Studies on the Regulation of Beta-Nerve Growth Factor Gene Expression in the Rat Iris: The Level of mRNA-Encoding Nerve Growth Factor Is Increased in Irises Placed in Explant Cultures In Vitro, But Not in Irises Deprived of Sensory or Sympathetic Innervation In Vivo

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Abstract. Beta-nerve growth factor (NGF) is a protein necessary for the survival and maintenance of sympathetic and sensory neurons that appears to be produced by the target tissues of these neurons in vivo. Both denervation and the culture of explants of one model target, the rat iris, leads to an increase in the NGF content, suggesting that innervating neurons may regulate a step in synthesis or turnover of NGF. To determine whether there is a change in synthesis controlled at the mRNA level, the rat iris has been assayed for its content of NGF mRNA after surgical and chemical denervation and after explant into culture.

Using a sensitive blot hybridization assay, a large, rapid increase in the content of NGF mRNA was observed upon explant of the rat iris. The increase was readily detectable within 1 h, reached a maximum increase of 10- to 20-fold by 6 to 12 h, and was still evident after 3 d in culture. The distribution of NGF mRNA in different areas of the iris does not change during this time. This rapid increase in NGF mRNA is also seen in the fully innervated iris in vivo after trauma to the anterior chamber.

In contrast, denervation to varying degrees in situ had no effect on NGF mRNA levels. Neither removal of sympathetic innervation by surgical or chemical methods nor combined surgical removal of sympathetic and sensory innervation detectably altered NGF mRNA content. Thus, denervation of the rat iris in situ does not cause the observed accumulation of NGF by increasing the level of NGF mRNA, and the increase in NGF content must be due to other factors.

During development and throughout life, neurons and their target cells exert trophic influences upon each other. For example, target cells produce and release factors which are required by innervating neurons for maintenance and survival (reviewed in Oppenheim, 1981). By far the best characterized of these factors is beta-nerve growth factor (NGF),[1] which is required for the normal development and maintenance of sensory and sympathetic neurons (reviewed in Yankner and Shooter, 1982).

NGF is required in culture medium for the survival and differentiation of sensory and sympathetic neurons in vitro (Levi-Montalcini et al., 1954), and must be present at the tips of elongating neurites in order to allow their continued extension (Gundersen, 1985; Campenot, 1977). Treatment of newborn mammals with antiserum to NGF causes an almost complete destruction of the sympathetic nervous system (Levi-Montalcini and Booker, 1960). Prenatal exposure to these antibodies results in a similar destruction of sensory neurons (Gorin and Johnson, 1979). These results demonstrate that endogenous NGF is also required to maintain these neurons in vivo. Responsive neurons are able to bind, internalize, and retrogradely transport NGF from their terminal fields to the cell bodies in vivo (reviewed in Thoenen and Barde, 1980). Interrupting the peripheral connection of these neurons either by nerve crush or blockage of retrograde transport leads to similar effects as treatment with antibodies to NGF (Hendry, 1975; Hendry and Campbell, 1976; Thoenen et al., 1978). These effects, which are particularly dramatic in neonates, can be reversed by exogenous NGF (Hendry, 1975; Hendry and Campbell, 1976). This strongly suggests that neurons, particularly in neonates, obtain most endogenous NGF from their peripheral targets.

Although a major feature of this model is that NGF is present in target organs, it has only recently been possible to

[1] *Abbreviations used in this paper:* DME-H21, Dulbecco's modified Eagle's medium with 4.5 g/liter glucose and no pyruvate; NGF, beta-nerve growth factor; NGF mRNA, mRNA-encoding NGF; 6-OHDA, 6-hydroxydopamine; TTX, tetrodotoxin.

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detect it there. The development of an extremely sensitive two-site enzyme-linked immunosorbent assay has allowed the unambiguous detection of NGF antigen in several tissues which contain a dense sympathetic innervation, such as iris and heart (Korsching and Thoenen, 1983). It appears that this NGF is synthesized locally, as we have recently demonstrated a strong positive correlation between the level of sympathetic innervation and the amount of mRNA–encoding NGF (NGF mRNA). In addition, we find sufficient NGF mRNA to account for the NGF content of tissue (Shelton and Reichardt, 1984).

The amounts of NGF present in many sympathetic effector organs have been shown to increase after culture of explants, using semi-quantitative bioassays (Harper et al., 1980; Ebendal et al., 1980). The amount of NGF which is present in the rat iris has also been shown to increase in vivo after denervation using both bioassays and radioimmune assays. With these assays, Ebendal et al. (1980, 1983) have detected large increases in NGF when irises are placed into organ culture and smaller increases when irises are surgically deprived of their sensory and sympathetic innervation. Still smaller, but significant increases are seen when irises are deprived only of sympathetic innervation (Ebendal et al., 1980). These findings have since been more precisely quantified by using the two-site enzyme-linked immunosorbent assay. The NGF content increases ~200-fold in cultured irises (Barth et al., 1984), and approximately twofold in irises which have been chemically sympathectomized with 6-hydroxydopamine (6-OHDA) (Korsching and Thoenen, 1985).

There are several mechanisms which might contribute to the increase in NGF content observed after explantation or denervation. Perhaps most obviously, these treatments might cause an increase in the synthesis of NGF, thereby leading to higher levels in the tissue. Such an increase might be caused by an increase in NGF gene expression, NGF mRNA stability, translational efficiency, or posttranslational processing. Alternatively, the increased NGF content of a tissue may reflect decreases in the degradation or removal of NGF. Combinations of these mechanisms might also regulate the changes in NGF content seen after experimental manipulations.

To determine if these changes in NGF content are due to changes in NGF mRNA, we have assayed irises for their content of NGF mRNA after denervation in situ and after explant culture, using a sensitive blot hybridization procedure (described in Shelton and Reichardt, 1984). The results, presented in this paper, show that there is no change in NGF mRNA levels after sympathetic and sensory denervations in vivo, suggesting that some other mechanism(s) must be responsible for the two- to threefold increases in NGF content seen after these treatments (Ebendal et al., 1983; Korsching and Thoenen, 1985). Irises cultured in vitro, however, do show a rapid, large increase in NGF mRNA levels, suggesting strongly that an increase in gene expression is an important contributor to the much more dramatic increases in NGF content observed after culture of explanted irises (Ebendal et al., 1980, 1983; Barth et al., 1984).

Materials and Methods

Materials

Sprague Dawley rats and New Zealand white rabbits were obtained from local suppliers. Enzymes were purchased from New England Biolabs, Beverly, MA. *Alpha*-[^{32}P]-dCTP (3,000 Ci/mmol) was purchased from Amersham Corp., Arlington Heights, IL. All other chemicals were reagent grade or the best commercially available.

Organ Culture

Rat irises with attached ciliary bodies were cultured in Dulbecco's modified Eagle's medium with 4.5 g/liter glucose and no pyruvate (DME-H21) in 8% CO_2, 92% air at 37°C with additives as described in individual experiments. 20 mM Na-Hepes (pH 7.4) was added in some experiments.

Denervation Procedures

Rats were anesthetized with ketamine HCl (100 mg/kg, i.m.) and pentobarbital (40 mg/kg, i.p.). Sympathetic denervation was performed by the unilateral removal of the superior cervical ganglion after a midline incision. Combined sensory and sympathetic denervation was achieved by the intracranial electrocoagulation of the ophthalmic branch of the trigeminal nerve under visual control as described (Ueda et al., 1982). Eyelids were sutured after this procedure to avoid corneal erosion. Complete retrobulbar denervation was accomplished by exposing the retroorbital region (Malmfors and Nilsson, 1964). The ciliary ganglion was removed and the long and short ciliary nerves and the optic nerve were cut. To chemically sympathectomize animals, they were injected with 6-OHDA (200 mg/kg, i.p.) which was dissolved in normal saline (0.9% NaCl) with 0.1% ascorbate immediately before use.

Anterior Chamber Manipulations

Rats were anesthetized as above, and a tracheotomy was performed to aid respiration. Anesthesia was maintained with pentobarbital for the 6-h duration of the experiment. For flushing the iris with culture fluid, two cannulae were implanted into the anterior chamber, with their openings at opposite sides of the chamber to provide a flow across the iris. The cannulae were made of PE10 polyethylene tubing which had been trimmed to a bevel. To avoid an increase in intraocular pressure during implantation, the cornea was first pierced with a 25-ga needle, which was then removed. The cannula was then inserted into the hole left by the needle, taking care not to injure the iris. After the two cannulae were implanted from opposite sides of the eye, the flow of culture medium was started at a rate of 200 μl/h with a syringe pump. The free end of the exit cannula was kept 15 cm above the eye to maintain normal intraocular pressure. Sham animals had two cannulae implanted which were sealed ~1 cm from the inserted end. These animals were also kept under anesthesia for 6 h and had a tracheotomy performed.

For anterior chamber injections, a solution of drug or vehicle (saline) was diluted 1:1 with a 4% solution of low melting temperature agarose in saline at 40°C. 0.2 μl of this solution was drawn into a 25-ga needle which was attached to a microliter syringe. After a few moments at room temperature to allow the agarose to solidify, the needle was inserted into a previously pierced hole in the cornea of an anesthetized rat and the agarose plug expelled with gentle pressure. The needle was then withdrawn, leaving the plug in the anterior chamber. When tetrodotoxin (TTX) was used, its dose was 1 μg per eye.

NGF mRNA Assay

RNA was prepared from irises by a modification of the method of Cheley and Anderson (1984). Individual irises were homogenized in 200 μl of 7.6 M GuHCl, 0.1 M K-Acetate (pH 5.0), 0.01% blue dextran by repeated trituration through a 23-ga needle. RNA was precipitated by addition of 120 μl of ethanol and storage at −20°C for at least 12 h. RNA was pelleted by centrifugation for 10 min at 13,000 *g*, and the resultant pellet was washed twice with 70% ethanol. The pellet was then resuspended directly in sample buffer for formaldehyde gels (Lehrach et al., 1977) and heated to 70°C for 10 min. These samples were then separated on 1.2% agarose-formaldehyde gels, transferred to nitrocellulose, and hybridized to ^{32}P-labeled single-stranded cDNA probe for mouse NGF (Scott et al., 1983) as described earlier (Shelton and Reichardt, 1984). The amount of NGF mRNA was then determined by densitometry of autoradiograms of the nitrocellulose transfers, which were quantified by comparison to standard curves of male mouse submaxillary gland poly (A^+) RNA which were run on the same gel. This submaxillary gland poly (A^+) RNA contained 0.08 ± 0.03% NGF mRNA, as determined by comparison to single-stranded cloned NGF cDNA (Shelton and Reichardt, 1984). To confirm this, known quantities of single-stranded NGF cDNA were also run on these gels and were used to construct similar standard curves.

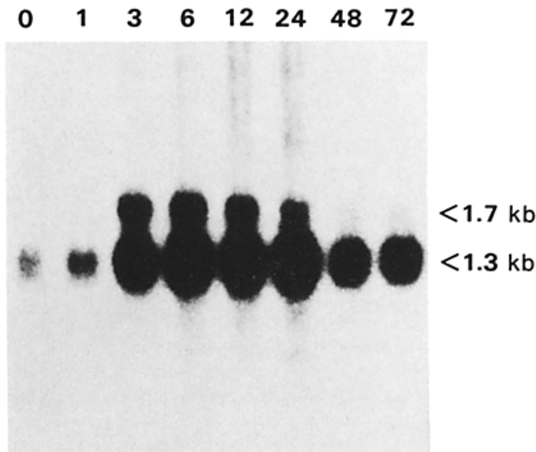

Figure 1. Autoradiogram of RNA blot hybridization of iris RNA after explant. Autoradiogram of RNA transfer after hybridization with ^{32}PDNA single-stranded probe. Each lane contains the RNA from a single iris after various times in vitro. Lanes (*left* to *right*) contain RNA from an iris at zero time, or 1, 3, 6, 12, and 24 h, 2 d, or 3 d after explant. Main hybridizing band is 1.3 kb. In darker lanes notice the existence of band at slightly higher molecular weight (1.7 kb).

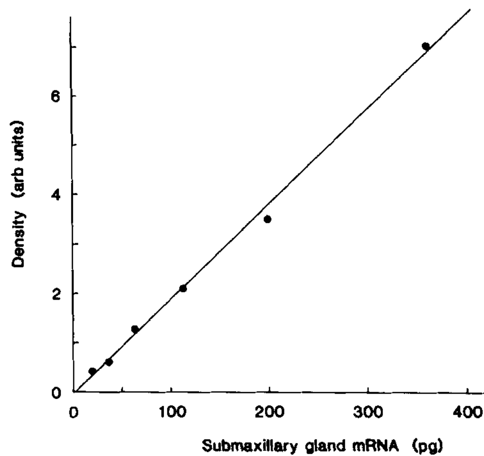

Figure 2. Standard curve obtained using densitometry of autoradiogram. Example of standard curve of NGF mRNA assay obtained by densitometry of autoradiogram. Autoradiographic density was obtained by an integration of scan of the band of interest. Line was drawn using linear regression analysis of the data.

Results

Assay for NGF mRNA

When RNA was prepared and assayed for NGF mRNA content as described, the major hybridizing band always comigrated with NGF mRNA from mouse submaxillary gland, with a molecular size of 1.3 kb (see Fig. 1). As previously described, there was a minor band that also hybridized under stringent conditions that corresponded to an RNA of molecular size 1.7 kb in addition to the major 1.3-kb band (Shelton and Reichardt, 1984). This band was detectable in all samples when autoradiograms were sufficiently exposed, but was not included during densitometry readings, except where measured separately. This band is not due to hybridization to 18S ribosomal RNA (Shelton and Reichardt, 1984).

When autoradiograms of serial dilutions of mouse submaxillary gland poly (A$^+$) RNA are scanned densitometrically, a standard curve such as that shown in Fig. 2 is obtained. From any one autoradiographic exposure, the usable linear range is slightly more than one order of magnitude. Exposure was varied so that measurements were always made from the linear portion of the standard curve.

Irises Placed In Vitro

When rat irises were cultured under the conditions described, they maintained a normal gross morphology for at least 3 d. They tended not to adhere to the culture dish except occasionally at a small, limited area of contact. When this occurred, there were cells which migrated out of the explant and onto the surface of the culture dish. If irises were dark adapted for several hours in the incubator and then exposed to bright light, there was a vigorous contraction. Since the irises were irregularly folded, it was not possible to determine whether this was due to a contraction of the sphincter, dilator, or both. Irises which were killed by freezing did not display this contraction.

The RNA from these irises, when analyzed with Northern blots, showed a large, rapid increase in NGF mRNA. The autoradiograph from one such experiment is shown in Fig. 1 and the data is summarized in Fig. 3. The increase was readily detectable after 1 h in culture (fivefold in the experiment shown in Fig. 3) and by 6 to 12 h reached a maximum increase which varied in different experiments from 6- to 20-fold. The increase in NGF mRNA appeared to start at the time of explantation, with no observable lag. The maximum level was maintained until 24 h, after which there was a slow decline in levels of NGF mRNA. Even at the longest times

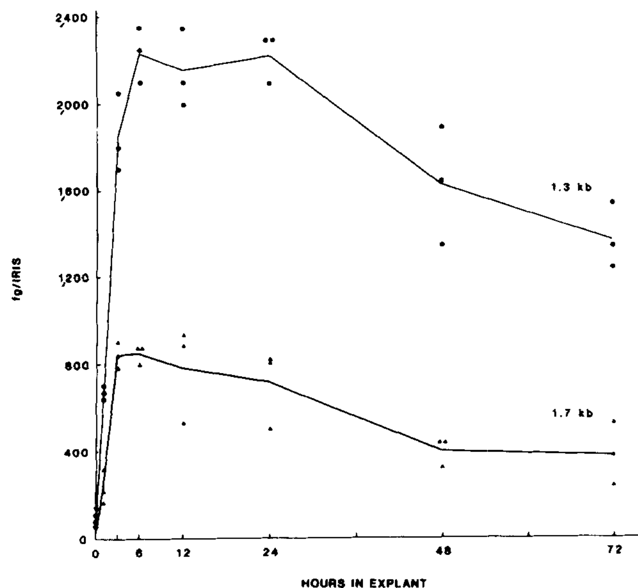

Figure 3. Content of NGF mRNA in irises cultured in vitro. The amount of hybridizing RNA migrating with the indicated size in individual irises after various times in culture. Irises were harvested after 0, 1, 3, 6, 12, 24, 48, and 72 h in culture. Lines are drawn through the means of the three individual determinations indicated by points. Points represent RNA of either (●) 1.3 kb, or (▲) 1.7 kb. Individual points for the 1.7-kb RNA at zero time have been omitted for clarity. The actual values obtained in this experiment were 12, 22, 30, and 34 fg/iris. The increase above the content at time zero is statistically significant at all times after explant for each band (P = 0.05, Mann-Whitney U test).

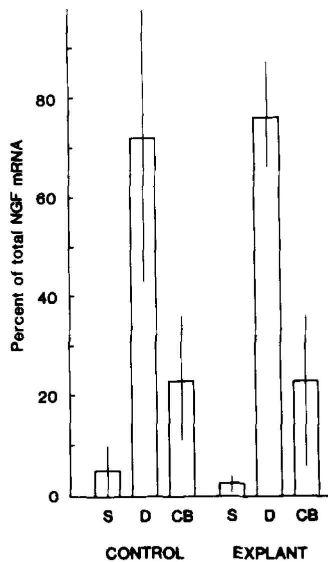

Figure 4. Regional distribution of NGF mRNA content of the normal and cultured rat iris. The NGF mRNA content of regions that were dissected from irises which were freshly removed (*CONTROL*) or had been cultured for 15 h in serum free DME-H21 (*EXPLANT*), was determined. Irises were dissected into regions containing the sphincter (*S*), dilator (*D*), and ciliary bodies (*CB*) and each region's contribution to the total NGF mRNA content of the iris was calculated. Vertical bars represent the means of two determinations and the vertical lines indicate the ranges of those determinations. In this experiment, the mean content of NGF mRNA was 90 fg NGF mRNA/iris before culture and 1,010 fg NGF mRNA/iris after culture.

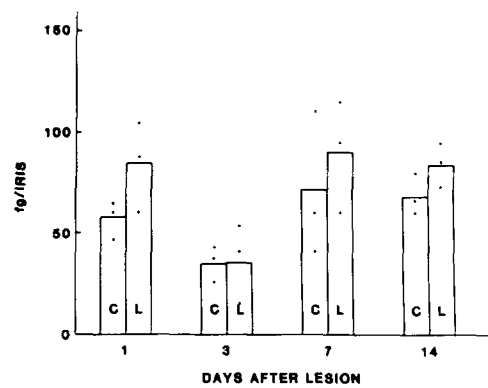

Figure 5. NGF mRNA content of irises after surgical sympathetic denervation. The content of NGF mRNA in individual irises at various times after removal of the superior cervical ganglion. Bars are the means of the three individual determinations shown by the dots. *C* is the iris from the control, unoperated side of the animal. *L* is the iris which has had its sympathetic ganglion removed. No statistically significant differences between control and lesion side by the Mann-Whitney U test.

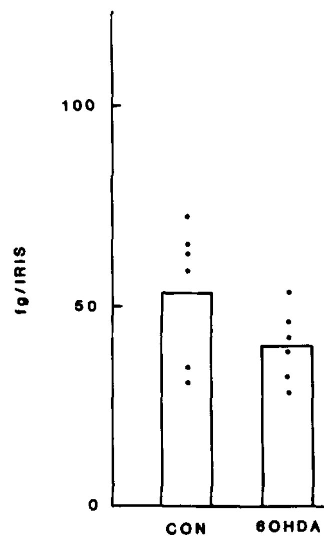

Figure 6. NGF mRNA content of irises after chemical sympathetic denervation. The content of NGF mRNA in irises from animals treated with 200 mg/kg 6-OHDA (*6OHDA*) or vehicle alone (*CON*) 18 h before. Bars represent the means of the six determinations shown by the individual points. There is a significant decrease in the content after treatment with 6-OHDA ($P = 0.02$, Mann-Whitney U test).

studied (72 h) the levels of NGF mRNA was still much higher than in a freshly dissected iris (15-fold in the experiment shown in Fig. 3). Experiments done in parallel have shown that the total RNA content of an iris falls to 40–50% of a freshly dissected iris over 1 to 2 d in vitro (data not shown). As shown in Fig. 3, the 1.7-kb band of hybridizing RNA increases and decreases in parallel with the 1.3-kb NGF mRNA.

To determine which areas of the iris were expressing the NGF gene and to determine if this distribution of expression might change during the large induction seen after explant, RNA was prepared from different regions of the iris. Freshly dissected irises and irises which had been in explant for 15 h were dissected into sphincter, dilator, and ciliary body regions, and these were assayed for their content of NGF mRNA. As seen in Fig. 4, the dilator region contained most of the NGF mRNA in the iris. The ciliary body contained most of the rest of the NGF mRNA and the sphincter region contained very little. This distribution did not change during explant even though there was an increase of an order of magnitude in the NGF mRNA content of the iris and is similar to the distribution of NGF antigen after culture (Barth et al., 1984).

Sympathetic and Sensory Denervation In Vivo

The above results suggest that increased NGF mRNA content contributes to the increased NGF content of the iris seen during the culture of explants. To determine if the NGF mRNA content of the rat iris increased in response to denervation in situ, the sympathetic innervation to the iris was interrupted by surgically removing the ipsilateral superior cervical ganglion. This procedure always led to immediate ptosis on the operated side. As shown in Fig. 5, analysis of these irises from 1 to 14 d after surgery revealed no significant change in NGF mRNA content compared to those from the control side. There is an increase in the NGF activity of irises 10 d after a sympathetic denervation (Ebendal et al., 1980).

Sympathetic denervation was also achieved by the use of 6-OHDA. Animals injected with 200 mg/kg developed piloerection and extreme exophthalmia within 5 min. By the time of sacrifice at 18 h post-injection, there was pronounced bilateral ptosis. Measurements of the NGF mRNA content of these irises, presented in Fig. 6, show that this RNA was not significantly increased from age-matched controls 18 h after treatment. In fact there was a slight decrease ($P = 0.02$, Mann-Whitney U test). This is in contrast to the approximately

twofold increase in NGF antigen seen 18 h after 6-OHDA treatment (Korsching and Thoenen, 1985).

As much larger increases in the NGF content of irises have been seen after combined sensory and sympathetic denervation than after sympathetic denervation alone (Ebendal et al., 1980, 1983), it seemed possible that sensory innervation might regulate NGF gene expression in the iris. To investigate this possibility, combined denervations were performed by intracranial electrocoagulation of the ophthalmic branch of the trigeminal nerve. After this treatment, a complete severance of all nerves leaving the anterior lacerated foramen was observed postmortem in all animals at all postoperative times studied. Immediately before sacrifice, absence of the corneal blink reflex on the operated side was verified in all animals used. As shown in Fig. 7, irises from both operated and control sides had elevated levels of NGF mRNA compared to unoperated controls at 4 d after surgery (compare to zero time in Fig. 3). However, there was no significant difference in NGF mRNA content between irises from operated and control sides at 4, 7, and 10 d after surgery. Increases in the NGF antigen and activity have been seen in irises 10 d after this operation (Ebendal et al., 1980, 1983).

The results in this section show that changes in NGF mRNA content are not sufficient to account for the increase in NGF content seen in the iris after sympathetic and sensory denervation in situ.

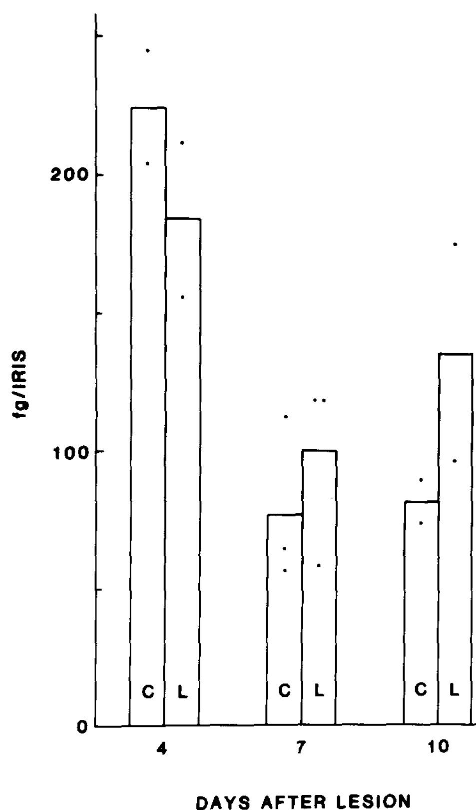

Figure 7. Content of NGF mRNA in irises after sympathetic and sensory denervation. The content of NGF mRNA in individual irises at various times after electrocoagulation of the ophthalmic branch of the trigeminal nerve. Bars are the means of the individual determination shown by the dots. *C* is the iris from the control, unoperated side of the animal. *L* is the iris which had the combined denervation. No statistically significant differences between the control and lesion sides by the Mann-Whitney U test.

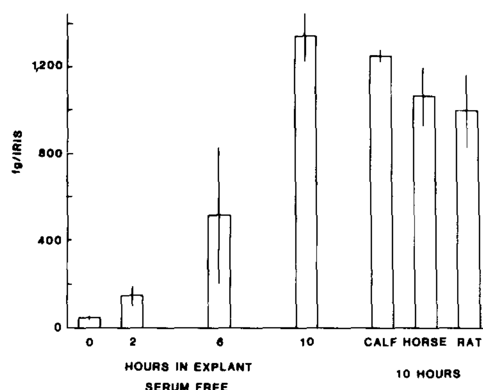

Figure 8. Effect of various culture media on induction of NGF mRNA in vitro. Left side of figure shows time course of increase in NGF mRNA content of irises cultured in DME-H21 with no serum or other additives. Right side of figure shows the increase obtained in parallel cultures after 10 h growth in DME-H21 with serum from the indicated species added to 10%. Bars show the means of two determinations. Vertical lines indicate the range.

Regulation of NGF mRNA by Culture Conditions

It now seemed unlikely that the large increase in NGF mRNA obtained in vitro was due to the denervation which occurs upon explant, and other aspects of the difference between growth in vitro and the normal situation in vivo were explored. In an attempt to define what might be causing the induction of NGF mRNA observed after placing irises in vitro, a variety of different culture conditions and additives were surveyed. Since the iris is normally bathed in aqueous humor, which is dissimilar to serum in its content of protein and some small molecules (Sears, 1981), the effects of varying serum source and concentration were examined. The results, presented in Fig. 8, show that the induction occurred in DME-H21 with 10% horse, calf, or rat serum, or in DME-H21 which was completely serum free. The concentration of ascorbate in aqueous humor is many times higher than that in plasma, sometimes exceeding 2 mM (Sears, 1981). Addition of ascorbate at 2.8 mM had no effect on the in vitro induction (data not shown). Since irises were explanted into a relatively large volume (usually 1 ml per iris) it seemed possible that dilution of endogenous NGF might be causing the increase in NGF gene expression. However, addition of NGF at 1 μg/ml or rabbit antiserum to NGF at 10% did not change the observed in vitro induction (data not shown).

To more directly test if the increase in NGF mRNA seen in vitro was due to differences between the culture media used and the normal physiological milieu of the aqueous humor, irises were cultured in media which contained various concentrations of freshly drawn rabbit aqueous humor. Initial results of this experiment showed very clearly that when present at concentrations of 40% or greater, aqueous humor prevented the increase in NGF mRNA after explant (data not shown). However, further work revealed that approximately half of the batches of aqueous humor tested could not prevent the increase in NGF mRNA seen after explant. This variability remained in the presence of attempts to standardize the method of aqueous humor collection and is not yet understood.

Because of these results which indicated that aqueous hu-

mor could reduce the level of induction of NGF mRNA when added to cultures, an experiment was performed to ascertain the effect of aqueous humor in vivo. To test this, two cannulae were placed into the anterior chamber and used to continuously wash the iris with DME-H21 in vivo. During the implantation of these polyethylene cannulae, most of the aqueous humor initially present in the anterior chamber was lost, as judged by leakage and the decrease in volume of the anterior chamber. This loss occurred both in the experimental case and when the plugged cannulae used for the sham experiments were implanted. The cornea quickly resealed around the polyethylene tubing, and the anterior chamber appeared to regain its original volume within 30 to 60 min. After 6 h of continuously flushing the anterior chamber, the iris was removed and assayed for content of NGF mRNA. As seen in Fig. 9, irises which were treated in this way had an increase in NGF mRNA of five- to sevenfold compared to the control side. This was similar to that seen after 6 h in vitro (compare with Fig. 8). Irises from sham animals, which had cannulae implanted but were not perfused, showed a similar increase compared to their control side. Increases in both perfused and sham animals were statistically significant ($P = 0.05$, Mann-Whitney U test). Therefore, either the implantation procedure or the presence of cannulae led to a large increase in the NGF mRNA content of the fully innervated rat iris. Continuously replacing the aqueous humor by washing with DHE-H21 did not result in further increases in NGF mRNA levels.

In concurrent experiments designed to ascertain the effect of complete blockage of Na^+-dependent action potentials in the iris, TTX was injected into the anterior chamber. To avoid losing all of the injected TTX in the outflow which followed needle withdrawal, the TTX was delivered in a plug of solidified low melting temperature agarose. Injection with TTX resulted in immediate relaxation of the iris, which lasted the 6 h until sacrifice. As seen in Fig. 9, there was a three- to fourfold increase in NGF mRNA in the treated iris compared to the control side. In this case also, however, sham animals, which received injections of saline in agarose, showed an equivalent increase when compared to the untreated contralateral side. Both these increases were significant ($P = 0.05$, Mann-Whitney U test). The NGF mRNA content of the control irises in this experiment, though high, are not outside the range seen in untreated animals. If the presence of TTX exerted any effect on the expression of the NGF gene, it was not discernable because again, in these experiments, paracentesis of the anterior chamber caused an increase in the NGF mRNA content of the iris. Such treatment is known to lead to a rapid breakdown of the blood-aqueous barrier (Raviola, 1974).

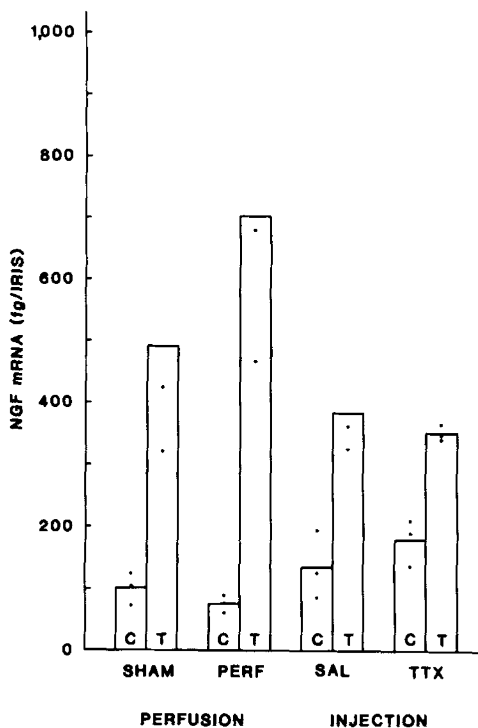

Figure 9. Content of NGF mRNA in irises subjected to anterior chamber insult. The content of NGF mRNA was determined in irises removed from eyes which were subjected to either cannulae implantation (*PERFUSION*) or injection (*INJECTION*) 6 h previously. Anterior chambers implanted with cannulae were either continuously perfused with DME-H21 (*PERF*) or not (*SHAM*). Anterior chambers were injected with 0.9% saline (*SAL*) or saline containing 1 μg tetrodotoxin (*TTX*). In all cases, the contralateral, untreated eye served as a control (*C*) for the eye which had been subjected to trauma (*T*). All treated irises have a statistically significant increase over the control ($P = 0.05$, Mann-Whitney U test).

Retrobulbar Denervation In Situ

Another difference between the in situ sensory and sympathetic denervation and growth in vitro is that the latter involves removal of all innervation to the iris, not just removal of sympathetic and sensory fibers. In an attempt to determine if complete removal of innervation to the iris in situ would show any effects on the expression of the NGF gene, surgery was performed which severed all known projections to the eye. After the retrobulbar section of the optic nerve and the long and short ciliary nerves, there was a response on the operated side similar to that described after lesion of the sensory projection to the eye (Moses and Feldman, 1969). The anterior chamber became cloudy, and there was a somewhat variable amount of bleeding into the anterior and posterior chambers and into the vitreous humor. Approximately 1 wk after surgery, the cornea became vascularized. The corneal blink reflex remained absent at all times studied, but a blink reflex could usually be elicited by brushing of the eyelash on the operated side. There was a significant increase in NGF mRNA content of the iris on the operated side between 36 h and 10 d after this treatment (Fig. 10) ($P = 0.05$, Mann-Whitney U test; control vs. operated at each time). The increase was variable, but averaged about fivefold at each post-operative time examined. The significance of these results is discussed below.

Discussion

The results in this paper show that sympathetic and/or sensory denervation of the rat iris in situ does not increase the synthesis of NGF by increasing the level of NGF mRNA. Further, we demonstrate that placing the iris into culture medium does cause an increase in NGF mRNA, and that this response is probably not due to denervation.

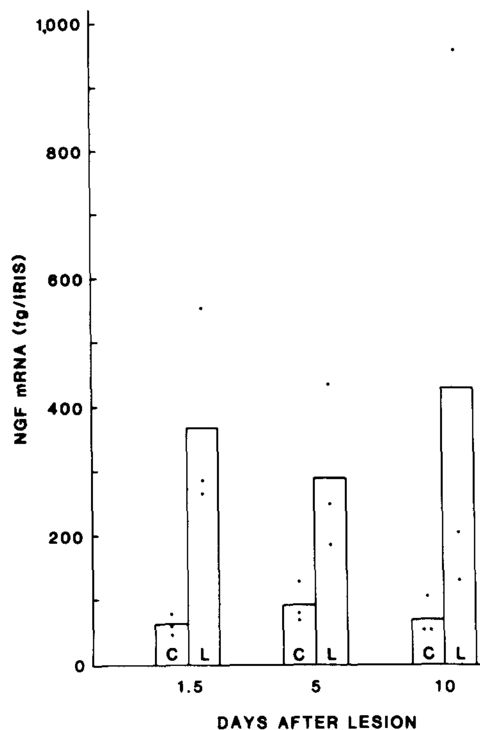

Figure 10. Content of NGF mRNA in irises after complete retrobulbar denervation. The content of NGF mRNA in individual irises at various times after the complete severance of all nerves leading to the eye. Bars are the means of the individual determination shown by the dots. *C* is the iris from the control, unoperated side of the animal. *L* is the iris which was denervated. There is a statistically significant increase in NGF mRNA in the operated iris at all times after surgery ($P = 0.05$, Mann-Whitney U test).

Assay for NGF mRNA

The assay used appears to detect authentic NGF mRNA for the following reasons. First, the hybridization and washing conditions were very stringent. Second, the major bands of RNA that hybridized under these conditions co-migrated with authentic NGF mRNA from male mouse submaxillary gland. Finally, under conditions of similar stringency, Southern blots of genomic DNA from the rat showed a single hybridizing band, implying that there is only one gene capable of hybridizing under these conditions (Shelton and Reichardt, 1984). Although we have not used pure NGF mRNA to calibrate this assay, the values obtained for mouse submaxillary gland mRNA agree closely with estimates obtained from the frequency of occurrence of NGF mRNA in a submaxillary gland cDNA library (Scott et al., 1983) and with the relative rate of synthesis of the protein in the same gland (Berger and Shooter, 1978). The standard curves obtained by densitometry using serial dilutions of RNA are linear over at least an order of magnitude for each autoradiogram (Fig. 2), and values were always determined from exposure times which yielded signals within this range. Therefore, the assay should give reliable estimates of the relative amounts of NGF mRNA in samples of iris RNA.

The identity of the band of hybridizing RNA that migrates equivalent to 1.7 kb is not known. It has only been observed in tissues from the rat, and not those from mouse, rabbit, or dog (Shelton and Reichardt, 1984). The results of the Southern blot argue that this RNA is also a transcription product of the NGF gene. Because the 1.7-kb and 1.3-kb transcripts appear to be coordinately regulated in explanted irises (Fig. 3), it seems unlikely that these transcripts are initiated at different promoters. Instead, it seems more likely that the two transcripts are generated by selective processing of the same primary transcript. Although the rat gene has not been characterized in detail, the human gene contains several alternative polyadenylation signals downstream of the normally used polyadenylation signal (Ullrich et al., 1983). The presence of such downstream alternate polyadenylation sites has also been described in the gene encoding human copper-zinc superoxide dismutase. These alternate sites are used in a percentage of cases, yielding a population of transcripts which are several hundred bases larger than normal (Sherman et al., 1984). The presence and use of multiple polyadenylation signals has also been shown to be the basis of some of the multiple transcripts of the *Drosophila* tropomyosin gene (Boardman et al., 1985). It seems likely that a similar process might explain the existence of the 1.7-kb band hybridizing with the NGF probe, but verification of this will require further characterization of the larger RNA.

Irises Cultured In Vitro

We have found that culturing the iris in vitro causes a rapid and large increase in the level of NGF mRNA. Although the level of specific message rises by an order of magnitude, the regional distribution of that RNA remains unchanged (Fig. 4). This is consistent with, but does not prove, that the same cells which contain NGF mRNA in vivo are the cells which contain the much larger amounts of NGF mRNA seen in vitro. This increase does not reflect a general increase in total RNA level of the tissue, nor does this increase seem to be caused by any special culture medium or serum factors, as it occurs in a variety of different media (Fig. 8). There is evidence which suggests that this induction is actually due to the absence of some activity which is normally present in aqueous humor, but this putative activity has proven to be either highly variable or very unstable, and therefore difficult to characterize. The frequent observation that culture in the presence of aqueous humor does not cause the induction of NGF mRNA suggests that denervation is not sufficient to increase the level of NGF mRNA in the iris. This is in agreement with results obtained when the iris is deprived of sympathetic or sensory innervation in vivo (Figs. 5–7). Further, the difference in response to denervation in vivo and growth in vitro raises serious questions about the use of the explant procedure as a model for denervation.

We also show that the large increase observed upon explant can be mimicked in the fully innervated iris in situ. A variety of insults to the anterior chamber results in a large increase in NGF mRNA (Fig. 9). This shows that denervation is not a necessary prerequisite for the induction of NGF mRNA.

The mechanism of this increase is not known. Although care was taken not to injure the iris, it is possible that this is due to some type of wound response, either because of undetectable damage to the iris itself or because of the injury to the cornea. Alternatively, the induction might be caused by the release and subsequent rapid replacement of the aqueous humor, or the breakdown in the blood-aqueous barrier which occurs in response to a corneal puncture (Raviola, 1974). One

consistent observation is that this increase occurs whenever the media bathing the iris is changed from the physiologically normal aqueous humor. Whatever the cause of the increase seen after these implantations, it may help to explain the sympathetic hyperinnervation of the iris which has been reported to occur after transplantation of tissue into the anterior chamber (Olson and Malmfors, 1970). This transient effect does not depend on the type of tissue transplanted.

The increase in NGF mRNA content obtained upon explant of the rat iris is very rapid and appears to be initiated immediately (Fig. 3). Any lag period, if it exists, must be much less than 1 h. This is in contrast to the rise in NGF immunoreactivity seen after explant, which only occurs after a lag time of ~4 h (Barth et al., 1984). This difference presumably reflects the time required for transport of RNA from nucleus to cytoplasm, translation, and posttranslational processing to occur.

In Vivo Denervation

In contrast to the increase in NGF mRNA seen in irises placed in explant culture, irises deprived of sympathetic or sensory innervation in vivo did not show an increase in their content of NGF mRNA (Figs. 5–7). Many of these denervation experiments have actually been repeated many more times than are reported. Technical problems in these experiments made it impossible to obtain complete time courses on age and sex-matched animals and so these have not been included. To summarize, our experiments have never detected a significant increase in NGF mRNA due to sympathetic or sensory denervation.

Despite our results, though, accumulations of NGF protein or bioactivity in the iris have been consistently seen in other studies following each of the sympathetic and sensory denervation techniques used in this paper. Surgically denervated irises have been examined using an NGF bioassay and a radioimmune assay, and although the assays used are only semi-quantitative, the increase in content appears to be at least an order of magnitude (Ebendal et al., 1980; 1983). Irises from animals treated with 6-OHDA have been shown to undergo a two- to fourfold increase in their content of NGF antigen with a two-site enzyme-linked immunosorbent assay (Korsching and Thoenen, 1985). At each of the times at which accumulations of NGF in the iris have been seen by others, we have searched for and failed to find significant increases in NGF mRNA levels.

Since no increase in NGF mRNA was observed after denervation, it was important to be certain of the efficacy of the denervation procedures. The surgical procedure for removing the sympathetic innervation is very simple and straightforward, as is the injection of 6-OHDA. The trigeminal nerve lesion, although more difficult, can be done under direct visual observation, and the ablation can be examined carefully after sacrifice. All of the procedures have easily visible physiological symptoms, which were verified in each animal, confirming the lesion.

Although there is not an increase in NGF mRNA in response to denervation, it is possible that there is an increase in synthesis of NGF, but that the regulation is occurring at the level of translational efficiency. Alternatively, there might be an increase in synthesis which reflects an increase in the efficiency of the processing of the initial translation product. It is also possible that there is no increase in the synthesis rate of NGF and that its accumulation after denervation is due to a decrease in breakdown. These hypotheses cannot be ruled out without further experimentation.

All of the above hypotheses involve the assumption that the observed change in NGF content is due to a change in some metabolic process of the iris in response to denervation. It is known that denervation can induce such changes in the iris, for example, receptor supersensitivity (Bourgon et al., 1978). However, it is possible that the change in NGF content is not due to denervation caused changes in the target tissue, but instead is a direct consequence of the loss of the nerve plexus on the equilibrium between production and removal of NGF. Loss of the innervation of the iris obviously removes the neuronal retrograde transport system, which is known to take up and remove NGF with high affinity and capacity (Dumas et al., 1979).

For the removal of the retrograde transport system alone to cause an increase in the content of NGF, the transport system must be important in establishing the level of NGF in the normal target. If this is the case, at least a portion of the NGF in a tissue must be extracellular, so that it would be accessible to the nerve. It is not difficult to imagine that NGF, a highly basic and notoriously 'sticky' protein, in the presence of the predominantly acidic extracellular matrix, could achieve a relatively high extracellular concentration. In fact, it has recently been demonstrated that NGF can be adsorbed in tracks onto polyanionic substrates to which sufficient factor remains attached for several days to promote selective neurite outgrowth along these tracks (Gundersen, 1985). There is evidence that some NGF is extracellular in normal tissue, as Korsching and Thoenen (1983) have noted that brief saline rinses of tissue, and especially the iris, lead to a loss of NGF. Thus, at this time, it seems that removal of the retrograde transport system can explain the build up of NGF after denervation without postulating denervation-induced changes in the metabolic processes of the iris tissue. Consistent with this hypothesis, it has recently been shown that treatment with colchicine leads to an increase in the NGF content of the iris (Korsching and Thoenen, 1985). This explanation predicts that the increase in NGF seen after denervation will be in an extracellular compartment.

Retrobulbar Lesions

The only denervation procedure which resulted in a significant increase in NGF mRNA was the complete retrobulbar section of all nerves entering the eye (Fig. 10). This caused a traumatic response in the eye, similar to that described after lesions of the trigeminal nerve (Moses and Feldman, 1969). In these experiments, the response following the retrobulbar procedure was always greater than any observed after the trigeminal lesions. Although it is possible that the increase in NGF mRNA was due to the denervation per se, this explanation seems unlikely. It seems more probable that the trauma following the surgical procedure led to the increase in NGF mRNA. The much less damaging procedures of cannula placement or anterior chamber injection cause a large, reproducible increase in NGF mRNA without denervation (Fig. 9). Furthermore, the other denervation procedures used, albeit giving less extensive denervations, give no increase in NGF mRNA (Figs. 5–7). The retrobulbar procedure does remove

input to the eye that is not removed by the sympathetic and sensory denervation procedures. Specifically, the parasympathetic innervation of the iris from the ciliary ganglion is eliminated. It is possible that other, unknown projections are also differentially affected by the different procedures and their removal contributes to the observed increase in NGF mRNA. Whatever the mechanism behind this increase, the results in this paper show that the increases in NGF content observed after sympathetic and sensory denervation of the iris (Ebendal et al., 1980, 1983; Korsching and Thoenen, 1985) are not due to an increase in the level of NGF mRNA.

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References

Barth, E. M., S. Korsching, and H. Thoenen. 1984. Regulation of nerve growth factor synthesis and release in organ cultures of rat iris. *J. Cell Biol.* 99:839–843.

Berger, E. A., and E. M. Shooter. 1978. Biosynthesis of *beta*-NGF in mouse submaxillary glands. *J. Biol. Chem.* 253:804–810.

Boardman, M., G. S. Basi, and R. V. Storti. 1985. Multiple polyadenylation sites in a *Drosophila* tropomyosin gene are used to generate functional mRNAs. *Nucleic Acids Res.* 13:1763–1776.

Bourgon, P., S. F. J. Pilley, and H. S. Thompson. 1978. Cholinergic supersensitivity of the iris sphincter in Adie's tonic pupil. *Am. J. Ophthalmol.* 85:373–377.

Campenot, R. B. 1977. Local control of neurite development by nerve growth factor. *Proc. Natl. Acad. Sci. USA.* 74:4516–4519.

Cheley, S., and R. Anderson. 1984. A reproducible microanalytical method for the detection of specific RNA sequences by dot-blot hybridization. *Anal. Biochem.* 137:15–19.

Dumas, M., M. E. Schwab, and H. Thoenen. 1979. Retrograde axonal transport of specific macromolecules as a tool for characterizing nerve terminal membranes. *J. Neurobiol.* 10:179–197.

Ebendal, T., L. Olson, and A. Seiger. 1983. The level of nerve growth factor (NGF) as a function of innervation. *Exp. Cell Res.* 148:311–317.

Ebendal, T., L. Olson, A. Seiger, and K. Hedlund. 1980. Nerve growth factors in the rat iris. *Nature (Lond.).* 286:25–28.

Gorin, P. D., and E. M. Johnson. 1979. Experimental autoimmune model of nerve growth factor deprivation: effects on developing peripheral sympathetic and sensory neurons. *Proc. Natl. Acad. Sci. USA.* 76:5382–5386.

Gundersen, R. W. 1985. Sensory neurite growth cone guidance by substrate adsorbed nerve growth factor. *J. Neurosci. Res.* 13:199–212.

Harper, G. P., A. M. Al-Safar, F. L. Pearce, and C. A. Vernon. 1980. The production of nerve growth factor in vitro by tissues of the mouse, rat, and embryonic chick. *Dev. Biol.* 77:379–390.

Hendry, I. A. 1975. The response of adrenergic neurones to axotomy and nerve growth factor. *Brain Res.* 94:87–97.

Hendry, I. A. 1977. The effect of the retrograde axonal transport of nerve growth factor on the morphology of adrenergic neurones. *Brain Res.* 134:213–223.

Hendry, I. A., and J. Campbell. 1976. Morphometric analysis of rat superior cervical ganglion after axotomy and nerve growth factor treatment. *J. Neurocytol.* 5:351–360.

Korsching, S., and H. Thoenen. 1983. Nerve growth factor in sympathetic ganglia and corresponding target organs of the rat: correlation with density of sympathetic innervation. *Proc. Natl. Acad. Sci. USA.* 80:3513–3516.

Korsching, S., and H. Thoenen. 1985. Treatment with 6-hydroxydopamine and colchicine decreases nerve growth factor levels in sympathetic ganglia and increase them in the corresponding target tissues. *J. Neurosci.* 5:1058–1061.

Lehrach, H., D. Diamond, J. M. Wozney, and H. Boedtker. 1977. RNA molecular weight determinations by gel electrophoresis under denaturing conditions, a critical reevaluation. *Biochemistry.* 16:4743–4751.

Levi-Montalcini, R., and B. Booker. 1960. Destruction of the sympathetic ganglia in mammals by an antiserum to a nerve-growth protein. *Proc. Natl. Acad. Sci. USA.* 46:384–391.

Levi-Montalcini, R., H. Meyer, and V. Hamburger. 1954. *In vitro* experiments on the effects of mouse sarcomas 180 and 37 on the spinal and sympathetic ganglia of the chick embryo. *Cancer Res.* 14:49–57.

Malmfors, T., and O. Nilsson. 1964. Parasympathetic past-ganglionic denervation of the iris and the parotid gland in the rat. *Acta Morphol. Neerl. Scand.* 6:81–85.

Moses, R. A., and M. E. Feldman. 1969. Ocular lesion following fifth nerve injury in rats. *Am. J. Ophthalmol.* 68:1082–1088.

Olson, L., and T. Malmfors. 1970. Growth characteristics of adrenergic nerves in the adult rat. *Acta Physiol. Scand. Suppl.* 348:1–112.

Oppenheim, R. W. 1981. Neuronal cell death and some related regressive phenomena during neurogenesis: a selective historical review and progress report. *In* Studies in Developmental Neurobiology. W. M. Cowan, editor. Oxford Press, New York. 74–133.

Raviola, G. 1974. Effects of paracentesis on the blood-aqueous barrier: an electron microscope study on *Macaca mulatta* using horseradish peroxidase as a tracer. *Invest. Ophthalmol.* 13:828–858.

Scott, J., M. Selby, M. Urdea, M. Quiroga, G. Bell, and W. J. Rutter. 1983. Isolation and nucleotide sequence of a cDNA encoding the precursor of mouse NGF. *Nature (Lond.).* 302:538–540.

Sears, M. L. 1981. The aqueous. *In* Adler's Physiology of the Eye. R. A. Moses, editor. C. V. Mosby Co., St. Louis. 204–226.

Shelton, D. L., and L. F. Reichardt. 1984. Expression of the *beta*-nerve growth factor gene correlates with the density of sympathetic innervation in effector organs. *Proc. Natl. Acad. Sci. USA.* 81:7951–7955.

Sherman, L., D. Levanon, J. Lieman-Hurwitz, N. Dafni, and Y. Groner. 1984. Human Cu/Zn superoxide dismutase gene: molecular characterization of its two mRNA species. *Nucleic Acids Res.* 12:9349–9365.

Thoenen, H., and Y.-A. Barde. 1980. Physiology of nerve growth factor. *Physiol. Rev.* 60:1284–1335.

Thoenen, H., M. Schwab, and U. Otten. 1978. Nerve growth factor as a mediator of information between effector organs and innervating neurons. *Symp. Soc. Dev. Biol.* 35:101–118.

Ueda, N., I. Muramatsu, H. Hayashi, and M. Fujiwara. 1982. Trigeminal nerve: the possible origin of substance P-ergic response in isolated rabbit iris sphincter muscle. *Life Sci.* 31:369–375.

Ullrich, A., A. Gray, C. Berman, and T. J. Dull. 1983. Human *beta*-NGF gene sequence highly homologous to that of mouse. *Nature (Lond.).* 303:821–825.

Yankner, B. A., and E. M. Shooter. 1982. The biology and mechanism of action of nerve growth factor. *Annu. Rev. Biochem.* 51:845–868.