# Neuroimmune Networks:

# Physiology and Diseases

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# Regulation of Nerve Growth Factor Synthesis: Role Played by Macrophages

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## INTRODUCTION

The physiological importance of nerve growth factor (NGF) for the development and maintenance of specialized properties of peripheral sympathetic and neural crest-derived sensory neurons is well established (Levi-Montalcini and Angeletti, 1968; Greene and Shooter, 1980; Thoenen and Barde, 1980). More recently a similar function of NGF became apparent for the cholinergic neurons of the basal forebrain nuclei (Thoenen et al., 1987; Whittemore and Seiger, 1987).

The function of NGF as a retrograde trophic messenger transferring information from the field of projection to the innervating NGF-responsive neurons is based on the observation that interference with the retrograde axonal transport has the same consequences as neutralization of endogenous NGF by specific antibodies (Schwab and Thoenen, 1983). This concept is further supported by the observation that the density of innervation by NGF-responsive neurons is well correlated with the levels of NGF and NGF mRNA in their fields of projection (Korsching and Thoenen, 1983; Heumann et al., 1984; Shelton and Reichardt, 1984; Korsching et al., 1985; Whittemore et al., 1986). Besides the elucidation of the nature of the signal transduction resulting from the interaction of NGF with its specific receptors in NGF-responsive neurons, the regionally differential regulation of NGF synthesis is the most essential and challenging question that remains to be established in the understanding of the physiological function of NGF. In the following, we will give a brief survey of our efforts to approach the resolution of this question 1) by analyzing the initiation of NGF synthesis during development in relation to the onset of innervation and 2) by analyzing the mechanisms responsible for the reactive changes in NGF synthesis after nerve lesion.

## RELATIONSHIP BETWEEN THE INITIATION OF NGF SYNTHESIS AND INGROWTH OF NGF-RESPONSIVE NERVE FIBERS DURING EMBRYONIC DEVELOPMENT

In previous experiments we have analyzed the developmental time course of NGF mRNA and NGF protein synthesis in the mouse whisker pad, and we have correlated the onset of NGF synthesis with the beginning of innervation (Davies et al., 1987). The ingrowth of fibers from trigeminal sensory neurons follows a very precise schedule and takes place within a narrow time window. Twenty-four hours after the initiation of the neurite outgrowth from the trigeminal ganglion the fibers have reached their terminal field of innervation. We demonstrated that the initiation of NGF mRNA and NGF protein synthesis in the maxillary process (which then develops to the whisker pad) does not precede the ingrowth of the trigeminal fibers. In addition,

it was shown that NGF synthesis was restricted to the target area and not present along the outgrowing fibers. These findings exclude an essential role for Schwann cells in NGF synthesis during the initial fiber outgrowth. Interestingly, in the initial phase of ingrowth the fibers do not even express NGF receptors. Thus, a chemotactic action of NGF on the ingrowing sensory fibers in this early developmental stage can be excluded. In the target area, NGF mRNA synthesis (followed by a delay of a few hours by NGF protein synthesis) started precisely at the timepoint when the first trigeminal sensory fibers reached their final target areas. The subsequent increase in the density of innervation correlated with the increase in NGF mRNA and NGF protein. Thus, the coincidence of the arrival of the ingrowing sensory fibers with the initiation of NGF synthesis in the target area was compatible with the assumption that the ingrowing sensory fibers initiate NGF synthesis (Davies et al., 1987). This correlation did not, however, exclude the possibility that the onset of NGF production is autonomously regulated and not dependent on the arriving nerve fibers.

To decide between these two alternatives an experimental approach was necessary that allowed the elimination of sensory neurons before they started to project to their peripheral target area. Since appropriate experimental manipulation of mouse embryos at this early developmental stage is not practical, we chose the chick embryo in which unilateral removal of the neuronal primordia (half of the neuronal tube including the neuronal crest) permitted the production of chick embryos with unilateral aneural legs (Rohrer et al., 1988). Thus, the development of NGF mRNA in the absence and the presence of innervation could be compared. The levels of NGF mRNA of the intact and denervated leg skin were found to be identical. These results indicate that the developmental regulation of NGF synthesis is not causally related to the ingrowing nerve fibers and that NGF synthesis has to be controlled by an autonomous neuron-independent mechanism (Rohrer et al., 1988).

## REGULATION OF NGF SYNTHESIS AFTER NERVE LESION

The goal of these investigations was to obtain information on the mechanisms involved in the control of NGF synthesis after nerve lesion, i.e., reactive regulatory mechanisms of NGF synthesis. These results might also be of relevance for the physiological regulation. The system chosen for these investigations was the rat sciatic nerve. In adult animals the contribution of the non-neuronal cells of the rat sciatic to NGF synthesis is negligible (Heumann et al., 1987a). The relatively high levels of NGF in the rat sciatic result from NGF transported retrogradely from the peripheral target areas in axons of NGF-responsive neurons (sympathetic and dorsal root ganglion (DRG) sensory neurons) that run in the sciatic nerve. Transection of sciatic nerve results in a large increase in local NGF synthesis, and thus this system represents an ideal model in which to analyze regulatory mechanisms.

### Transection of the Sciatic Nerve

Transection of the sciatic nerve evokes a dramatic increase in the level of NGF mRNA, which is followed by a corresponding increase in local NGF protein synthesis. The changes in NGF mRNA show a very characteristic biphasic time course. The initial peak was reached 6 hr after the lesion (Fig. 1). The relapse to intermediate levels was followed by a steady increase from 24 to 48 hr, remaining at these elevated levels for several weeks (Heumann et al., 1987a,b). The time courses of the changes in NGF mRNA levels in all the distal segments after transection were essentially the same. In contrast, the changes in NGF mRNA levels proximal to the lesion were strictly confined to the lesion area. In the more proximal segments there were no changes in NGF mRNA levels.

The enhanced local synthesis of NGF protein in the lesioned area partially compensated for the interrupted supply from the periphery. This can be deduced from the observation that

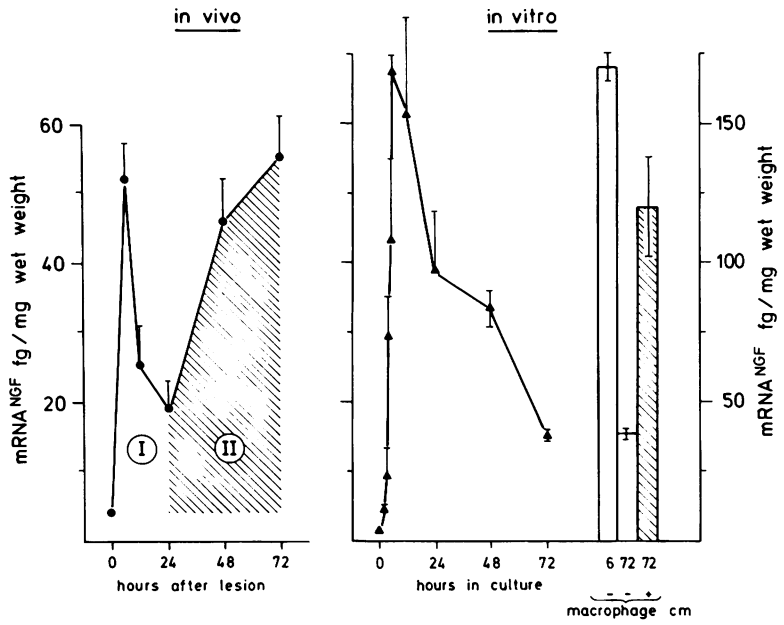


**Fig. 1.** *Time course of changes in NGF mRNA levels after transection in vivo. The initial rapid peak (phase I) and the later increase of NGF mRNA (phase II, hatched) are indicated (***left***). Nerves were kept in culture (***right***). Where indicated, activated macrophage-conditioned medium (cm) was added at the beginning of the incubation period. (Reproduced from Heumann et al., 1987b, with permission.)*

in the proximal segments (where no changes in NGF mRNA were observed), after an initial decrease to less than 15% of control (because of interrupted supply from the periphery), the NGF protein levels re-increased to about 40% of control levels, indicating that local synthesis of NGF at the lesion site can only partially compensate for the lack of supply from the periphery. This incomplete compensation is conceivable since in spite of the high levels of NGF and NGF mRNA present in the "neuroma" forming at the lesion site by dividing and immigrating non-neuronal cells the volume of this "substitute target organ" is much smaller than the sum of the physiological target tissues. The insufficient compensation by local synthesis also offers a rational explanation for the beneficial effects of the local administration of NGF for the maintenance of function and prevention of neuronal degeneration after sciatic nerve lesion (Johnson et al., 1986).

## Comparison Between the Changes in NGF mRNA Levels In Vivo and In Vitro

To analyze further the mechanisms underlying the changes in NGF mRNA occurring after nerve lesion, we brought pieces of sciatic nerve into culture and compared the changes in NGF mRNA with those occurring in vivo after transection (Heumann et al., 1987b). In distinct contrast to the situation in vivo, there was only a rapid initial increase but not a re-increase after 24 hr (Fig. 1). There was no evidence for a functional deterioration of the sciatic nerve kept in culture (e.g., the levels of 18S ribosomal RNA did not decrease), which could have explained the absence of the re-increase after 24 hr. Thus, we questioned whether the absence of immigrating macrophages could be responsible for the absence of the second phase of NGF mRNA in vitro. Indeed, in the presence of activated macrophages the NGF mRNA levels in cultured

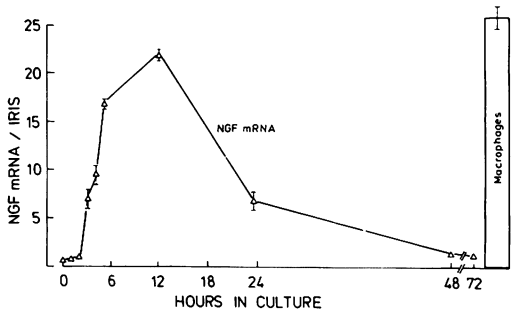


**Fig. 2.** *Time course of changes in NGF mRNA levels in cultured iris. Where indicated, irides were kept in the presence of activated macrophages. (Modified from Heumann and Thoenen, 1986.)*

sciatic nerves came close to those observed in vivo after 72 hr (Fig. 1). This initial observation was followed by the demonstration that the macrophages themselves were not necessary but that the same effect could be obtained by medium conditioned by activated macrophages.

## NGF Synthesis in (Cultured) Rat Iris Affected by Activated Macrophages

The time course of NGF mRNA changes observed in organ cultures of rat sciatic nerve kept in culture were reminiscent of the time course of NGF mRNA changes determined in rat irides kept in culture (Heumann and Thoenen, 1986). As in the rat sciatic, there was a rapid initial increase in NGF mRNA, which then was followed by a gradual decrease within the following days, remaining at slightly elevated levels at 48 and 72 hr. In agreement with the observations made with rat sciatic cultures, the addition of activated macrophages to rat irides elevated the NGF mRNA levels after 72 hr to the peak levels observed in the iris after 12 hr (Fig. 2). Thus, the capability of activated macrophages to increase NGF mRNA levels is not restricted to the nonneuronal cells of the sciatic nerve but seems to be a more general phenomenon shared also by the peripheral target cells of NGF-responsive neurons. The mechanisms underlying the initial rapid increase in NGF mRNA in rat sciatic and rat iris kept in culture seem to result from a poorly defined "lesioning effect" (Shelton and Reichardt, 1986), which seems to result in the activation of signal transduction mechanisms that, at least partially, seem also to come into play during the macrophage-mediated increase in NGF mRNA.

## Identification of the Molecule Released by Activated Macrophages, Which Is Responsible for the Increase in NGF mRNA

Macrophages are known to produce a great variety of biologically active molecules (Nathan, 1987). Of all the molecules investigated, such as acidic and basic fibroblast growth factors, platelet-derived growth factor, tumor necrosis factor, and epidermal growth factor, by far the strongest effect was obtained by interleukin-1 (IL-1) (Lindholm et al., 1987). Platelet-derived growth factor and tumor necrosis factor resulted in a doubling of the NGF mRNA levels as compared with a more than 10-fold increase by IL-1. That IL-1 is indeed the responsible agent can be deduced from the fact that antibodies to IL-1 virtually completely abolished the effect of medium conditioned by activated macrophages on NGF mRNA in sciatic nerves in cultures.

## Mechanisms Responsible for the IL-1–Mediated Increase in NGF mRNA

To analyze further the mechanism of action of IL-1 in the macrophage-mediated regulation of NGF synthesis, we evaluated whether the effect on intact tissue could also be obtained in constituents of the adult rat sciatic nerve. Indeed, cultures of fibroblast-like cells obtained after dissociation of adult rat sciatic nerves responded to IL-1 in a manner similar to those in rat sciatic tissue cultures. Since these fibroblast-like cells can easily be expanded in culture, they were suitable objects for evaluating the mechanisms responsible for the increase in NGF

mRNA levels by IL-1. Run-on experiments demonstrated that IL-1 slightly enhanced the rate of transcription of NGF mRNA. However, the predominant effect of IL-1 on NGF mRNA resulted from mRNA stabilization (Lindholm et al., 1988). Experiments are in progress to determine if cis elements and trans-acting factors are responsible for the IL-1–enhanced transcription and whether an evolutionary conserved consensus sequence (UUAUUUAU) in the 3′ untranslated region is responsible for the stabilization-destabilization of NGF mRNA as seems to be the case for other rapidly regulated mRNAs (Caput et al., 1986; Shaw and Kamen, 1986).

Since the IL-1–mediated NGF mRNA increase is preceded by an increase in c-fos mRNA (Lindholm, unpublished observations), the question arises whether there is a causal relationship between the increase in c-fos and the subsequent increase in NGF mRNA or whether the increase in c-fos is only a nonrelated epiphenomenon in view of the fact that a great variety of stimuli lead to an increase in c-fos (Müller, 1986). In this context it will be essential to evaluate whether the increase in c-fos is also related to an activation of phospholipase $A_2$ as seems to be the case for the IL-1–mediated increase in NGF mRNA. This assumption is based on the observation that the IL-1–mediated increase in NGF mRNA could be abolished by mepacrine and high concentrations of indomethacin, both inhibitors of phospholipase $A_2$. This interpretation is further supported by the fact that the preincubation of the sciatic fibroblast cultures with glucocorticoids abolished the IL-1–mediated increase in NGF mRNA, whereas the simultaneous addition of glucocorticoids did not interfere with the IL-1 effect. The necessity to preincubate the cultures with glucocorticoids suggests but does not prove that their effect results from an induction by glucocorticoids of lipocortin, a substrate competitor for phospholipase $A_2$ (Lindholm et al., 1988).

## POSSIBLE LINK BETWEEN MACROPHAGE-MEDIATED INCREASES IN NGF SYNTHESIS AFTER NERVE LESION AND REGULATORY MECHANISMS DURING DEVELOPMENT

The consequences of nerve lesion represent in many respects a functional relapse of the affected neurons and non-neuronal cells into earlier developmental stages (Heumann et al., 1987b). Thus, we asked the question whether non-neuronal cells of the rat sciatic nerve at early developmental stages would express properties of cells after lesion, i.e., high NGF synthesis. Indeed, the NGF mRNA levels of sciatic nerve at the first postnatal days were as high as those reached after nerve lesion in adult animals. Moreover, in situ hybridization experiments have demonstrated that all the non-neuronal cells, in particular all the Schwann cells (ensheathing axons of NGF-responsive and nonresponsive neurons), express NGF mRNA, as is the case after nerve lesion in adult animals (Bandtlow et al., 1987; Heumann et al., 1987a). During the first postnatal week the NGF mRNA levels decreased slowly, and, after a more rapid decay during the second postnatal week, they approached adult levels after the third postnatal week. Interestingly, intact sciatic nerves of newborn rats are populated by a relatively large number of macrophages (Stoll and Müller, 1986), suggesting that the control of NGF synthesis during development could also be controlled by IL-1. It is not clear whether the presence of these macrophages is related to ongoing neuronal cell death and concomitant degeneration of the corresponding axons in the sciatic nerve leading to a chemotactic attraction and activation of macrophages, as is the case after nerve lesion in adult animals (Perry and Gordon, 1988). Unfortunately, the scarcity of available tissue did not allow us to compare NGF mRNA levels and macrophage numbers in prenatal developmental stages before the beginning of neuronal cell death.

Experiments are in progress to study the

possible relationship between the expression of IL-1 and NGF during normal embryonic development. It seems to be very likely that the discovery of a role for IL-1 in the control of NGF synthesis is the beginning rather than the end of the elucidation of a sophisticated and complex regulatory system responsible for the regional differential regulation of NGF synthesis, explaining also the marked regional differences in the levels of NGF.

## CONCLUSIONS

The various target tissues of the peripheral nervous system display different degrees of innervation. There is strong evidence that the agent that determines the density of innervation by sympathetic and neural crest-derived sensory neurons is nerve growth factor, since the density of innervation correlates with the levels of NGF in the corresponding target areas. The elucidation of the mechanisms responsible for the differential regulation of NGF synthesis in the different target areas is one of the most essential and challenging open questions in the understanding of the physiological function of NGF. The analysis of the developmental changes of NGF synthesis in a target tissue of sensory neurons, the mouse whisker pad, demonstrated that NGF synthesis starts precisely at the time when the innervating fibers arrive in the target area. This finding was compatible with the assumption that NGF synthesis is initiated by the arrival of the sensory fibers in the target area. However, it is now demonstrated that the NGF synthesis in the developing chick skin, another target of sensory neurons, is initiated and proceeds to similar levels also in the complete absence of innervation, indicating autonomous control mechanisms intrinsic to the skin. Marked changes in NGF synthesis are not only observed during embryonic development but also in adult animals after lesioning of peripheral nerves or during culturing of nerves or peripheral target tissues. Under these experimental conditions, NGF synthesis increases dramatically and the elucidation of the mechanisms involved in the reactive regulation of NGF synthesis may also provide information on the physiological regulation. The persistent increase of NGF in the sciatic nerve after lesion depends on the presence of activated macrophages. The same is true for rat irides kept in culture that also exhibit a prolonged increase in NGF mRNA levels if activated macrophages are added to the culture medium. It was demonstrated that the effect of macrophages is mediated by the release of the lymphokine IL-1, which regulates the levels of NGF mRNA by a slight increase in the rate of transcription but predominantly by a marked stabilization of NGF mRNA. The observation that in the early postnatal period both the NGF mRNA levels and the number of macrophages in the rat sciatic nerve are considerably increased as compared with those in the adult nerve supports the hypothesis that macrophages could also be involved in the physiological regulation of NGF synthesis.

Please note: Because of space limitations, referencing was very limited so that many contributions in the area could not be cited.

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