects of combining LIF and NGF were at least additive and all of the neurons plated at these ages could be rescued in the presence of LIF plus NGF. At E14, slightly more neurons were observed after culturing with LIF plus NGF than were initially plated (Fig. 6). Possibly a small number of neuronal precursors were still present even at E14, which differentiated in the presence of LIF plus NGF.

To determine whether the response to LIF and NGF was synergistic or whether the factors were acting independently, an NGF titration in the presence and absence of LIF was undertaken with E15 DRG neurons (Fig. 7). This shows that the incremental effect of LIF was approximately the same at all concentrations of NGF, indicating an additive response. Thus, at this developmental stage LIF and NGF act on discrete, non-overlapping, populations of sensory neurons.

#### Effects of LIF and NGF at later stages of sensory development

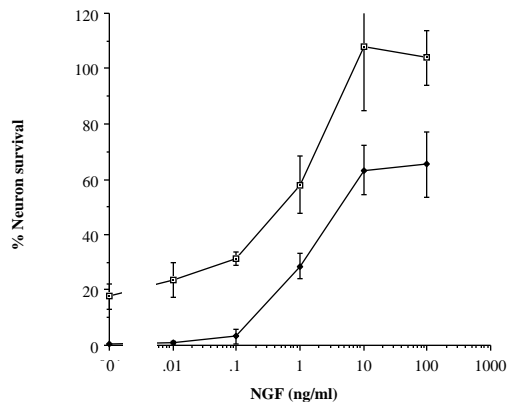
After the period of innervation and natural neuronal death,



**Fig. 6.** Response of differentiated DRG neurons to LIF and NGF through embryonic development. DRG cells from embryos aged E14-P2 were plated as described in Materials and methods and incubated in the presence or absence of growth factors and anti-NGF antibodies. Initial cell numbers plated/well in these experiments were as follows: E14-E15, 1000; E16, 300; E17-E19, 200; P2, 100. The percentage neuronal survival was determined by counting the same individual wells after 2-3 days as described in Materials and methods. (A) Bar graph showing full experimental results. (B) Line graph to show the time course of responsiveness to LIF and NGF alone and in combination.

neurons continue to die when placed in culture without added factors, so they are presumably still dependent on factors for their survival. Assays conducted on DRG cells isolated after E15 showed a different response to the factors than that seen at E14 and E15 (Fig. 6A,B). In the case of NGF, far fewer neurons could be rescued between E16 and E18; only 25-35% of the neurons survived in the presence of NGF. However, at E19 and postnatally, the number of NGF responsive neurons again increased to approximately 70-80% of plated neurons (Fig. 6A,B).

The number of neurons that responded to LIF continued to increase from less than 20% at E16, up to 90% postnatally, and this response was not significantly reduced by



**Fig. 7.** Effect of NGF titration in the presence and absence of LIF on the survival of E15 DRG neurons. DRG cells were plated at 1000 cells/well in the presence of the indicated concentrations of NGF and in the presence (open square) or absence (closed diamond) of  $10^4$  U/ml LIF. Neuronal survival was determined as in Fig. 3 after 3 days.

anti-NGF antibody. As a result, the additive effect of LIF plus NGF decreased with developmental age, indicating that the population of sensory neurons responding to both LIF and NGF was increasing. By E19, there was almost complete overlap between the LIF and the NGF responsive populations.

#### Expression of LIF during embryonic development

In order to examine the expression of LIF *in vivo* during the time of sensory development, we have isolated tissues from mice at E9 through to P2 and extracted RNA to look for LIF transcripts. Previously, using standard RNA detection methods, such as northern blotting, we have not been able to detect LIF expression in any tissues from the adult mouse (Metcalf and Gearing, 1989; M. A. Brown, D. Metcalf and N. M. Gough, unpublished data), suggesting that LIF mRNA is only produced at very low levels, if at all, *in vivo*. Using the more sensitive polymerase chain reaction (PCR), however, we and others have previously detected LIF mRNA in a number of tissues (Croy et al., 1991; Allan et al., 1990; Yamamori, 1991; M. A. Brown, D. Metcalf and

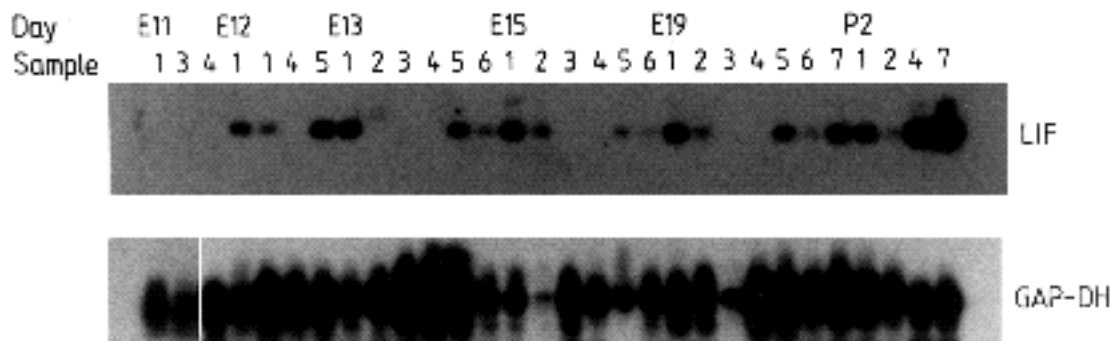
N. M. Gough, unpublished data) suggesting that constitutive LIF gene expression does occur, albeit at very low levels. Given this and the small amounts of tissue available from mouse embryos, PCR appeared to be the method of choice. We were confident that detection of transcripts by PCR could be equated with biological activity as a number of northern blot-negative, PCR-positive cell lines and tissues secrete sufficient LIF protein to elicit a biological response (M. Brown and D. Metcalf, unpublished data).

The areas that were chosen for analysis were the DRG themselves as well as the whole spinal column within which the DRG develop (including DRG, spinal cord, meninges, and developing somites, or vertebrae after E13). In addition, the major regions of DRG neuron innervation, which are skin, muscle (limbs), gut and spinal cord were investigated, as well as the brain. As shown in Fig. 8, using PCR, we were able to detect LIF expression in a number of these tissues. All tissue samples were taken at least twice and analysed up to 5 times and only those samples that always gave a signal in the PCR assay were taken as positive. In studies of LIF expression from adult tissue (M. A. Brown, D. Metcalf and N. M. Gough, unpublished data), the amount of PCR products generated from some tissues were similar to those seen in this analysis. However in the former study, cDNA was generated from 5  $\mu$ g of poly(A)<sup>+</sup> RNA, compared to approx. 100 ng total RNA at E12 and E15, or 100 ng poly(A)<sup>+</sup> RNA from E19 and P2 mice. These comparisons suggest that LIF expression during embryogenesis is considerably higher than in the adult.

The results from these analyses are summarized in Table 1. The earliest time at which LIF expression could be

**Table 1. Summary of LIF expression data**

	E11	E12	E13	E15	E19	P2
Dorsal root ganglia	-	-	+	+	+	-
Spinal column	-	+	+	+	+	+
Spinal cord	-	-	-	-	-	+
Limbs (excluding feet)	-	+	+	+	+	+
Feet	-	-	-	+	+	-
Skin	-	-	-	+	+	-
Gut	-	-	-	+	+	+
Brain - fore + mid	-	-	-	-	-	+
- hind	-	-	-	-	-	-



**Fig. 8.** Detection of LIF mRNA in embryonic and neonatal tissues by PCR. First-strand cDNA was synthesized from RNA extracted from tissues shown and used as a template for PCR using primers specific for LIF or GAP-DH, as described in Materials and methods. This figure shows the results from a single experiment. Numbers refer to the following tissues: 1, whole spinal column; 2, dorsal root ganglia; 3, spinal cord alone; 4, brain (fore- and mid-); 5, limbs; 6, skin; 7, gut. Each embryonic age starts at sample 1.

detected by this method was at E12, where it was found in spinal column and limbs. Expression in these areas was seen throughout embryonic development and into early postnatal life. Soon after (E13), LIF mRNA could be detected in the DRG itself and this was also maintained up until birth. By E15, expression was detectable in feet, skin and gut and was also maintained until birth. Interestingly, the PCR fragment derived from E13 DRG appeared to be larger than bands from other tissues; the significance of this finding, however, is unclear at this stage. Samples containing early postnatal forebrain and midbrain also showed consistent expression of LIF.

## DISCUSSION

### Early sensory development

We first reported that LIF stimulates the differentiation of sensory neurons from non-dividing precursors in neural crest cultures and in early DRG cultures (Murphy et al., 1991). The data presented here show that, in addition to LIF, there is a requirement for NGF to be present in order to generate well-differentiated neurons from their precursors in developing DRG cultures. We propose that LIF and NGF have two separate activities in the early development of sensory neurons. LIF acts at the stage from  $NF^-$  neuronal precursor to  $NF^+$  immature sensory neuron and NGF at the stage of survival and further maturation of these immature neurons.

The proposed activity of NGF within this system is entirely consistent with its proposed activity as a selective maintenance factor for terminally differentiated but not fully mature sensory neurons (Levi-Montalcini, 1982; Thoenen and Barde, 1980). Further, in cultures where only NGF was added, the immature neurons in the cultures ( $NF^+$  cells) both survived and continued to develop into morphologically mature neurons, but there was little if any increase in the total number of neurons. The requirement of the neurons for NGF for continued maturation could either be a permissive one, resultant from NGF's survival activity, or an active one, driven by NGF.

The neural crest experiments show that there is a phase after initial differentiation from the  $NF^-$  precursor cell when the neurons are not dependent on NGF for their survival, as is the case for chick sensory neurons (Ernsberger and Rohrer, 1988). In related experiments, the development of sensory neurons in the mouse trigeminal ganglion is independent of NGF up until the time just prior to target innervation (Davies and Lumsden, 1984; Vogel and Davies, 1991). Further, NGF receptors are not present on developing trigeminal sensory neurons until their fibres reach their targets, which is the same time as NGF synthesis begins in developing skin (Bandtlow et al., 1987; Davies et al., 1987).

The finding of NGF activity in the DRG cultures may reflect synthesis of NGF in the DRG *in vivo* at this time or it may be an artefact of the culture conditions as Schwann cells have been previously demonstrated to produce NGF *in vitro* (Richardson and Ebendal, 1982; Ferguson et al., 1987). Developing sensory ganglia do have NGF *in vivo* (Korsching and Thoenen, 1985), although this probably

arises, at least in part, from retrograde transport from target tissues.

The proposal that LIF acts at the stage of differentiation of the sensory precursor cells is supported by the following observations. First, in the DRG cultures, new neurons only appear in cultures containing LIF. Second, LIF does not appear to be a survival factor for these immature neurons. Third, in early neural crest cultures (up until 5 days of culture), the differentiation of neurons from their precursors is stimulated by LIF independently of the presence of NGF, and it is not influenced by NGF. From previous studies of the action of LIF on neural crest cells (Murphy et al., 1991), these neurons are in the sensory lineage. Finally, these neurons do not mature further into large sensory neurons in the presence of LIF alone; this step is dependent on the presence of NGF.

These observations do not reveal the precise action of LIF and at least two possibilities can account for its early action: LIF promotes the survival of the precursor cells and differentiation into immature sensory neurons is constitutive or LIF actively promotes differentiation of the precursor cells. In order to distinguish between these possibilities, it will be necessary to identify the sensory precursors; however, markers for murine sensory precursors have so far not been described and thus these experiments await the characterization of suitable antibodies for use in identification of the precursors.

The finding of mRNA for LIF in early DRG (E13), as well as within the whole spinal column (E12), is consistent with an action of LIF in sensory neuron development *in vivo*. Although differentiation of sensory precursors commences before these times, the limits of detection of the technique, particularly at the early embryonic ages when developing tissues might be very small, may preclude the reproducible detection of LIF at these stages. In addition, this technique cannot determine the cell type that synthesizes LIF mRNA or whether there is a general production of LIF in the tissues rather than a small number of strongly positive cells. These questions may be resolved by *in situ* hybridization, although this technique is still relatively insensitive and has only convincingly identified LIF expression in the endometrial glands of the uterus at E4 (Bhatt et al., 1991), a period when LIF mRNA is also detectable in the uterus by northern blot analysis.

The question of the level of LIF that is biologically significant is difficult to answer. Several cell lines that produce biologically active LIF, including F9 cells and STO fibroblasts, are positive for LIF mRNA by PCR but negative by northern blot (M. A. Brown and D. Metcalf, unpublished data), suggesting that low levels of LIF mRNA may still reflect biologically significant levels of LIF protein and that PCR is a valid method for its detection. This is not wholly analogous to the situation *in vivo* where, for example, protein half-life cannot be easily determined and thus a positive signal by PCR is not direct evidence for bioactive LIF *in vivo*, a problem that it shares with any other mRNA detection technique.

Overexpression of LIF leads to multiple effects in adult mice (Metcalf and Gearing, 1989), indicating that it has multiple targets *in vivo*. However, mice with LIF gene knockout reveal only a single specific defect in implanta-

tion (Stewart et al., 1992), which implies that the other described activities of LIF may not be functionally important. It may be that a single gene knockout cannot reveal all the activities of a growth factor, or for that matter, the full function of any gene product. Another explanation for this discrepancy comes from the finding that LIF belongs to a structurally related family of molecules, which may have overlapping activities (Bazan, 1991). In particular, it has recently been found that LIF and ciliary neurotrophic factor (CNTF) share the same signalling mechanism along with another factor, oncostatin M (OSM; Gearing et al., 1992; Ip et al., 1992). Another factor that will probably be added to this list is the newly cloned growth promoting activity (GPA), which has 50% homology with CNTF (Leung et al., 1992). Thus, LIF may activate a series of pathways normally activated by other members of this family; one of the pathways being in sensory neuron development. We have tested both OSM and Interleukin-6 (IL-6), a relative, in survival assays on postnatal DRG cultures and found that OSM, but not IL-6, is active in these assays (M. Murphy and K. Reid, unpublished observations).

Other factors, such as the other neurotrophins, have also been implicated in the developmental process. 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). It has recently been shown that either of the neurotrophins, BDNF or NT-3, can promote the maturation of immature (NF<sup>+</sup>) neurons before they become dependent on NGF for survival (Wright et al., 1992). Thus, these neurotrophins may act at an intermediate stage in the differentiation of sensory neurons, prior to target innervation. The sequence of events in sensory neuron differentiation could then be described as follows: the step from sensory neuron precursor cell to immature neuron is promoted by LIF; the next step from immature neuron to a more mature stage may be promoted by BDNF or NT-3; target innervation and continued maturation of these neurons follows and is dependent on NGF.

### Period of target innervation and natural neuron death

From E14 to E15, the major events in sensory development in the DRG are probably target innervation and natural cell death, as neurogenesis is mainly complete. At this stage, LIF and NGF act independently and do not show the synergism observed at E12. The majority of neurons respond to NGF but a smaller, but significant, percentage respond to LIF independent of the presence of NGF. The subpopulations of neurons that respond to each factor are discrete and together account for approximately 100% of the plated neurons.

The observations with NGF are consistent with its role as a target-derived survival factor for sensory neurons as discussed above and with the experiments showing that the administration of NGF antibodies to the developing embryo in guinea pigs results in the destruction of up to 85% of their DRG neurons (see Johnson et al., 1986). More recently, it has been shown that more discrete subpopulations of DRG neurons are susceptible to NGF deprivation and it has been suggested that the other neurons in the DRG

may depend on the other neurotrophins for survival (Ruit et al., 1992).

Why discrete populations of neurons respond to LIF and NGF at these developmental ages is unclear. The responses may be explained in either a spatial or temporal context. It is possible that the sensory neurons at this stage develop along divergent pathways so that one population becomes dependent on NGF and another on LIF (or other factors as described above) as the target-derived factor. The mRNA expression pattern at E15 suggests that LIF is beginning to be expressed in many areas of the embryo, including the DRG, which is not consistent with it being spatially confined to areas innervated by NGF non-responsive neurons (e. g. it is expressed in skin, where NGF is also synthesized). Thus, the lack of differential distribution of target-derived factors does not account for the survival pattern of DRG neurons.

Alternatively, since the populations of cells in these cultures are not developmentally synchronized (that is, the neurons have been isolated from sacral to cervical DRG and are a heterogenous population of neurons at slightly different stages of development), some of the neurons may have already passed through this phase of NGF dependency (during target innervation) and have become responsive to LIF. This is consistent with the observation that DRG neurons become increasingly responsive to LIF throughout embryogenesis, from 0% just postdifferentiation (at E12) to 90% by birth.

The probability that DRG neurons isolated from one particular embryonic age are not developmentally synchronized leads to the question of the relationship between the cells isolated at different embryonic ages. In vivo, the neurons that are born at the early developmental ages (up to E14) represent all possible DRG neurons and, as the DRG develop, the total number decreases by approximately 50%, specifically during the period of target innervation and natural neuron death. Thus, the older DRG neurons represent a subpopulation of all neurons that have been born and were present at E12 and E13. The sampling of DRG cells at different times reflects the response of the particular population of neurons present at that developmental stage. In addition, correlations can be made between the development of the neurons in vivo and that seen in vitro. For example, after 5 days in culture, the neurons in E12 cultures that have been treated with LIF+NGF begin to die, which corresponds to a declining response of E16 DRG neurons to NGF after 1 day in culture. Interestingly, neurons are present in E12 cultures treated with NGF alone for longer periods (at least 9 days in culture) than those treated with LIF+NGF (most of the neurons in these cultures have died by 6 days; data not shown). The reasons for this are unclear, but suggest that E12 cultures treated with LIF+NGF have greater similarities to cultures of DRG isolated later in development than those treated with NGF alone. Other trends emerge such as the increasing survival response of the neurons to LIF as discussed below.

### Sensory development to postnatal day 2

From E16 to E18, the neuronal population goes through a period of relative non-responsiveness to NGF. Similar patterns of response to NGF have also been reported for



chicken sensory neurons at similar developmental ages (Barde et al., 1980). At this time, only a small proportion are responsive to LIF. These data would suggest that the neurons require other factors at this stage (E16-E18) in their development, such as the other neurotrophins or even non-soluble factors.

The increasing survival response of the neuronal population to LIF throughout embryonic development, to the stage where nearly all sensory neurons are maintained in its presence, suggests that LIF responsiveness is associated with neuronal maturity. In addition, one of the surprising features of these studies is that after a period of NGF insensitivity, nearly all the sensory neurons become acutely responsive to NGF around birth. This finding suggests that neurons are sensitive to NGF at other times apart from the period of target innervation and is consistent with nerve ligation studies that show rescue of postnatal neurons with NGF (Yip et al., 1984). The observations also indicate that the populations of neurons converge with respect to growth factor sensitivity at the time of birth.

The widening pattern of LIF mRNA expression during this period is consistent with LIF being available to most DRG neurons, either within the DRG, or via their target tissue. We have recently shown that LIF may be an effective peripheral molecule as it can be retrogradely transported from the footpad to DRG neurons in adult and neonatal mice (Hendry et al., 1992). The observation that the retrograde transport of <sup>125</sup>I-LIF could be completely inhibited by an excess of unlabelled LIF also shows that transport of LIF is receptor mediated and thus that LIF receptors are present on sensory axons in vivo (Hendry et al., 1992).

The expression of LIF in some of the tissues examined may also be related to other activities of LIF (see Hilton and Gough, 1991, for review). However, it is also possible that the expression of LIF at a particular site may serve multiple functions, e.g. LIF expression in the footpad may be involved in the switching of adrenergic sympathetic neurons to a cholinergic phenotype (Yamamori et al., 1989; Yamamori, 1991) as well as acting as a survival factor for the innervating sensory neurons.

We thank Dr Nic Nicola for the gift of LIF and Dr Nicholas Gough for helpful discussions. This work was funded by the AMRAD Corporation (Melbourne), the National Health and Medical Research Council of Australia and The Cooperative Research Center for Cellular Growth Factors.

## REFERENCES

- Allan, E. H., Hilton, D. J., Brown, M. A., Evely, R. S., Yumita, S., Metcalf, D., Gough, N. M., Ng, K. W., Nicola, N. A. and Martin T. J. (1990). Osteoblasts display receptors for and responses to Leukemia Inhibitory Factor. *J. Cell Physiol.* **145**, 110-119.
- Bandtlow, C. E., Heumann, R., Schwabb, M. E. and Thoenen, H. (1987). Cellular localization of nerve growth factor synthesis by in situ hybridization. *EMBO J.* **4**, 891-899.
- Barde, Y.-A., Edgar, D. and Thoenen, H. (1980). Sensory neurons in culture: changing requirements for survival factors during embryonic development. *Proc. Natn. Acad. Sci. USA* **77**, 1199-1203.
- Barde, Y.-A., Edgar, D. and Thoenen, H. (1982). Purification of a new neurotrophic factor from mammalian brain. *EMBO J.* **1**, 549-553.
- Bazan, J. F. (1991). Neuropoietic cytokines in the hemopoietic fold. *Neuron* **7**, 197-208.
- Bhatt, H., Brunet, L. J. and Stewart, C. L. (1991). Uterine expression of leukemia inhibitory factor coincides with the onset of blastocyst implantation. *Proc. Natn. Acad. Sci. USA* **88**, 11408-11412.
- Chomczynski, P. and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analyt. Biochem.* **162**, 156-159.
- Cochar, P. and Paulin, D. (1984). Initial expression of neurofilaments and vimentin in the central and peripheral nervous system of the mouse embryo in vivo. *J. Neurosci.* **4**, 2080-2094.
- Croy, B. A., Guilbert, L. J., Brown, M. A., Gough, N. M., Stinchcomb, D. T., Reed, N. and Wegmann T. G. (1991). Characterization of cytokine production by the metrial gland and granulated metrial gland cells. *J. Reprod. Immunol.* **19**, 149-166.
- Davies, A. M., Bandtlow, C., Heumann, R., Korsching, S., Rohrer, H. and Thoenen, H. (1987). Timing and site of nerve growth factor in developing skin in relation to innervation and expression of the receptor. *Nature* **326**, 353-358.
- Davies, A. and Lumsden, A. (1984). Relation of target encounter and neuronal death to nerve growth factor responsiveness in the developing mouse trigeminal ganglion. *J. Comp. Neurol.* **223**, 124-137.
- Ernsberger, U. and Rohrer, H. (1988). Neuronal precursor cells in the chick dorsal root ganglion: differentiation and survival in vitro. *Dev. Biol.* **126**, 420-432.
- Ferguson, I. A., Williams, R. and Rush, R. A. (1987). Chicken NGF and non-NGF synthesis and release by sciatic nerves in vitro. *J. Neurosci. Res.* **22**, 408-417.
- Gearing, D. P., Comeau, M. R., Friend, D. J., Gimpel, S. D., Thut, C., McGourty, J., Brasher, K. K., King, J. A., Gillis, S., Mosley, B., Ziegler, S. F. and Cosman, D. (1992). The IL-6 signal transducer, gp130: an oncostatin M receptor and affinity converter for the LIF receptor. *Science* **255**, 1434-1437.
- Gonda, T. J., Sheiness, D., K. and Bishop, J. M. (1982). Transcripts from the cellular homologs of retroviral oncogenes: Distribution among chicken tissues. *Mol. Cell. Biol.* **2**, 617-624.
- Hendry, I. A., Murphy, M., Hilton, D. J., Nicola, N. A., and Bartlett, P. F. (1992). Binding and retrograde transport of leukemia inhibitory factor by the sensory nervous system. *J. Neurosci.* **12**, 3247-3434.
- Hilton, D. J. and Gough, N., M. (1991). Leukemia Inhibitory Factor: A biological perspective. *J. Cell Biochem.* **46**, 21-26.
- Hohn, A., Leibrock, J., Bailey, K. and Barde, Y.-A. (1990). Identification and characterization of a novel member of the nerve growth factor/brain-derived neurotrophic factor family. *Nature* **344**, 339-341.
- Ip, N. Y., Nye, S. H., Boulton, T. G., Davis, S., Taga, T., Li, Y., Birren, S. J., Yasukawa, K., Kishimoto, T., Anderson, D. J., Stahl, N. and Yancopoulos, G. D. (1992). CNTF and LIF act on neuronal cells via shared signaling pathways that involve the IL-6 signal transducing receptor component gp130. *Cell* **69**, 1121-1132.
- Johnson, E. M. Jr., Rich, K. M. and Yip, H. K. (1986). The role of NGF in sensory neurons in vivo. *Trends Neurosci.* **9**, 33-37.
- Kalcheim, C. and Gandreau, M. (1988). Brain-derived neurotrophic factor stimulates survival and neuronal differentiation in cultured avian neural crest. *Brain Res.* **469**, 79-86.
- Korsching, S. and Thoenen, H. (1983). Nerve growth factor in sympathetic ganglia and corresponding target organs of the rat: correlation with density of sympathetic innervation. *Proc. Natn. Acad. Sci. USA* **80**, 3513-3516.
- Korsching, S. and Thoenen, H. (1985). Nerve growth factor supply for sensory neurons: site of margin and competition with the sympathetic nervous system. *Neurosci. Lett.* **54**, 201-205.
- Lawson, S. N., Caddy, K. W. T. and Biscoe, T. J. (1974). Development of rat dorsal root ganglion neurones. Studies of cell birthdays and changes in mean cell diameter. *Cell Tiss. Res.* **153**, 399-413.
- Le Douarin, N. M. (1986). Cell line segregation during peripheral nervous system ontogeny. *Science* **231**, 1515-1522.
- Le Douarin, N. M. and Smith, J. (1988). Development of the peripheral nervous system from the neural crest. *Ann. Rev. Cell. Biol.* **4**, 375-404.
- Leibrock, J., Lottspeich, F., Hohn, A., Hofer, M., Hengerer, B., Masiakowski, P., Theonen, H. and Barde, Y.-A. (1989). Molecular cloning and expression of brain-derived neurotrophic factor. *Nature* **341**, 149-152.
- Leung, D. W., Parent, A. S., Cachienes, G., Esch, F., Coulombe, J. N., Nikolics, K., Eckenstein, P. P. and Nishi, R. (1992). Cloning, expression during development, and evidence for release of a trophic factor for ciliary ganglion neurons. *Neuron* **8**, 1045-1053.

- Levi-Montalcini, R.** (1982). Developmental neurobiology and the natural history of nerve growth factor. *Annu. Rev. Neurosci.* **5**, 341-362.
- Metcalfe, D. and Gearing, D. P.** (1989). Fatal syndrome in mice engrafted with cells producing high levels of the leukemia inhibitory factor. *Proc. Natn. Acad. Sci. USA* **86**, 5948-5952.
- Murphy, M., Reid, K., Hilton, D. J. and Bartlett, P. F.** (1991). Generation of sensory neurons is stimulated by leukemia inhibitory factor. *Proc. Natl. Acad. Sci. USA* **88**, 3498-3501.
- Neet, K. E., Fanger, M. W. and Baribault, T. J.** (1987). Derivation of monoclonal antibody to nerve growth factor. *Methods Enzymol.* **147**, 186-194.
- Purves, D.** (1988). *Body and Brain. A Trophic Theory of Neural Connections*. Cambridge, Massachusetts: Harvard University Press.
- Reed, K., C. and Mann, D. A.** (1985). Rapid transfer of DNA from agarose to nylon membranes. *Nucl. Acids Res.* **13**, 7207-7221.
- Richardson, P. M. and Ebendal, T.** (1982). Nerve growth factor activities in rat peripheral nerve. *Brain Res.* **246**, 57-64
- Rohrer, H., Hofer, M., Hellweg, R., Korsching, S., Stehle, A. D., Saadat, S. and Thoenen, H.** (1988). Antibodies against mouse nerve growth factor interfere in vivo with the development of avian sensory and sympathetic neurons. *Development* **103**, 545-552.
- Ruit, K. G., Elliot, J. L., Osborne, P. A., Yan, Q. and Snider, W. D.** (1992). Selective dependence of mammalian dorsal root ganglion neurons on nerve growth factor during embryonic development. *Neuron* **8**, 573-587.
- Sieber-Blum, M.** (1991). Role of the neurotrophic factors BDNF and NGF in the commitment of pluripotent neural crest cells. *Neuron* **6**, 949-955.
- Stewart, C. L., Kasper, P., Brunet, L. J., Bhatt, H., Gadi, I., Kontgen, F. and Abbondanzo, S. J.** (1992). Blastocyst implantation depends on maternal expression of leukemia inhibitory factor. *Nature* **359**, 76-79.
- Theiler, K.** (1989). *The House Mouse. Atlas of Embryonic Development*. New York: Springer-Verlag.
- Thoenen, H. and Barde, Y.-A.** (1980). Physiology of nerve growth factor. *Physiol. Rev.* **60**, 1284-1335.
- Vogel, K. S. and Davies, A. M.** (1991). The duration of neurotrophic factor independence in early sensory neurons is matched to the time course of target field innervation. *Neuron* **7**, 819-830
- Wright, E. M., Vogel, K. S. and Davies, A. M.** (1992). Neurotrophic factors promote the maturation of developing sensory neurons before they become dependent on these factors for survival. *Neuron* **9**, 139-150.
- Yamamori, T., Fukada, K., Aebersold, R., Korsching, S., Fann, M. J. and Patterson P. H.** (1989). The cholinergic neuronal differentiation factor from heart cells is identical to leukemia inhibitory factor. *Science* **246**, 1412-1416.
- Yamamori, T.** (1991). Localization of cholinergic differentiation factor/leukemia inhibitory factor mRNA in the rat brain and peripheral tissue. *Proc. Natn. Acad. Sci. USA* **88**, 7298-7302.
- Yip, H. K., Rich, K. M., Lampe, P. A. and Johnson, E. M. Jr.** (1984). The effects of nerve growth factor and its antiserum on the postnatal development and survival after injury of sensory neurons in rat dorsal root ganglia. *J. Neurosci.* **4**, 2986-2992.

(Accepted 24 November 1992)