DEMONSTRATION OF RETROGRADELY TRANSPORTED ENDOGENOUS NERVE GROWTH FACTOR IN AXONS OF SYMPATHETIC NEURONS¹

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Received June 2, 1983; Revised October 13, 1983; Accepted October 14, 1983

Abstract

The presence of retrogradely transported endogenous nerve growth factor (NGF) in sympathetic nerves of the guinea pig was demonstrated directly by fluorescent and peroxidase immunohistochemistry in the ligated superior postganglionic nerve of the superior cervical ganglion. Fixed, frozen sections of previously ligated nerve were incubated with either rabbit antiserum against guinea pig NGF (gpNGF), rabbit antibodies against gpNGF purified on a mouse NGF (mNGF) affinity column, the portion of rabbit antiserum against gpNGF that did not bind to the mNGF affinity column, or nonimmune rabbit serum. Positive staining on the peripheral side of the ligation was obtained only with unfractionated antiserum against gpNGF and with purified antibodies against gpNGF. The staining properties of the various antiserum preparations correlated with their ability to block gpNGF- and mNGF-induced neurite outgrowth in the embryonic chick dorsal root ganglion bioassay and in the PC12 bioassay. Homogenates of superior postganglionic nerve supported growth of embryonic chick dorsal root ganglia and differentiation of PC12 cells. This support was blocked by the specific antisera against NGF used in the immunohistochemistry experiments. These experiments demonstrate that endogenous NGF, presumably released by peripheral target tissues, is retrogradely transported *in vivo*.

Nerve growth factor (NGF) has been shown to be necessary for the maintenance and survival of peripheral sympathetic and some sensory neurons *in vivo* and *in vitro* (for review see Thoenen and Barde, 1980). The biological importance of NGF has been demonstrated by its ability to support sympathetic and sensory neurons *in vitro* and through *in vivo* experiments showing the destruction of sympathetic and sensory neurons by NGF deprivation after exposure to antibodies to NGF. *In vivo*, NGF is believed to reach its site of action via a series of processes involving its release by sympathetic and sensory target organs, uptake into nerve terminals via spe-

cific receptors, and retrograde transport of NGF to the neuronal cell body where NGF exerts its trophic effects. Various data support this general scheme of NGF action. Exogenous NGF can be retrogradely transported, as demonstrated by the appearance of [¹²⁵I]NGF in sympathetic and sensory ganglia after peripheral administration (Hendry et al., 1974; Johnson et al., 1978). NGF biological activity has been shown to be released by a denervated sympathetic target organ *in vitro* and *in situ* (Ebendal et al., 1980). Exogenous NGF, administered into an end organ, can exert trophic effects on the neurons innervating that organ; this result appears to involve retrograde transport of the added NGF (Hendry, 1977; Goedert et al., 1981). These experiments are highly supportive of a physiological role for NGF. An important missing link in this scheme is a direct demonstration that endogenous NGF is indeed being constantly retrogradely transported *in vivo*.

Based on the proposed scheme of NGF action *in vivo*, we attempted to demonstrate the presence of endogenous NGF in the superior postganglionic nerve of the guinea pig using indirect immunohistochemistry. The fibers within this nerve transport exogenous [¹²⁵I]NGF to sympathetic neurons located in the superior cervical ganglion

¹ We would like to thank Pokay Ma for his critical suggestions and Sandra Kalmbach for helpful advice. This work was supported by National Institutes of Health Grants NS12311 (B. K. H.) and NS18071 (E. M. J.), Research Scientist Development Award MH70451 (B. K. H.), the March of Dimes Birth Defects Foundation (E. M. J.), and National Institutes of Health Training Grant 5T32 NS07071 (M. A. P.). E. M. J. is an Established Investigator of the American Heart Association.

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(SCG) which are known to be dependent on NGF for survival. Initial experiments on unligated nerves failed to produce observable staining. In order to increase the concentration of any retrogradely transported NGF we performed a ligation of the superior postganglionic nerve for a short period of time. Ligation of the nerve prevents retrograde transport of material to the cell body and causes an accumulation of exogenously administered NGF at the peripheral side of the ligation. Using this approach, we have now visualized endogenous NGF retrogradely transported in axons of guinea pig sympathetic neurons. Part of this material has been presented in abstract form (Palmatier et al., 1982).

Materials and Methods

Sixty guinea pigs were anesthetized with 3.5% chloral hydrate (1.0 ml/100 gm body weight). The left SCG and superior postganglionic nerve were exposed and the nerve was ligated close to the ganglion. The right SCG and superior postganglionic nerve were also exposed and served as a control. In other animals, one vagus, median, or sciatic nerve was ligated and the contralateral nerve was exposed as a control. An attempt was made to minimize bleeding in and around the ligation as red blood cells emit a yellow autofluorescence after formaldehyde fixation which obscures the specific bright green fluorescence of the fluorescein-conjugated antibodies. The animals were allowed to recover for 20 hr, after which they were again anesthetized and perfused using a modification of the technique reported by Berod et al. (1981). The circulatory system was purged under pressure by administration of 150 ml of phosphate-buffered saline through the heart. The perfusate was changed to the fixative beginning with a solution of 10% formalin, 0.015% glutaraldehyde in 0.2 M sodium acetate, pH 6.5, followed by 10% formalin, 0.015% glutaraldehyde in 0.1 M potassium phosphate, pH 8.5. At this point, the appropriate ligated nerve and contralateral control nerve were removed and processed for frozen sections. The prostates of male guinea pigs were removed and processed identically for positive controls (see below). Fifteen-micrometer cryostat sections were mounted on coated slides, dehydrated, and stored under polyethylene glycol or used immediately for immunohistology. The primary antiserum was raised in rabbit against purified guinea pig prostate NGF and was kindly provided by Drs. Jeffrey Rubin and Ralph Bradshaw (1981). Nonimmune rabbit serum was used as a control. Two fractions of the primary antiserum were obtained by passing the antiserum against guinea pig NGF (gpNGF) over a mouse NGF (mNGF) affinity column as described by Stoeckel et al. (1976). Fraction 1 did not bind to mNGF and passed directly through the column. Fraction 2 bound to the mNGF affinity column and was eluted with either high salt or low pH (Stoeckel et al., 1976). Both fractions were used at lower dilution than the unfractionated antiserum for staining and bioassay procedures in order to compensate for dilution during processing on the mNGF affinity column. The secondary antibody was fluorescein isothiocyanate or peroxidase-conjugated goat anti-rabbit IgG from Cappel Laboratories. The peroxidase immunohistochemistry was developed using diaminobenzidine as

the reaction product. The peroxidase reaction was enhanced by the addition of the heavy metals cobalt and nickel as described by Adams (1981).

The chick embryonic dorsal root ganglion bioassay (Fenton, 1970) and the NGF-primed PC12 bioassay developed by Greene (1977) were used to detect NGF biological activity. Guinea pig superior postganglionic nerves that had been ligated for 20 hr or contralateral control nerves were homogenized in 125 μ l of cold tissue culture media in ground glass homogenizing vessels. Homogenates were left on ice for 1 to 2 hr and then filtered with 0.2- μ m filters and used immediately or after being frozen for up to 3 weeks. Antisera used in the assays were the whole rabbit antiserum against gpNGF, the two fractions of this antiserum used in the immunohistochemical studies, nonimmune rabbit serum, and guinea pig anti-mNGF serum known to have a high titer against gpNGF (Johnson et al., 1980). No attempt was made to quantitate precisely the amount of NGF retrogradely transported in these nerves. Photomicrographs of the fluorescence immunohistochemistry were taken on a Leitz Orthoplan microscopic system with a xenon lamp. Photomicrographs of the peroxidase immunohistochemistry were taken on an American Optical microscope and those of the PC12 bioassay were taken on a Leitz inverted phase microscope.

Results

Since prostate gland was the source of the gpNGF used for the preparation of the specific antiserum, the prostates of these experimental animals were removed post-fixation and used as positive controls for the immunohistochemical methodology and for the degree of fixation. Nonspecific staining was controlled for by substituting nonimmune rabbit serum for specific antiserum in the staining procedure. In guinea pig prostate nonspecific staining was negligible (Fig. 1A). The prostate tissue showed specific staining with each of the antiserum preparations: the rabbit antiserum against gpNGF (not shown) and fraction 2 (Fig. 1B), from the mNGF affinity column which contains only those antibodies that bind to determinants on both the gpNGF and mNGF molecules. Fraction 1 also stained guinea pig prostate tissue specifically, although not as well. The inability to completely block specific staining in prostate by adsorption against the heterologous mNGF is most probably due to the presence of antibodies to gpNGF that do not cross-react with determinants on the mNGF molecule. Because of the very high concentration of NGF in the prostate, these remaining antibodies are sufficient to produce a positive and specific signal. Alternately, the specific reaction could be due to antibodies to any impurities that might have been present in the guinea pig prostate NGF antigen preparation. In order to exclude the possibility that the antibodies were binding to guinea pig insulin or an insulin-like molecule, guinea pig pancreas was stained; the results using all four serum preparations were negative. Guinea pig skeletal muscle and liver also exhibited no specific immunohistochemical staining.

Experiments using peroxidase immunohistochemistry on sections of prostate tissue gave results similar to those seen in Figure 1, i.e., intense staining with rabbit anti-

Figure 1. Nonspecific and specific staining for NGF in guinea pig prostate. *A*, Guinea pig prostate stained with nonimmune rabbit serum as the first antiserum at 1:1000 dilution plus fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG as the secondary antibody at 1:1000 dilution. Magnification $\times 500$. *B*, Guinea pig prostate stained with fraction 2 as the first antibody at a dilution equivalent to the dilution of the original antiserum, plus FITC-conjugated goat anti-rabbit IgG as the secondary antibody at 1:1000 dilution. Magnification $\times 500$. Photomicrographs were taken with Kodak Tri-X pan film, ASA 400.

serum against gpNGF and no staining with nonimmune rabbit serum. Ligated superior postganglionic nerve stained positively for NGF with rabbit antiserum against gpNGF and fraction 2 (Fig. 2, *A* and *D*) and did not stain with nonimmune rabbit serum (Fig. 2*B*). Unligated nerves did not stain with any of the serum preparations (for example, see Fig. 2*C*). Nonspecific staining similar to that seen with nonimmune rabbit serum was observed in ligated superior postganglionic nerve with fraction 1 (not shown), indicating that adsorption against mNGF was successful in reducing the antibodies which reacted with the transported NGF to below the limits of detection.

The results obtained with fluorescent immunohistochemistry were identical to those shown with peroxidase immunohistochemistry. Of the various nerves examined, the superior postganglionic contained by far the greatest number of fibers stained positively for NGF with either rabbit antiserum against gpNGF or fraction 2. Other nerves examined, the vagus, median, and sciatic nerves, had varying numbers of fibers peripheral to the ligation which also stained positively for NGF with either rabbit antiserum against gpNGF or fraction 2 (Fig. 3).

Since ligated superior postganglionic nerve contained the greatest number of axons that stained specifically for NGF-like immunoreactivity peripheral to the ligation, homogenates of ligated and unligated superior postganglionic sympathetic nerves were used in the bioassays for NGF activity. For the PC12 bioassay, ligated and unligated superior postganglionic nerves were removed from guinea pigs. The portion of ligated nerve between the ligation and the point where the nerve enters the skull was homogenized in 125 μ l of cold tissue culture medium. Fifty microliters of the homogenate were added to 450 μ l

of tissue culture media in each culture well of PC12 cells. The corresponding segment of the unligated nerve was treated the same way. Initially the proximal portions of the nerve and the attached SCG were also homogenized separately and bioassayed. No NGF activity was detectable in homogenized proximal nerve plus SCG. Also, no NGF activity was observed in homogenates of unligated nerves. NGF biological activity was seen in all homogenates of ligated superior postganglionic nerve that had been frozen for up to 3 weeks (Fig. 4*A*). This NGF activity was completely blocked by the rabbit antiserum against gpNGF (Fig. 4*B*) and fraction 2, which contains antibodies against gpNGF that cross-react with mNGF, used in the immunohistological studies and by other guinea pig antisera against mNGF known to have a high titer against gpNGF. The NGF activity was not blocked by nonimmune rabbit serum or fraction 1, which does not bind to mNGF and passes through the mNGF affinity column. In the chick embryonic dorsal root ganglion bioassay, homogenates of ligated superior postganglionic nerve induced neurite outgrowth. This NGF activity was blocked by rabbit antiserum against gpNGF and fraction 2 and was not blocked by nonimmune rabbit serum or fraction 1. These results are in agreement with those obtained in the PC12 bioassay.

Discussion

The objective of this series of experiments was to provide a direct demonstration of the retrograde transport of endogenous NGF in axons of NGF-dependent neuronal types. If endogenous NGF is normally in retrograde transit in axons of sympathetic and sensory neurons of neural crest origin, NGF would be expected to

Figure 2.

Figure 3. Peroxidase immunohistochemistry in ligated median nerve. *A*, Area of ligation and distal nerve stained with nonimmune rabbit serum at 1:1000 dilution plus peroxidase-conjugated goat anti-rabbit IgG at 1:200 dilution. Magnification $\times 160$. *B*, Area of ligation and distal nerve stained with fraction 2 at a dilution equivalent to the dilution of the original antiserum, plus peroxidase-conjugated goat anti-rabbit IgG at 1:200 dilution. Magnification $\times 160$. Photomicrographs were taken with Kodak Tri-X pan film, ASA 400.

Figure 4. PC12 bioassay of homogenized ligated superior postganglionic sympathetic nerve. *A*, PC12 cells plus 50 μ l (1:10) of homogenized ligated nerve. Positive results were scored by neuritic outgrowth. Magnification $\times 300$. *B*, PC12 cells, 50 μ l (1:10) of homogenized ligated nerves, plus 100 μ l (1:5) of rabbit anti-gpNGF serum. Magnification $\times 300$. Photomicrographs were taken with Kodak Ektachrome, ASA 400.

accumulate on the peripheral side of a constriction of these axons. In the superior postganglionic nerve, most of the NGF accumulated on the peripheral side of a ligation of 20 hr duration was probably in transit at the time the ligation was made, if the rate of retrograde

transport of NGF in sympathetic nerves of the guinea pig is comparable to NGF transport in the same nerves of the rat. In the rat, retrogradely transported [125 I]NGF travels at a rate of 2 to 3 mm/hr and peaks in the SCG at 16 hr after injection into the anterior eye chamber

Figure 2. Peroxidase immunohistochemistry in ligated and unligated superior postganglionic sympathetic nerve (arrows) of the SCG (*g*). *A*, Ligated nerve stained with rabbit antiserum against gpNGF as the first antiserum at 1:1000 dilution plus peroxidase-conjugated goat anti-rabbit IgG at 1:200 dilution. Magnification $\times 60$. *B*, Ligated nerve stained with nonimmune rabbit serum as the first antibody at 1:1000 dilution plus peroxidase-conjugated goat anti-rabbit IgG at 1:200 dilution. Magnification $\times 60$. *C*, Unligated nerve stained with rabbit antiserum against gpNGF as the first antiserum at 1:1000 dilution plus peroxidase-conjugated goat anti-rabbit IgG at 1:200 dilution. Magnification $\times 60$. *D*, Ligated nerve stained with fraction 2 as the first antibody at a dilution equivalent to the dilution of the original antiserum, plus peroxidase-conjugated goat anti-rabbit IgG at 1:200 dilution. Magnification $\times 60$. Photomicrographs were taken with Kodak Tri-X pan film, ASA 400.

(Hendry et al., 1974; Johnson et al., 1978). The estimated length of the superior postganglionic nerve in the rat is 2 cm and in the guinea pig it is 4 cm. Furthermore, it seems likely that 20 hr of ligation is not time enough for the NGF output of the target tissue to have changed significantly as a result of denervation. Therefore, the NGF-like immunoreactivity observed in ligated nerves is most probably due to NGF in transit at the time of the ligation. In any case, these experiments do demonstrate the retrograde transport of endogenous NGF.

It is interesting that the median, sciatic, and vagus nerves, which are mixed nerves, all contain fibers that stain specifically for NGF. The median and sciatic nerves contain fibers originating from sympathetic and sensory neurons, of neural crest origin. Sympathetic neurons are known to be dependent on NGF for survival during development and for maintenance *in vitro*. Neural crest-derived sensory neurons in the dorsal root ganglia transport [¹²⁵I]NGF in adult animals, and NGF deprivation in adult animals results in decreased substance P levels in the sensory ganglia (Schwartz et al., 1982). It is probable that the few fibers stained specifically in ligated vagus nerve arise from cells of neural crest origin in the jugular ganglion of the vagus, since placodally derived neurons in the nodose ganglion are not NGF dependent (Pearson et al., 1983). Thus, it seems likely that these neurons would also require NGF for maintenance *in vivo* and would transport it from the target organs to the cell body.

Specific immunological localization of NGF molecules in guinea pig nerves, particularly with antibodies against gpNGF purified by binding to heterologous mNGF, in addition to the demonstration of the presence of NGF biological activity in ligated sympathetic nerves of the guinea pig, fulfill the requirements necessary to establish the presence of endogenous NGF. These data thus provide a direct demonstration of the retrograde transport of endogenous NGF, previously assumed on the basis of the behavior of exogenous NGF, and further strengthen the general working model that trophic factors elaborated by target organs are responsible for the maintenance of the neurons which innervate them.

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