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REGULATION OF THE SYNTHESIS OF NERVE GROWTH FACTOR

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SUMMARY

Through nearly 40 years of research nerve growth factor (NGF) has become a paradigm for neurotrophic factors. NGF is synthesized and released from innervated target tissues in limiting amounts, thereby regulating the cell number and the differentiated properties of responsive neurones. Three distinct cell types are responsive to NGF: peripheral sensory and sympathetic neurones and certain types of central cholinergic ones. The effects of NGF are mediated through interaction with a specific receptor which activates a transmembrane second-messenger system. NGF synthesis is regulated during development and in the adult animal. In the developing whisker pad, NGF synthesis commences with its sensory innervation, but sensory neurones lack NGF receptors at the stage when their fibres are growing to their target. These findings indicate that NGF does not attract sensory nerve fibres chemotactically to their target fields during development, but is involved in target-controlled neuronal cell death and in regulation of the density of innervation of target tissues. In non-neuronal cells of the sciatic nerve, NGF synthesis is up-regulated during development and after nerve lesion. Thus the changes in NGF levels after lesion in the adult animal are consistent with the hypothesis that the non-neuronal cells relapse into an earlier developmental stage. Regenerating fibres penetrating into the distal nerve stump restore the low adult levels of NGF. Recent evidence indicates that macrophages invading the nerve after transection produce signals which increase NGF synthesis.

INTRODUCTION

Essential functions of nerve growth factor (NGF) were recognized very early in the history of NGF research in transplantation experiments on the influence of the volume of target tissues on the survival of innervating neurones. Mouse sarcoma tissue transplanted into the vicinity of the chick sciatic nerve during embryonic development released a substance which deflected the growth of part of the sciatic fibres, leading to the apparent innervation of the tumour instead of the normal target organ (reviewed by Levi-Montalcini & Angeletti, 1968; Purves & Lichtman, 1985). Moreover, hypertrophy of the sympathetic chain and of the dorsal root ganglia was also observed: owing not to a mitogenic action of NGF but to the prevention of normally occurring cell death in conjunction with an increase of neuronal cell

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volume. This was demonstrated clearly in subsequent experiments by the lack of incorporation of [^{3}H]thymidine into neuronal DNA (Hendry, 1976). These and a number of additional results suggested that NGF was synthesized and released from sympathetic and sensory target organs in limiting amounts, thereby regulating the neuronal survival, the density of innervation of NGF-responsive target tissues, and the maintenance of differentiated functions (see Thoenen & Barde, 1980; Greene & Shooter, 1980). More recently it has been shown that NGF has a similar effect on subpopulations of cholinergic neurones in the brain (Korsching, 1986).

The advanced state of our knowledge of the physiological function of NGF (it is the only neurotrophic factor whose physiological function is established) is based on the fact that it accumulates in very large quantities in accessory organs of various species and in exocrine glands (e.g. the submandibular gland of the male mouse). This rich source allowed the purification of NGF which was the pre-requisite for the production of anti-NGF antibodies (see Levi-Montalcini & Angeletti, 1968) and the determination of its amino acid sequence (Hogue-Angeletti & Bradshaw, 1971). This information was used for the molecular cloning and establishment of the genomic organization of NGF (Scott *et al.* 1983; Ullrich, Gray, Berman & Dull, 1983).

THE ROLE OF NGF AS A (RETROGRADE) MESSENGER BETWEEN PERIPHERAL TARGET ORGANS AND INNERVATING NEURONES

Besides controlling neuronal survival, NGF is also essential in the regulation of expression of specific properties of neurones which synthesize substances such as catecholamines, substance P, somatostatin or cholecystokinin (see Otten, 1984; Thoenen, Korsching, Heumann & Acheson, 1985). The functions of NGF are mediated by interaction of NGF with its specific cell surface receptor (see Misko, Radeke & Shooter, 1987). Subsequently, NGF is taken up from responsive cells by a saturable receptor-mediated mechanism followed by retrograde transport to the perikarya in membrane-confined compartments (see Hendry, 1980; Thoenen & Barde, 1980; Schwab, Heumann & Thoenen, 1982; Schwab & Thoenen, 1983). A prerequisite for NGF's regulatory function *in vivo* is that it should be available only in limiting concentrations, such that the receptors are only partially saturated. This explains why exogenous sources of NGF effectively increase neuronal survival, for example the transplantation of an NGF-producing tissue (Levi-Montalcini & Hamburger, 1951) or the application of NGF during the period of naturally occurring cell death (Hamburger & Yip, 1984). Conversely, deprivation of NGF *in vivo* by application of NGF antibodies leads to a degeneration of the developing sympathetic and sensory neurones (see Levi-Montalcini & Angeletti, 1968; Johnson, Gorin, Brandeis & Pearson, 1980; Johnson, Rich & Yip, 1986; Thoenen & Barde, 1980). Neurone-specific enzymes and peptides are also physiologically regulated by NGF (see Hendry, 1980; Thoenen & Barde, 1980; Schwab & Thoenen, 1983). In the adult animal, NGF antibodies cause a transient reduction in synthesis of both tyrosine hydroxylase and dopamine-β-hydroxylase (see Thoenen & Barde, 1980; Schwab & Thoenen, 1983). Furthermore, the administration of antibodies to adult

Table 1. *Comparison between NGF and mRNA^{NGF}*

Organ	NGF (ng g^{-1} wet mass)	mRNA^{NGF} (ng g^{-1} wet mass)	Ratio NGF/mRNA^{NGF}
Heart atrium	1·0	0·08	13
Iris	1·9	0·24	8
Vas deferens	2·1	0·45	5
Mouse whisker pad (E12·5)*	4·3	1·54	3
Sciatic nerve	1·2	0·004	308

Values are taken from Heumann, Korsching, Bandtlow & Thoenen (1987), if not otherwise indicated.

* Values are taken from Davies *et al.* (1987*a*).

animals resulted in a reduction of neurone-specific peptides in the spinal sensory neurones (see Otten, 1984). The requirement of the retrograde action of NGF for the maturation and differentiation of responsive neurones has been demonstrated by the observation that interference with retrograde axonal transport had the same effect as the neutralization of endogenous NGF by anti-NGF antibodies (see Hendry, 1980; Thoenen & Barde, 1980; Schwab & Thoenen, 1983).

During the last few years new methodological approaches have allowed direct analysis of the retrograde messenger function of NGF between innervated target organs and NGF-responsive neurones. NGF protein could be determined by the development of a highly sensitive enzyme immunoassay (accurate within 0·01 fmol NGF per assay) (Korsching & Thoenen, 1983*a*). In addition, a quantification of mRNA^{NGF} in total message preparations was possible using single-stranded DNA probes (Shelton & Reichardt, 1984) or single-stranded RNA probes (Heumann, Korsching, Scott & Thoenen, 1984*a*). The inclusion of RNA recovery standards permitted reliable mRNA^{NGF} determinations, for example in sensory target organs (see below) during various developmental stages using less than 1 mg of tissue (Heumann & Thoenen, 1986).

Comparison of the ratios between NGF and its mRNA in various densely innervated sympathetic or sensory organs revealed that they were relatively constant (Table 1). However, tissues containing neuronal cell bodies, such as sympathetic (Heumann *et al.* 1984*a*) or sensory ganglia (Davies *et al.* 1987*a*), or those containing mainly Schwann cells and axons, such as the sciatic nerve (R. Heumann and others, in preparation), showed an increase of up to 100-fold in the ratio between NGF and its mRNA (Table 1) reflecting the very low levels of local synthesis by associated non-neuronal cells in these tissues. Moreover, direct measurement of the accumulation of endogenous NGF after nerve lesions (Korsching & Thoenen, 1983*b*) provided evidence that the high levels of NGF in the nerve are almost exclusively due to uptake and retrograde transport. In addition, interruption of retrograde transport in sympathetic neurones by chemical (6-hydroxydopamine) treatments resulted in a rapid decay of NGF levels in the sympathetic ganglia (Korsching & Thoenen, 1985), with a concomitant increase of NGF in the target organs.

STRUCTURE–FUNCTION RELATIONSHIP AND MECHANISM OF ACTION OF NGF

The experimental transplantation of mouse tumour tissue into chick embryo mentioned above had previously demonstrated that mouse NGF could act on chick neurones, implying that NGF effects are mediated by mechanisms that evolution has conserved. Such an assumption was further substantiated when it became possible to purify NGFs from evolutionary distant species, such as the venom of the snake *Naja naja* (Hogue-Angeletti *et al.* 1976) and bovine seminal plasma (Harper, Glanville & Thoenen, 1982). The establishment and comparison of dose–response relationships for the effects of mouse and bovine NGF indicated that their biological activities were virtually the same. However, there was no or very little cross-reactivity between the antisera produced against these NGFs (Harper *et al.* 1983). To elucidate which domains of the NGF molecule were conserved, and how this conservation related to biological activity or antigenicity, the amino acid sequences derived from the cloned cDNAs and genomic sequences coding for chick, bovine and human NGF were compared with that of mouse NGF (Meier *et al.* 1986). Hydrophylicity plots indicated a remarkable conservation in the overall structure of the various NGFs which was reflected by an evolutionary conservation of, for example, 99 out of 118 amino acids between bovine and chick NGF. It was surprising that in spite of this extensive homology many polyclonal antisera against NGF were monospecific, suggesting that minor differences in the amino acid sequences were responsible for antigenic specificity. Consistently, the amino acid changes were clustered, virtually exclusively in hydrophilic regions of NGF thought to represent antigenic determinants (Meier *et al.* 1986). The evolutionary conservation of action of NGF may be extended even to amphibians because mouse NGF is able to regulate neuronal differentiation in *Xenopus* (Levi-Montalcini & Aloe, 1985).

Although a variety of long- and short-term effects of NGF have been described, on NGF-responsive cells, the exact sequence of events, following the receptor–ligand interaction and leading finally to the physiological response, remains to be determined. An important step to facilitate the elucidation of the intracellular transduction mechanisms is the molecular cloning and establishment of the structure of the NGF receptor (Radeke *et al.* 1987) described by Misko *et al.* (1987). Previously, injection of NGF into the cytoplasm or the nucleus of NGF-responsive PC12 cells had not elicited the characteristic effects of NGF (Heumann, Schwab & Thoenen, 1981; Seeley, Keith, Shelanski & Greene, 1983). Conversely, injection of anti-NGF antibodies could not prevent the receptor-mediated effects of NGF on fibre outgrowth and induction of choline acetyltransferase (Heumann, Schwab, Merkl & Thoenen, 1984*b*). These experiments indicated that neither the cytoplasm nor the nucleus of NGF-responsive cells expresses functional NGF receptors and, therefore, a transmembrane second-messenger mechanism must be involved (Schwab *et al.* 1982). Unfortunately, a number of previously proposed mechanisms including changes in the levels of cAMP or the activation of sodium/potassium-activated ATPase, are now considered not to be linked directly to the intracellular

transduction mechanism (see Thoenen *et al.* 1985). Recently, however, morphological differentiation has been induced in PC12 cells by infection with *ras*-expressing viruses (Noda *et al.* 1985), by transfection with activated *ras* genes (Guerrero, Wong, Pellicer & Burstein, 1986) and by micro-injection of the *ras* oncogene protein (Bar-Sagi & Feramisco, 1985). Consistently, the effect of NGF was prevented by injection of anti-*ras* antibodies (Hagag, Halegoua & Viola, 1986), suggesting that a *ras*-like protein could act as an intracellular transducer of NGF action. There is now some evidence that the *ras* oncogene might be involved in regulation of the phosphoinositide metabolism: expression of transfected *ras* proto-oncogene in cultured cells strongly enhanced the accumulation of inositoltrisphosphate by bombesin as well as its mitogenic action (Wakelam *et al.* 1986). Similarly, the *ras* oncogene increased the breakdown of phosphatidylinositide diphosphate in cultured cells, raising the possibility that the proto-oncogene product p21, found in normal tissue, could subserve the function of a G-protein in regulating the activity of phospholipase C. However, at present these findings are still a subject of controversy (Dolphin, 1987; Benjamin, Tarpley & Gorman, 1987). Recently, it has been shown that NGF potentiates the accumulation of inositolphosphates elicited by bradykinin or carbachol (van Calker & Heumann, 1987). The effect of NGF, but not that of epidermal growth factor (EGF), was inhibited when the cells were preincubated with 5′-deoxy-5′-methyladenosine, an inhibitor of S-adenosylhomocysteine hydrolase (Clarke, 1985). Although the exact site of action remains to be determined, this inhibitor was able to block all effects of NGF both in PC12 cells (Seeley, Rukenstein, Connolly & Greene, 1984) and in primary cultures such as adrenal chromaffin cells and sympathetic neurones (Acheson, Vogl, Huttner & Thoenen, 1986; Acheson & Thoenen, 1987). The specificity of action of the inhibitor was further demonstrated by the observation that the dephosphorylation of a 70 kDa protein by NGF was abolished whereas the dephosphorylation by high levels of potassium remained unaffected [high potassium levels are known to mimic (Wakade & Thoenen, 1984) the survival effect of NGF]. Thus, in view of the recently described rapid changes of Ca^{2+} concentrations after addition of NGF to chromaffin cells (Pandiella-Alonso, Malgaroli, Vicentini & Meldolesi, 1986), a new and fascinating perspective has appeared in the field of the mechanism of action of NGF which should lead to the identification and characterization of the protein(s) transducing the effects of NGF in the cellular cytoplasm and to the nucleus.

REGULATION OF NGF SYNTHESIS IN THE PERIPHERAL SENSORY AND SYMPATHETIC SYSTEM

Synthesis of the NGF precursor

Biologically active mature NGF is synthesized by proteolytic cleavage from a 33 kDa precursor, as predicted from the $cDNA^{NGF}$ (Darling *et al.* 1983). Recently, the precursor protein has been identified in the thyroid and parathyroid glands of the rat (Dicou, Lee & Brachet, 1986). It does not, however, contain any detectable biological NGF activity. This observation substantiates previous assumptions, based

on Western blot analysis and electron microscope immunohistochemistry, that the NGF precursor is antigenically different from the mature protein (Meier, 1985) and that the precursor is not biologically active. Experiments in the cultured iris have shown that blockade of the passage of NGF precursor through the Golgi apparatus, by using monensin, prevented the appearance of mature NGF (Heumann & Thoenen, 1986). It remains to be determined whether the only evolutionary conserved signal peptide for *N*-linked glycosylation (-Asn-Arg-Thr) located in the NGF precursor (Meier *et al.* 1986) plays a role in regulation of the synthesis of the biologically active β-subunit of NGF. A glycosylation-dependent proteolytic functional activation of the haemagglutinin precursor molecule has recently been shown to regulate the infectivity of the influenza virus (Deshpande, Fried, Ando & Webster, 1987).

Synthesis of NGF during sensory innervation of the whisker pad

NGF might be essential for regulation of the neuronal cell number and for the development and stabilization of fibre structure in the peripheral nervous system. These issues have recently been directly investigated in the mouse trigeminal ganglion and in part of its cutaneous target field (the maxillary process and the whisker pad which develops from it) at closely staged intervals before and after innervation (Davies *et al.* 1987*a*).

In the normal development of the mouse, nerve fibres emerge from the trigeminal ganglion at 9·5 days of gestation and increase in number, reaching a maximum at E13. The earliest of these fibres reach the epithelium of the maxillary process by E11 and the latest reach their peripheral targets shortly after E15. Between E13 and E15 over half of the fibres and neurones are lost as a result of cell death.

NGF was first detectable at E11, the stage at which the earliest nerve fibres contact the target field epithelium, and increased in concentration to reach a maximum level by E12·5, after which there was a 10-fold fall in levels. Similarly, the $mRNA^{NGF}$ was not detectable in the maxillary process at E10. It was detectable only at very low levels at E10·5, about 12 h before the NGF protein was first detected in the maxillary process. The concentrations of $mRNA^{NGF}$ increased to reach a maximum at E12·5, the stage at which innervating neurones express NGF receptors and become dependent on NGF. Thereafter, $mRNA^{NGF}$ decreased to levels three-fold lower than at E12·5 (Davies *et al.* 1987*a*).

Several conclusions can be drawn from these results.

(a) The observation that the synthesis of mature NGF did not precede the advent of the initial fibres (which in addition did not express NGF receptors at that stage) indicates that NGF cannot act as a chemotactic agent attracting the initial fibres to grow into their target. Accordingly, a molecule different from NGF has been proposed previously to be responsible for the initial fibre outgrowth of the trigeminal ganglion (Lumsden & Davies, 1983).

The close correlation between the commencement of target field innervation and the onset of $mRNA^{NGF}$ raises the question of whether NGF synthesis is regulated by

the arrival of nerve fibres. This issue can be approached by measuring $mRNA^{NGF}$ in denervated target organs.

(b) The temporal sequence of the appearance of $mRNA^{NGF}$ and its protein in the target field, together with the parallel increases in the levels of these molecules during the early stages of innervation, indicate that the synthesis of mature NGF during development is governed by the level of its messenger and that post-translational mechanisms seem not to be rate-limiting.

(c) The decrease of NGF protein levels in the whisker pad after E12·5 was due both to a decrease in $mRNA^{NGF}$ levels and to its removal by uptake and retrograde transport by the trigeminal fibres. A corresponding increase of NGF levels was found in the trigeminal ganglion in the absence of any measurable $mRNA^{NGF}$. This indicates that the increased levels of NGF in the ganglion resulted from uptake and retrograde transport rather than from local synthesis.

(d) *In situ* hybridization demonstrated that $mRNA^{NGF}$ was found both in cutaneous epithelium and in the mesenchyme, but the concentration in the former was 5- to 10-fold higher, correlating with its denser innervation. This finding further substantiates our observations that the predominant source of NGF is the innervated target and not the Schwann cells ensheathing the nerve fibres.

Previous experiments in the developing dorsal root ganglia (DRG) have indicated that normally occurring cell death could be prevented by systemic injection of NGF, suggesting that the number of sensory neurones is regulated by limited NGF availability (Hamburger & Yip, 1984). Therefore, the correlations between $mRNA^{NGF}$, NGF, the density of innervation and the number of sensory neurones are consistent with the hypothesis that NGF regulates the number of sensory neurones (Davies *et al.* 1987*a*).

It might be worth considering the possibility that fibres, as they become very dense at the target, could down-regulate NGF synthesis. We approached these questions in the adult animal by surgical interruption of the neuronal input and by studying the subsequent effects of the regenerating fibres on the expression of NGF (see below).

Regulation of NGF synthesis in peripheral target organs after nerve transections: the iris as a model system

In a first approach to test whether the state of innervation (Ebendal, Olson & Seiger, 1983) could determine the levels of $mRNA^{NGF}$, the time course of changes of $mRNA^{NGF}$ was measured in cultured iris (Heumann & Thoenen, 1986). As a result of the dissection procedure, the cultured iris is deprived of its neuronal input. After an initial time lag of 1–2 h there was a rapid 35-fold increase of $mRNA^{NGF}$, reaching a maximum after 12 h and thereafter decreasing to only three- to four-fold elevated levels at 48–72 h. The initial increase of $mRNA^{NGF}$ was followed by sequential increases of cellular NGF and NGF released into the culture medium (Barth, Korsching & Thoenen, 1984), reaching half-maximal levels at 9 and 12 h, respectively. It is worth mentioning that similar sequential increases of $mRNA^{NGF}$ and NGF were also observed in the developing whisker pad (see previous section).

The rapidly increasing levels of $mRNA^{NGF}$ during the initial 12 h suggest a mechanism resulting from a 'traumatic' response to the denervation procedure. This response is inhibited by the presence of actinomycin D (Heumann & Thoenen, 1986). An involvement of transcriptional mechanisms rather than an exclusive stabilization of $mRNA^{NGF}$ is therefore suggested by these experiments. Interestingly, the initial increase in $mRNA^{NGF}$ is dependent on protein synthesis, since it is inhibited by the presence of cycloheximide. This is in contrast to the 'superinduction' of the mRNA of the cellular proto-oncogene c-*fos* by cycloheximide in cultured 3T3 fibroblasts (Müller, Bravo, Burckhardt & Curran, 1984) and cultured nerve segments (Lindholm, Heumann & Thoenen, 1987).

In this context it is worth mentioning that the $mRNA^{NGF}$ carries an evolutionary conserved consensus sequence (UUAUUAU) in its 3'-untranslated region which seems to be specific for inflammatory mediators such as tumour necrosis factor, interleukin-1, interferons, etc. (Caput *et al.* 1986). Such AT-rich regions have been shown to be involved in rapid degradation of mRNAs (Shaw & Kamen, 1986). Thus, it would be interesting to test the possible function of the consensus sequence during the phase of decreasing $mRNA^{NGF}$ levels occurring after the initial 12 h in cultured iris (see also next section).

Whether the residual nerve terminals, which are thought to degenerate virtually immediately after placing the iris into culture, will release substances influencing the synthesis of NGF has been investigated in some detail (R. Hellweg, unpublished results). A large number of substances known to be stored in cholinergic, adrenergic and sensory nerve terminals (the main types of innervation of the iris) failed to exhibit a regulatory function. As an exception, noradrenaline decreased $mRNA^{NGF}$ and NGF levels *in vitro*, but the physiological role of noradrenaline during development and degeneration remains unresolved. Thus, the substances known to be present in the degenerating nerve terminals do not seem to influence NGF synthesis. However, it should be clearly stated that these *in vitro* results do not account for the possible interaction of degenerating nerve endings with non-resident haematogenous cells *in vivo*, which are known to be attracted by chemotactic peptides (Smith, Cox & Snyderman, 1986).

The investigation on the regional distribution of NGF in the intact iris indicated that the dilator area contained three-fold higher levels of NGF than the sphincter area (Barth *et al.* 1984). A similar distribution was confirmed for $mRNA^{NGF}$ (Shelton & Reichardt, 1986). *In situ* hybridization of $mRNA^{NGF}$ performed on cryostat sections showed, in addition, that the area of the epithelial cell layer was more prominently labelled than the remaining organ (Bandtlow, Heumann, Schwab & Thoenen, 1987), a situation reminiscent of the developing whisker pad. In the iris kept in culture for 12 h the pattern of distribution of $mRNA^{NGF}$ was apparently maintained, but the signals were much stronger: all cell layers were labelled and increased $mRNA^{NGF}$-specific grain density was observed over the dilator muscle and over the epithelial cell layer, which is in agreement with the quantitative $mRNA^{NGF}$ determinations (Shelton & Reichardt, 1986; Heumann & Thoenen, 1986).

Because the resolution obtained in cryostat sections was not sufficient to identify individual cell types, *in situ* hybridizations were performed on dissociated iris cell cultures (Bandtlow *et al.* 1987). These experiments demonstrated that all cell types (i.e. smooth muscle cells, fibroblasts, epithelial cells and Schwann cells) showed NGF-specific labelling. Thus, $mRNA^{NGF}$ is not only expressed in those cell types which have direct cell surface contacts with nerve fibres, such as the Schwann cells. It appears rather that the synthesis of NGF could also be regulated in cells which can interact with fibres only *via* diffusible factors. In contrast to the increase in NGF synthesis in all the non-neuronal cell types of the target organ, a re-expression of NGF receptors after denervation was restricted to Schwann cells (Taniuchi, Clark & Johnson, 1986; Bandtlow *et al.* 1987). Normally NGF receptors are seen on Schwann cells only at earlier stages of development (Zimmermann & Sutter, 1983; Rohrer, 1985). The previously suggested predominant (if not exclusive) site of NGF synthesis in Schwann cells in the iris was based on immunological data alone. The assumption that NGF synthesis takes place exclusively in Schwann cells (Rush, 1984) (which constitute only 5 % of the total cell population) has thus been seriously challenged by the *in situ* hybridization experiments, which demonstrate that NGF is produced in all the non-neuronal cells investigated. The locally synthesized NGF could be bound to, or even accumulated selectively in, the Schwann cells, resulting in their staining by anti-NGF antibodies.

Regulation of NGF synthesis in the sciatic nerve after transection

To study the regulation of NGF synthesis *in vivo* and to further analyse the effects of fibres – not only on cells of the innervated target tissues but, more specifically, on large populations of Schwann cells – we investigated the consequences of transection in the sciatic nerve. The relative contribution of Schwann cells in the nerve sheath to the total NGF supply of NGF-responsive neurones was a matter of debate (Rush, 1984; Finn, Ferguson, Renton & Rush, 1986; Korsching & Thoenen, 1983*b*) until it was demonstrated that in adult animals the sciatic nerve (whose sheath includes mainly Schwann cells and some fibroblast-like cells) contains negligible levels of $mRNA^{NGF}$ as compared with sympathetic and sensory target tissues (see Table 1). The small contribution of locally synthesized NGF is further demonstrated by the absence of a proximodistal NGF gradient in the sciatic nerve. Such a gradient would have been expected if production of NGF along the nerve sheath were to contribute substantially to the NGF transported from the periphery (as suggested by Rush and collaborators).

This situation changes dramatically after sciatic nerve transection, which results in altered levels of NGF and its mRNA proximal and distal to the transection site (Heumann, Korsching, Bandtlow & Thoenen, 1987).

Changes in $mRNA^{NGF}$ levels under conditions precluding regeneration

The events taking place distal to a nerve transection are of interest because of the generation of a tropic and trophic environment which allows the directed regrowth of

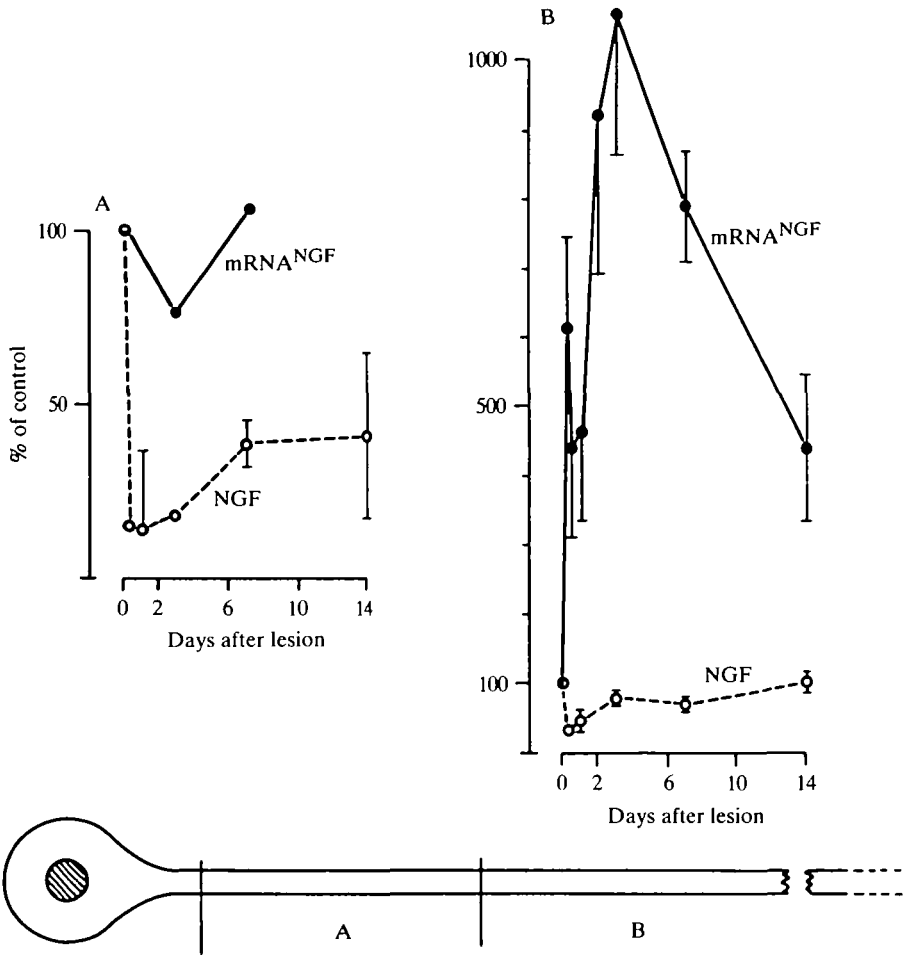

Fig. 1. Levels of NGF and $mRNA^{NGF}$ in the proximal segments A and B of the chronically transected sciatic nerve (taken from Heumann, Korsching, Bandtlow & Thoenen, 1987).

fibres into the distal stump and promotes rapid axonal elongation within the bands of Büngner (Cajal, 1928; Varon & Bunge, 1978). It is of particular interest to relate the events at the tip of the proximal stump to the initiation of sympathetic and sensory nerve fibre outgrowth.

Proximal nerve stump. After transection, the NGF supply from the periphery is interrupted, which leads to a rapid drop in NGF levels (Heumann *et al.* 1987). Within 6h NGF drops to 15% of control levels (i.e. in intact contralateral nerve) in the most proximal region of the stump (segment A in Fig. 1). However, between days 2 and 6, NGF levels recover to about 40% of control and remain elevated for up to 14 days. The observation that $mRNA^{NGF}$ in segment A persists at the very lo control levels ($3{\cdot}9\,pg\,g^{-1}$ wet mass) throughout the period investigated suggests the presence of a newly induced source of NGF. In fact, *in situ* hybridization

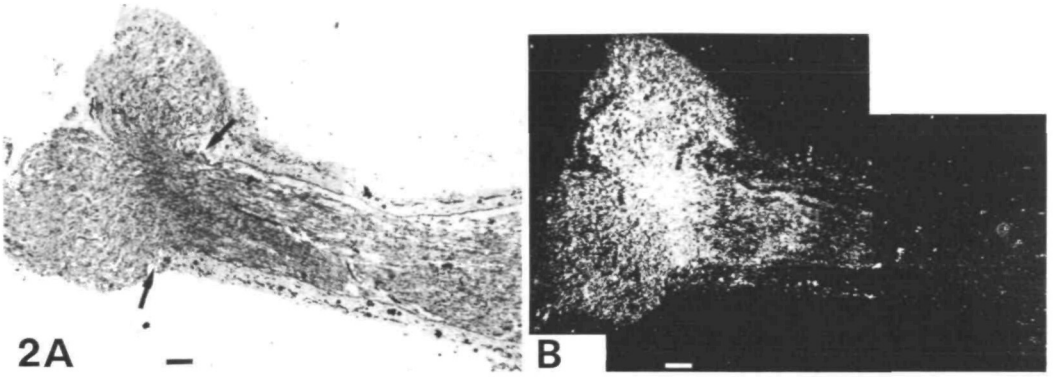

Fig. 2. Localization of $mRNA^{NGF}$ in longitudinal sections of adult rat sciatic nerve 4 days after transection. (A) Dark-field photographs; (B) *in situ* hybridization with a single-stranded ^{35}S-labelled $cRNA^{NGF}$ probe showing dense labelling over the neuroma structure (left of the arrows in A at the proximal tip of the epineurium) and over the adjacent 2 mm of the proximal stump (taken from Heumann, Korsching, Bandtlow & Thoenen, 1987). Scale bars, 0·4 mm.

experiments in conjunction with quantitative $mRNA^{NGF}$ determinations identified such a 'substitute target organ', which is confined to the neuroma structure at the tip of the proximal stump and the adjacent 2 mm, which undergo retrograde Wallerian degeneration (as shown in Fig. 2). There, the NGF concentrations correspond to those of densely innervated target organs, but the volume of the tissue is far too small to compensate for the interrupted NGF supply. That the newly synthesized NGF is fully available for uptake and retrograde axonal transport is suggested by preliminary experiments demonstrating that in the proximal segments there is relatively little increase of the NGF-receptor-mRNA ($mRNA^{rec}$). This is in distinct contrast to the drastic increase of NGF receptor (Taniuchi *et al.* 1986) and of $mRNA^{rec}$ (R. Heumann and others, in preparation) occurring in Schwann cells located in the distal stump. Detailed *in situ* hybridization experiments on dissociated sciatic nerve indicate that *all* the non-neuronal cells are induced to synthesize NGF. It follows from this that Schwann cells in the tip of the proximal stump are not able to compete for the NGF they secrete (due to the low levels of expression of NGF receptors). A further implication of the *in situ* hybridization experiments is that Schwann cells ensheathing NGF-unresponsive motoneurone axons are also involved in the reactive synthesis of NGF. This conclusion is strongly supported not only by the homogeneous distribution of $mRNA^{NGF}$ in cross-sections of the transected nerve but also by the observation that the distal portions of the nervus facialis, when cut after its branching into the marginalis and buccalis, show similar changes in $mRNA^{NGF}$ (Heumann *et al.* 1987). These nerve segments are thought to contain almost exclusively motoneurone axons. It is tempting to speculate that after nerve transection all Schwann cells (and fibroblast-like cells) attain a 'rescue function' by producing, in addition to NGF, a spectrum of other neurotrophic molecules which are able to attenuate neuronal cell death resulting from the interruption of the

peripheral supply. In the case of NGF, when it was exogenously applied to the proximal stump the lesion-induced degeneration of sensory neurones could be prevented (Fitzgerald, Wall, Goedert & Emson, 1985; Johnson *et al.* 1986). Thus, injured neurones apparently express non-saturated and functional NGF receptors, and these have been shown to transport NGF retrogradely (Stoeckel, Guroff, Schwab & Thoenen, 1976).

The question whether Schwann cells are also needed for the migration of growth cones has been investigated by Hall (1986). Injection of mitomycin C into the proximal stump of the nerve inhibited Schwann cell proliferation locally but did not prevent axons from penetrating into a live distal stump. However, if the distal stump was freeze-killed and injected proximally with mitomycin C regeneration was strongly inhibited. These results are compatible with the hypothesis that the trophic support provided by Schwann cells is essential for axonal regeneration.

Distal nerve stump. Unlike the proximal stump, where the changes in NGF synthesis were strictly localized to a defined region adjacent to the transection site, in the distal stump the whole nerve was affected over a large distance (>12 mm). Previous work on the production of NGF in the distal sciatic nerve *in vivo* suffered from the inability to discriminate between NGF derived from uptake and axonal transport on the one hand, and newly synthesized NGF on the other hand (Richardson & Ebendal, 1982). More recent NGF determinations have shown a rapid and steep increase of NGF level at a site immediately adjacent to the transection. This increase was obviously a result of the initially still intact retrograde transport and subsequent accumulation of target-derived NGF. As Wallerian degeneration proceeded (Hallpike, Adams & Bayliss, 1970), NGF levels decreased again but, interestingly, remained five-fold higher than the control levels for at least 2 weeks. This 'long-term' increase, but not the initial burst of NGF levels, was also detectable in more distally located regions of the nerve. This observation is most easily explained by an induction of NGF synthesis in the distal stump.

Consistently, 10- to 15-fold increased levels of $mRNA^{NGF}$ were found in the distal segments and persisted for at least 3 weeks. It is worth mentioning that the increases of $mRNA^{NGF}$ were biphasic, the peak of the initial rapid phase being reached after 6 h (Fig. 3) while the second phase reached maximal levels at 3–7 days.

While the data from the proximal stump strongly suggest a trophic function of the locally synthesized NGF, the significance of the increased NGF levels all along the distal stump is much less obvious. The finding that Schwann cells re-express NGF receptors (Taniuchi *et al.* 1986) and $mRNA^{rec}$ (R. Heumann and others, in preparation) after nerve transection raises the possibility of an autocrine function of NGF for Schwann cells located distal to the transection site. However, it remains to be determined whether NGF has any function in Schwann cells. In the case of neurones, apparently non-functional NGF receptors have recently been described (Rohrer & Barde, 1982; Davies, Lumsden & Rohrer, 1987*b*). Thus, regulation of the expression of the intracellular machinery responsible for coupling receptor activation to its (so far unknown) second-messenger mechanism could become an important aspect in the regulation of NGF effects.

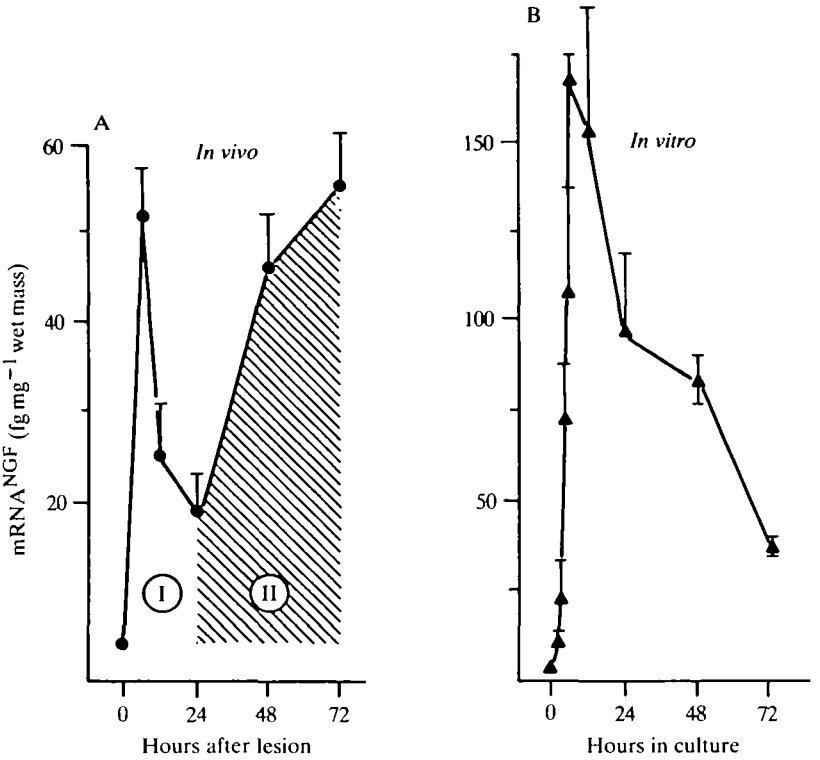

Fig. 3. Levels of mRNA^{NGF} in a distal segment immediately adjacent to the cut (A) and in nerves kept in culture (B). In A the initial rapid peak (phase I) and the later increase of mRNA^{NGF} (phase II, hatched) are indicated.

Changes of mRNA^{NGF} levels during development and under conditions that allow regeneration

The idea that the events taking place during regeneration may be considered in part as a regression into an earlier developmental stage was put forward very early (Cajal, 1928). During the first postnatal week the number of axons in the sciatic nerve is still increasing, this net increase being due to a balance between axonal growth (Webster, Martin & O'Connell, 1973) and some developmentally regulated neuronal death occurring simultaneously (see Purves & Lichtmann, 1985). Possibly as a consequence of this partial degeneration of neurones, a relatively large number of cells carrying the macrophage-specific marker ED1 have recently been detected in the sciatic nerve in the newborn rat (Stoll & Müller, 1986). As the nerve matures during the first 3 postnatal weeks, the very low numbers of macrophages characteristic of the adult nerve are found (Stoll & Müller, 1986). After lesion of the adult nerve, a large number of macrophages invade the sciatic nerve. It is these macrophages which seem to be responsible for at least some of the events known as Wallerian degeneration (Abercrombie & Johnson, 1946): prevention of the immigration of macrophages into the lesioned nerve by using suitable Millipore chambers almost completely inhibits the 'normal' degradation of myelin (Scheidt, Waehneldt,

Beuche & Friede, 1986; Beuche & Friede, 1986). In addition, the induction of Schwann cell mitosis does not occur.

In a manner similar to these changes in the numbers of macrophages, the levels of $mRNA^{NGF}$ are relatively high during the first postnatal week of normal development, as well as in adult distal sciatic nerve segments after lesioning (R. Heumann and others, in preparation). In the first postnatal week $mRNA^{NGF}$ levels remain nearly constant, they then decrease relatively rapidly during the second postnatal week to approach adult levels by the third postnatal week. To test a possible functional relationship between the levels of $mRNA^{NGF}$ and the presence of macrophages we cultured isolated segments of the sciatic nerve. The changes in $mRNA^{NGF}$ levels in the cultured sciatic nerve (in the absence of non-resident haematogenous cells) were very similar to those described above in the cultured iris (Heumann & Thoenen, 1986). There was a transient rapid increase of $mRNA^{NGF}$ (Fig. 3B) but, in contrast to the *in vivo* situation (Fig. 3A), a second phase of increase in $mRNA^{NGF}$ was not observed. The time course of the $mRNA^{NGF}$ increase during the second phase *in vivo* correlated well with that of the macrophage invasion. Addition of activated macrophages to the cultured nerve segments resulted in an increase in the levels of $mRNA^{NGF}$. The activated macrophages themselves did not contain any measurable levels of $mRNA^{NGF}$. This indicates that they must produce a signal(s) which regulates $mRNA^{NGF}$ levels in non-neuronal cells (R. Heumann and others, in preparation).

Thus, there are several functions associated with activated macrophages: they present antigens, and stimulate T-cell proliferation and antigen production (Unanue & Allen, 1987). In the lesioned sciatic nerve macrophages are apparently also involved in the degradation of cell debris and myelin and in the regulation of Schwann cell mitosis (Beuche & Friede, 1986). In addition, we now present evidence that they contribute to the establishment of a trophic environment which may be necessary for regeneration (R. Heumann and others, in preparation).

We then considered the possible role of regenerating fibres in the regulation of NGF synthesis. After nerve crush the axons are destroyed, but the epineurium remains intact. This allows the regenerating neurones easily to find the distal degenerating nerve stump, where they rapidly elongate their axons within the bands of Büngner (i.e. the preserved Schwann cell tubes). Some days after the axons have passed through the tubes the levels of $mRNA^{NGF}$ decrease. Again, there is an obvious correlation between the disappearance of macrophages (Müller, Gebicke-Härter, Hangen & Shooter, 1985) and the decrease of $mRNA^{NGF}$ levels in the regenerating nerve. It remains to be investigated whether one of the many secretory products of macrophages is responsible for the up-regulation of $mRNA^{NGF}$, or whether cell–cell contact between macrophages and Schwann cells is a necessary step.

The role of NGF in the central nervous system has not been discussed here, as this topic was reviewed recently by Korsching (1986).

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