Differential regulation of mRNA encoding nerve growth factor and its receptor in rat sciatic nerve during development, degeneration, and regeneration: Role of macrophages

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ABSTRACT   In newborn rats the levels of nerve growth factor (NGF) mRNA (mRNA^{NGF}) and NGF receptor mRNA (mRNA^{rec}) in the sciatic nerve were 10 and 120 times higher, respectively, than in adult animals. mRNA^{rec} levels decreased steadily from birth, approaching adult levels by the third postnatal week, whereas mRNA^{NGF} levels decreased only after the first postnatal week, although also reaching adult levels by the third week. Transection of the adult sciatic nerve resulted in a marked biphasic increase in mRNA^{NGF} with time. On the proximal side of the cut, this increase was confined to the area immediately adjacent to the cut; peripherally, a similar biphasic increase was present in all segments. mRNA^{rec} levels were also markedly elevated distal to the transection site, in agreement with previous results obtained by immunological methods [Taniuchi, M., Clark, H. B. & Johnson, E. M., Jr. (1986) *Proc. Natl. Acad. Sci. USA* 83, 4094–4098]. Following a crush lesion (allowing regeneration), the mRNA^{rec} levels were rapidly down-regulated as the regenerating nerve fibers passed through the distal segments. Down-regulation of mRNA^{NGF} also occurred during regeneration but was slower and not as extensive as that of mRNA^{rec} over the time period studied. Changes in mRNA^{NGF} and mRNA^{rec} occurring *in vivo* after transection were compared with those observed in pieces of sciatic nerve kept in culture. No difference was found for mRNA^{rec}. Only the initial rapid increase in mRNA^{NGF} occurred in culture, but the *in vivo* situation could be mimicked by the addition of activated macrophages. This reflects the situation *in vivo* where, after nerve lesion, macrophages infiltrate the area of the Wallerian degeneration. These results suggest that mRNA^{NGF} synthesis in sciatic non-neuronal cells is regulated by macrophages, whereas mRNA^{rec} synthesis is determined by axonal contact.

Nerve growth factor (NGF) is a protein produced in limiting quantities by the target tissues of NGF-responsive neurons and acts as a retrograde neurotrophic messenger. In this role NGF is essential for the development and the maintenance of specific properties of peripheral sympathetic and neural-crest-derived sensory neurons (1–3). A similar role for NGF recently became apparent for the cholinergic neurons of the basal forebrain nuclei (4).

In peripheral targets of sympathetic and sensory neurons, several cell types produce NGF: fibroblasts, epithelial cells, smooth muscle cells, and Schwann cells ensheathing the corresponding nerve fibers (5). The relative contribution of Schwann cells to the total NGF supply of NGF-responsive neurons was a matter of debate (6–8) until it was demonstrated that, at least in adult animals, Schwann cells ensheathing the axons of sensory and sympathetic neurons in sciatic nerve produce negligible NGF (9). However, after

transection of the sciatic nerve, local NGF synthesis increases dramatically. Augmented NGF synthesis is observed in all segments distal to the transection site but is confined proximally to those parts of the nerve stump immediately adjacent to the lesion (9). This part of the nerve stump may be considered as a "substitute target" for the axotomized and then regenerating axons of the sympathetic and sensory neurons. Although the NGF concentrations in the proximal nerve stump correspond to those of a densely innervated peripheral target organ (10, 11), the volume of the "substitute organ" is too small to fully replace the interrupted supply from the peripheral physiological target tissues. This is apparent from the fact that the NGF levels in the proximal unlesioned part of the sciatic nerve reach only 40% of their normal values (9). Simultaneously with the enhanced synthesis of NGF, the transection of the sciatic nerve leads also to reexpression of NGF receptors by Schwann cells (12), receptors normally seen only in earlier stages of development (13, 14).

In the present study we asked whether the reexpression of NGF receptors and the enhanced synthesis of NGF by Schwann cells are mediated by a common mechanism. We first followed the developmental changes in the levels of mRNA encoding NGF (mRNA^{NGF}) and of mRNA encoding NGF receptor (mRNA^{rec}) from birth to adulthood and then compared the time course of the levels of these two mRNAs after sciatic nerve transection both distally and proximally to the lesion. In experiments using a crush injury rather than transection, we studied whether neuronal regeneration brought about the return of mRNA^{NGF} and mRNA^{rec} to normal levels.

MATERIALS AND METHODS

Preparation of Sciatic Nerves. Wistar rats (male or female, 150–200 g) were anesthetized with diethyl ether and the sciatic nerve was cut or crushed at the sciatic notch. After cutting, the distal stump of the nerve was diverted into muscle tissue in order to minimize regrowth of fibers. For crushing of the nerve, forceps were cooled in liquid nitrogen and the crush site was marked by a thread.

At various times after nerve injury, animals were killed, the nerves were cut into three segments [B (proximal) and C and D (distal)], each 4 mm long, and each segment was frozen immediately in Eppendorf tubes on dry ice. For the developmental studies, intact nerves were taken from postnatal rats. Before further processing the frozen nerves were weighed and homogenized as described previously for the quantitative determination of mRNA^{NGF} levels (15).

Organ Cultures of Sciatic Nerve Segments. Wistar rats were killed and five segments (4 mm each) were placed into culture

Abbreviations: NGF, nerve growth factor; mRNA^{NGF}, mRNA encoding NGF; mRNA^{rec}, mRNA encoding NGF receptor.

in 1 ml of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum; the medium was changed daily. The same results were obtained when the serum-free, defined N2 medium of Bottenstein and Sato (16) as modified by Acheson *et al.* (17) was used. Routinely, a total of 2 cm of nerve was used for a single determination of mRNA^{NGF} and mRNA^{rec}.

Preparation of Macrophages. Rat peritoneal macrophages were produced and purified according to Mosier (18), cultivated in DMEM containing 10% fetal bovine serum, and activated with lipopolysaccharide (*Escherichia coli* 026B6, Sigma) at 70 μg/ml for 12–20 hr. At the beginning of the organ culture of sciatic nerves, 2×10^6 macrophages were added per ml of medium; macrophages remained present throughout the whole culture period. The purity of the macrophage preparation was determined by indirect immunofluorescence staining with the monoclonal antibody ED1 (19). At least 95% of all cells were positively stained. Among the other 5% of the cells, no granulocytes were present, as demonstrated by the absence of staining with the monoclonal antibody 3F12/F2 (20).

Determination of mRNA^{NGF} and mRNA^{rec}. mRNA levels were determined by a quantitative RNA gel blot procedure using a calibration standard of 3.4 kilobases (kb) for mRNA^{rec} [derived from a 3.4-kb insert (21) subcloned in plasmid pGEM] and of 0.92 kb for mRNA^{NGF}. A shorter, synthetic RNA^{NGF} fragment (0.51 kb) was added to the tissue samples before homogenization to assess the recovery in total RNA preparations after hybridization with cRNA probes (for details, see ref. 15). All mRNA^{rec} and mRNA^{NGF} values are expressed in terms of pg or fg, respectively, per mg of wet weight. The values given are the mean ± standard error of the mean (SEM).

RESULTS

Developmental Changes of mRNA^{rec} and mRNA^{NGF} Levels in Rat Sciatic Nerve. Previous experiments had shown that transection of the sciatic nerve resulted in a rapid increase in the synthesis of mRNA^{NGF} (9) and a reexpression of NGF receptors by Schwann cells (9, 12). To determine whether the expression of mRNA^{NGF} is also higher in earlier developmental stages and to explore the regulation of the synthesis of mRNA^{NGF} and mRNA^{rec} in sciatic non-neuronal cells, we compared the levels of these two mRNAs in the sciatic nerve from birth to adulthood.

The mRNA^{rec} levels at birth were 120-fold higher than in the intact adult sciatic nerve (Fig. 1). However, they were distinctly lower than the levels in the segments distal to the lesion (see Fig. 3), which reached 76 pg/mg of wet weight. There was a steady decrease in the levels of mRNA^{rec} from birth to the end of the third week after birth. The further decrease to adult levels was relatively small (Fig. 1).

The difference between the mRNA^{NGF} levels in newborn and adult nerves was smaller than that of mRNA^{rec}. At birth the mRNA^{NGF} levels were 10-fold higher than in adult animals, approaching the highest values reached after lesioning of the nerve (Figs. 1 and 2). The mRNA^{NGF} levels remained nearly constant during the first postnatal week and then decreased relatively rapidly during the second postnatal week to approach adult levels by the third postnatal week (Fig. 1).

Delineation of the Experimental Procedure for Sciatic Nerve Lesion and Regeneration Experiments. In view of the complexity of the results evolving from the experimental goal to compare, on the one hand, the changes in mRNA^{NGF} and mRNA^{rec} after nerve transection (cut) and, on the other hand, the changes occurring under experimental conditions that either minimize regeneration (cut) or permit regeneration
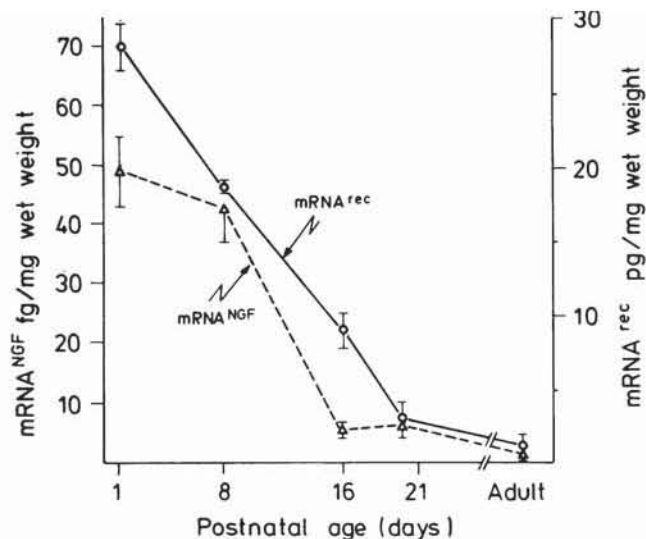

FIG. 1. Developmental changes in mRNA^{NGF} and mRNA^{rec} levels in rat sciatic nerve. Values given are means ± SEM of three or four experiments. Adult level of mRNA^{rec} was 400 ± 20 fg/mg of wet weight; that of mRNA^{NGF} was 5 ± 2 fg/mg of wet weight.

(crush), we give a brief summary of the way in which the data will be discussed.

After preliminary experiments had shown that the initial (6 hr and 12 hr) changes in the levels of mRNA^{NGF} after crush or cut were the same (data not shown) and that, in contrast to mRNA^{NGF}, the mRNA^{rec} levels did not show an initial rapid increase, the comparison between the short-term (first 3 days) changes of the two mRNAs was confined to experiments in which the nerve was cut (Figs. 2 and 3). For evaluating changes in mRNA^{NGF} and mRNA^{rec} on a longer time scale under experimental conditions minimizing (cut) or permitting (crush) regeneration, the comparison started at 3 days after the corresponding lesions. At this time point the values for both mRNA^{NGF} and mRNA^{rec} in experiments involving a cut or crush did not differ significantly ($P > 0.05$) in any of the segments studied.

Comparison Between the Changes in mRNA^{NGF} and mRNA^{rec} After Transection of the Sciatic Nerve. *Proximal segment.* As reported previously (9), the mRNA^{NGF} changes were confined to the segment immediately adjacent to the lesion (segment B, Fig. 2). A further segment, A, proximal to B (not shown in the present study) did not show any changes in mRNA^{NGF} (9). The same was true for mRNA^{rec} (data not shown). The initial changes in mRNA^{NGF} of segment B (adjacent to the lesion) were essentially the same as in the immediately adjacent distal segment C (see next section). However, the changes in segment B were in general smaller because they did not extend throughout the whole segment but were restricted to the region immediately adjacent to the lesion (9).

In contrast, the mRNA^{rec} changes in segment B as compared to segment C were distinctly different (Fig. 3) with respect to both the time course and the extent of the changes. The maximal (14-fold) increase in segment B was already reached 24 hr after nerve transection and remained at this level throughout the remaining 3 weeks (Fig. 3). In contrast, in the distal segment C, the maximal increase was not reached before 7 days after transection, but the increase was 370-fold.

Distal segments. In segments C and D the changes in mRNA^{NGF} showed a very rapid initial and transient increase peaking at 6 hr and then increasing again over the next 2 days (Fig. 2 and Fig. 4 *Left*). In contrast, the changes in mRNA^{rec} were distinctly different from those in the proximal segment B. The first consistent (6-fold) increase was detectable only after 24 hr, after which there was a steady further increase up
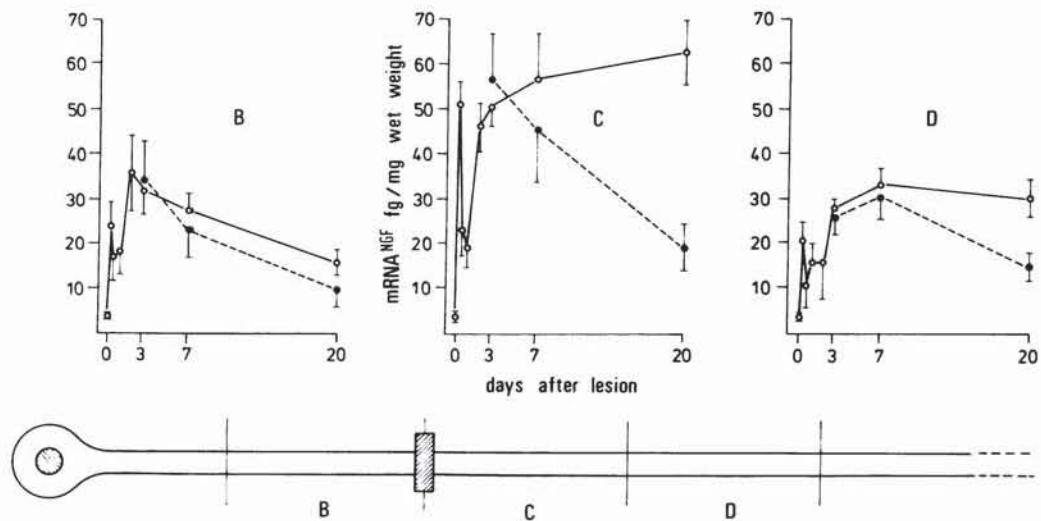
FIG. 2. Changes in sciatic mRNA^{NGF} levels proximal (segment B) and distal (segments C and D) to a transection (○) or crush (●). Values represent means ± SEM of three or four experiments. Level of mRNA^{NGF} for intact sciatic nerve was 3.4 ± 0.6 fg/mg of wet weight.

to 7 days in both segment C and segment D (Fig. 3). The values remained elevated until day 20 after transection.

Comparison Between Changes in mRNA^{NGF} and mRNA^{rec} After Cut and Crush. Here we will predominantly consider the changes occurring in the distal segments starting 3 days after a crush or a cut. At this time the levels of mRNA^{NGF} and mRNA^{rec} did not differ significantly ($P > 0.05$) between a crush or a cut in segments C and D. Thereafter the time course of the changes in mRNA^{NGF} and mRNA^{rec} levels differed markedly. In segment C the levels of mRNA^{rec} started to decrease between days 3 and 7, going from 170-fold to 54-fold control levels, and reached 25-fold control levels at day 20 in the crush experiment. In the more distal segment D there was a further increase in the crush experiment between days 3 and 7, from 138-fold at day 3 to 250-fold at day 7. However, this increase was followed by a decrease to 38-fold control levels at day 20. After a cut, the levels of mRNA^{NGF} and mRNA^{rec} remained elevated at the maximal levels reached at day 7.

Although the changes in mRNA^{NGF} and mRNA^{rec} in segments C and D started at the same time in crush and cut experiments (Figs. 2 and 3), the relative decreases for mRNA^{NGF} were smaller and were delayed as compared to those for mRNA^{rec}. At day 7 the ratio between mRNA^{rec} levels in cut and crush experiments in segment C was 6, whereas that for mRNA^{NGF} was 1.2. Although the main emphasis in comparing regenerating and nonregenerating nerves was put on the distal segments, a distinct difference between the two types of experiments was also observed for the proximal segment B. After the initial increase in mRNA^{rec} resulting from a cut, its level stayed the same throughout the whole observation period. After a crush, a decrease from 3.0 pg/mg of wet weight at day 7 to 1.0 at day 20 occurred.

Comparison Between the Time Courses of the Changes in mRNA^{NGF} and mRNA^{rec} *in Vivo* and *in Vitro*. As a prerequisite for the further analysis of the mechanism(s) underlying these changes in the mRNA^{NGF} and mRNA^{rec} after lesioning, the levels of these two mRNAs were also determined in pieces of
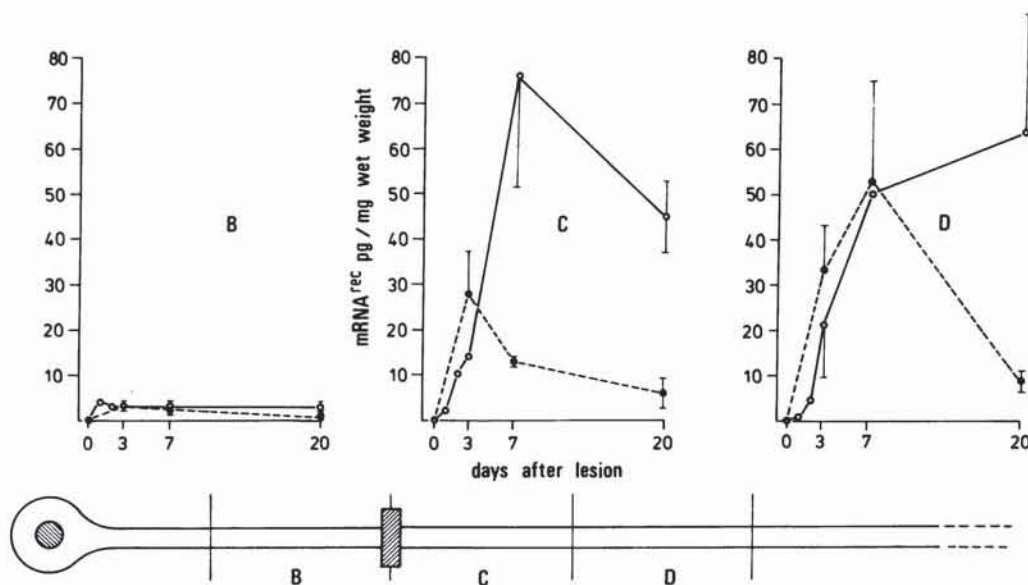

FIG. 3. Changes in mRNA^{rec} levels proximal (segment B) and distal (segments C and D) to a transection (○) or crush (●). Values represent means ± SEM of three or four experiments. Level of mRNA^{rec} in intact sciatic nerve was 240 ± 100 fg/mg of wet weight in this series of experiments.
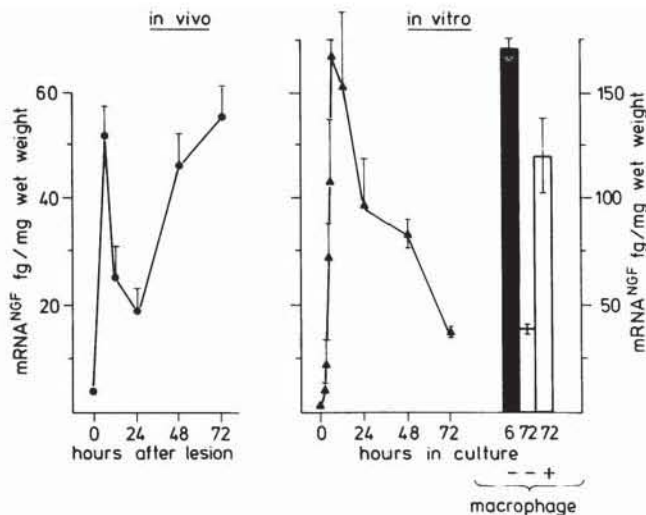
FIG. 4. Time course of changes in sciatic mRNA^{NGF} levels after transection *in vivo* (*Left*) and in nerves kept in culture (*Right*). Where indicated, activated macrophages were added to the cultures at the beginning of the incubation. Values are means ± SEM of three independent experiments.

sciatic nerve brought into culture for 6, 12, 24, 48, and 72 hr. The time course in the changes in mRNA^{rec} in culture (data not shown) was found to be essentially the same as that observed *in vivo* (Fig. 3)—i.e., there was a steady increase up to 72 hr.

In contrast, the changes in mRNA^{NGF} *in vivo* and *in vitro* were different, particularly at the 48 hr and 72 hr time points. The initial increase in mRNA^{NGF} *in vitro* occurred at the same time as *in vivo* but was even greater (Fig. 4). The following decrease between 12 hr and 24 hr was slower *in vitro* than *in vivo*. However, the most impressive difference was that observed between 48 hr and 72 hr. In contrast to the second increase seen *in vivo* (Fig. 4 *Left*), there was a further decrease *in vitro* (Fig. 4 *Right*).

Effect of Macrophages on mRNA^{NGF} and mRNA^{rec} Levels *in Vitro*. Since the changes in mRNA^{rec} levels *in vitro* reflected the situation *in vivo*, it was reasonable to assume that the decrease observed for mRNA^{NGF} was not due to a general deterioration of the sciatic nerve preparation kept in culture. However, an essential difference between the *in vitro* and *in vivo* situation is the absence of (infiltrating) macrophages *in vitro*. When activated macrophages (2×10^6 per ml of culture medium) were added to the sciatic nerve, they augmented the levels of mRNA^{NGF} 3- to 4-fold at 72 hr, reflecting the situation *in vivo* (Fig. 4). Addition of activated macrophages had no effect on the levels of mRNA^{rec} (data not shown). Special care was taken to check whether the added activated macrophages themselves contained measurable levels of mRNA^{NGF}. The 18S ribosomal RNAs of 16×10^6 macrophages and of three 1-cm pieces of (distal) sciatic nerve removed 3 days after transection were quantified as described previously (15). Despite the 4-fold greater amount of macrophage RNA (derived from 16×10^6 cells) loaded onto the gel, there was no detectable signal for mRNA^{NGF} in the macrophages. Thus, even if the sciatic nerve consisted entirely of macrophages, a signal for mRNA^{NGF} would remain undetectable.

DISCUSSION

The present experiments have demonstrated that the increase in mRNA^{NGF} after sciatic nerve lesion is compatible with the hypothesis of a relapse into an earlier developmental stage as shown previously for the reexpression of NGF receptors (12).

Furthermore, we have examined the role played by regenerating nerve fibers in restoring the original (low) levels of mRNA^{NGF} and mRNA^{rec} in adult rat sciatic nerve. From these experiments, it is clear that although there were similarities between the changes in mRNA^{NGF} and mRNA^{rec} during development, lesioning, and regeneration, there were also distinct differences that preclude a common mechanism of regulation of the two mRNAs. In particular the changes in mRNA^{NGF} and mRNA^{rec} *in vitro* differed fundamentally. Whereas the time courses of the changes in mRNA^{rec} *in vitro* and in the distal lesioned segments *in vivo* were the same, this was not true for mRNA^{NGF}. In order to mimic the *in vivo* situation for the changes in mRNA^{NGF}, the organ cultures of rat sciatic nerve had to be supplemented with activated macrophages.

Differences in the Developmental Changes Between mRNA^{NGF} and mRNA^{rec}. The levels of mRNA^{NGF} at birth (Fig. 1) correspond to the highest levels reached in distal segments after lesioning (Fig. 2). In contrast, the levels of mRNA^{rec} at birth were only half those found after sciatic nerve lesion. Although it would have been of great interest to determine the levels of mRNA^{NGF} and mRNA^{rec} in the prenatal period, this was not practical because of the very large number of pooled embryonic sciatic nerves needed to obtain reliable data.

The time courses of the postnatal changes in mRNA^{NGF} and mRNA^{rec} were also different from each other, although adult levels were approached at about the same time [i.e., the third week after birth (Fig. 1)]. The significance of the high levels of these two mRNAs early in development, particularly with respect to the presence of macrophages and the extent of contact between axons and Schwann cells, is discussed below.

Differences Between the Changes in mRNA^{NGF} and mRNA^{rec} Proximally and Distally to the Lesion Site (Crush and Cut). After transection of the sciatic nerve, the changes in the mRNA^{NGF} levels in the segments immediately adjacent to the lesion showed similar time courses (Fig. 2). In this context it should be noted that previous *in situ* hybridization experiments demonstrated that the increase in mRNA^{NGF} in the proximal segment was restricted to the region immediately adjacent to the lesion (9). Both proximal and distal segments showed biphasic alterations in mRNA^{NGF}: first, a rapid increase and decrease, which were independent of the presence of macrophages, and second, a slower increase, which correlated in time with the infiltration of macrophages (22) (Fig. 4). The same responses were observed for mRNA^{NGF} up to 3 days after lesion irrespective of whether the nerve was cut or crushed. Beyond this time point the two different types of lesions produced different responses. In the absence of nerve fiber regeneration, mRNA^{NGF} remained high in distal segments but fell markedly when regeneration was allowed. In the proximal segment, where uptake and retrograde flow of NGF remained viable, little difference was observed between a crush or a cut and the mRNA^{NGF} decreased constantly. In contrast, mRNA^{rec} levels had already reached maximal values after 24 hr in the proximal segment B, whereas in the distal segments (C and D) much higher maximal levels were reached, but not before 7 days. In the absence of regeneration, mRNA^{rec} levels remained at the elevated levels for at least 20 days in both proximal and distal segments. When nerve fiber regeneration was permitted, mRNA^{rec} levels fell continuously. The response of both mRNA^{rec} and mRNA^{NGF} in distal segments to regeneration or nonregeneration was, therefore, similar, although the return of mRNA^{rec} to normal low levels started earlier (Fig. 3) than for mRNA^{NGF} (Fig. 2) and was also greater in extent.

Possible Mechanisms Underlying the Differential Regulation of mRNA^{NGF} and mRNA^{rec}. The results summarized in the previous section demonstrated that regenerating nerve fibers reversed, although to a different extent and with a different

time course, the increases in mRNA^{NGF} and mRNA^{rec} produced by injury. However, in contrast to mRNA^{rec}, the changes in mRNA^{NGF} observed *in vivo* were distinctly different from those observed *in vitro*. Indeed, it was this difference that led to those further studies aimed at evaluating the possible role of macrophages in the regulation of mRNA^{NGF} *in vivo*. The fact that the addition of activated macrophages to pieces of cultured sciatic nerve mimicked the *in vivo* situation (Fig. 4) supports this explanation. Comparing the number of macrophages that regulate mRNA^{NGF} levels *in vitro* (2×10^6 macrophages per 2 cm of sciatic nerve in 1 ml of medium) and the number of macrophages needed to accomplish similar changes *in vivo*, it should be noted that macrophage-conditioned medium has the same effect as macrophages themselves (unpublished observation). Since the volume of the sciatic segments represent 0.3% of the culture medium, it can be deduced that 6×10^3 macrophages per 2 cm of sciatic nerve are sufficient to produce this effect. Moreover, in subsequent experiments, we have observed that the medium conditioned by 2×10^6 macrophages could be diluted 1:10 and still elicit a maximal response. The possible involvement of macrophages in the second phase of the increase in mRNA^{NGF} *in vivo* is supported further by the observation that infiltration of macrophages is known to occur during Wallerian degeneration (22–26). If the augmented synthesis of apolipoprotein E is taken as a quantitative measure for the entry and activation of macrophages, then it is clear that the time course of the second phase of the changes in mRNA^{NGF} levels correlates rather precisely with these processes (23). The fact that the increased level of apolipoprotein E persists longer than the increased level of mRNA^{NGF} probably results from the binding of apolipoprotein E and its associated lipid to the extracellular matrix of the nerve and a consequent decrease in its turnover rate (27). It is reasonable to assume that the elevated levels of mRNA^{NGF} seen in nonregenerating nerve result from the continued presence of macrophages, whereas the decrease seen during regeneration results from the slowly decreasing population of macrophages (27).

Intact sciatic nerve of newborn rats, in contrast to adult animals, is populated by a relatively large number of macrophages (24). This is also reflected in the observation that apolipoporotein E synthesis in the intact sciatic nerve of newborn rats is higher than in adults (25). It is not clear whether the presence of these macrophages is related to ongoing neuronal cell death and concomitant degeneration of corresponding axons in the sciatic nerve (1–3). Such a phenomenon could lead to a chemotactic attraction and activation of macrophages and a subsequent up-regulation of mRNA^{NGF} by a mechanism that remains to be established.

In contrast to the effect of macrophages on mRNA^{NGF}, there was no effect on the time course of the changes in mRNA^{rec}. Thus, although there are similarities between changes in mRNA^{NGF} and mRNA^{rec}, the mechanism of their regulation must be different.

With respect to possible regulatory mechanisms for mRNA^{rec}, it is possible that the synthesis of receptors is regulated by the contact of the Schwann cells with axons. Preliminary *in situ* hybridization experiments in combination with the immunohistochemical staining of neurofilaments (demonstrating the forefront of regenerating axons) showed that the down-regulation of mRNA^{rec} was closely correlated with the ingrowing regenerating axons (C.B., unpublished observation). The finding of a high level of mRNA^{rec} early in development is also compatible with this hypothesis, since in the early postnatal phase the relative contact between axons and Schwann cells is less extensive than in later stages (28).

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