MUSCARINIC RECEPTOR BINDING AND OXIDATIVE ENZYME ACTIVITIES IN THE ADULT RAT SUPERIOR CERVICAL GANGLION: EFFECTS OF 6-HYDROXYDOPAMINE AND NERVE GROWTH FACTOR¹

ANTHONY M. DOMBROWSKI, ANN A. JERKINS, AND FREDERICK C. KAUFFMAN²

*Department of Pharmacology and Experimental Therapeutics, University of Maryland School of Medicine,
Baltimore, Maryland 21201*

Received December 13, 1982; Revised March 17, 1983; Accepted April 28, 1983

Abstract

Administration of 6-hydroxydopamine to adult rats results in changes in the superior cervical ganglion similar to those noted after axotomy; namely, a decrease in muscarinic receptor binding and increases in activities of the oxidative enzymes of the pentose phosphate pathway. These changes were either prevented or attenuated markedly by the systemic administration of nerve growth factor. Administration of nerve growth factor alone did not significantly increase *N*-methylscopolamine binding in the ganglion or reduce the activities of the oxidative enzymes. Explants of the ganglion maintained in serum-free medium over a period of 3 days demonstrated increases in oxidative enzyme activity and a decrease in *N*-methylscopolamine binding. Addition of 20 nM nerve growth factor to the culture medium prevented the decline in *N*-methylscopolamine binding in ganglion explants. The increases in oxidative enzyme activities were unaltered. Addition of high amounts of nerve growth factor, 200 nM, resulted in a significant increase in tyrosine hydroxylase activity but no further increase in *N*-methylscopolamine binding in ganglion explants. Glucocorticoids added to the culture medium did not affect the muscarinic binding or enzyme activities. Thus, decreases in muscarinic binding activity which occur in the superior cervical ganglion after axotomy or 6-hydroxydopamine treatment may be explained by a loss of nerve growth factor supplied to the ganglion. Increases in the oxidative enzymes of the pentose phosphate pathway that occur in the ganglion after axonal injury appear to involve additional factors.

The capacity of nerve growth factor (NGF) to stimulate neurite fiber outgrowth from sensory and sympathetic neurons and clones of rat pheochromocytoma cells (Levi-Montalcini and Angeletti, 1968; Thoenen and Barde, 1980) has spurred the investigation of the mechanism of action of this hormone-like peptide in the hope of better understanding the regulation of neuronal growth and regeneration. The possibility that NGF or related substances influence biochemical changes in nerve cell bodies during the retrograde response to axonal injury is suggested by a number of studies using the

superior cervical ganglion as a model. Uptake of both exogenous and endogenous NGF at nerve terminals and retrograde transport to the superior cervical ganglion has been established (Hendry et al., 1974a, b; Johnson et al., 1978; Palmatier et al., 1982). Although sympathetic neurons may not have an absolute requirement for this peptide after 3 weeks of postnatal life (Hendry, 1975), NGF reaching the ganglion via retrograde transport has been strongly implicated in the induction of tyrosine hydroxylase and the production of a general hypertrophic response (Hendry, 1977). Increased utilization of glucose, as indexed by 2-deoxyglucose uptake, is one of the earliest biochemical changes noted in axotomized neural tissue, and this change is prevented by inhibition of retrograde axoplasmic flow (Singer et al., 1982).

In addition to affecting the biochemical properties of the ganglion, NGF also has significant actions on the response of this tissue to neurotransmitters. Halstead and Larrabee (1972) demonstrated that a single dose of NGF antiserum caused a significant reduction in the

¹ Gratitude is expressed to Mrs. Margaret Shimkaveg for her excellent help in preparing the manuscript and to Drs. David Burt and Martin Larrabee for their critical comments. This work was supported in part by United States Public Health Service Grant HD-16596 and the United States Army Medical Research Development Command.

² To whom correspondence should be addressed, at Department of Pharmacology and Experimental Therapeutics, University of Maryland School of Medicine, 660 W. Redwood Street, Baltimore, MD 21201.

postganglionic response to acetylcholine in superior cervical ganglia of neonatal mice as early as 48 hr after injection. Using guinea pig superior cervical ganglia, Purves (1975) and Purves and Nja (1976) demonstrated that the depression of the fast EPSP amplitude following surgical postganglionic axotomy was reversed with locally applied NGF. Axotomy or treatment with anti-NGF antibodies was also shown to produce a loss of synapses and presynaptic fibers in rat and guinea pig superior cervical ganglia (Matthews and Nelson, 1975; Nja and Purves, 1978). Thus, changes in ganglionic transmission that occur with axotomy may be mediated through decreases in the number of cholinergic receptors as well as the loss of presynaptic elements.

The present series of experiments was performed to determine whether selected biochemical changes brought about by axonal injury are influenced by NGF. Since surgical axotomy produces a fall in muscarinic ligand binding that correlates temporally with increases in 6-phosphogluconate dehydrogenase (EC 1.1.1.44) (Siniropi et al., 1979) and glucose-6-phosphate dehydrogenase activity (Harkonen and Kauffman, 1974), we examined the influence of axonal injury and NGF on the activities of the oxidative enzymes of the pentose phosphate pathway and muscarinic receptor content in the superior cervical ganglion. 6-Hydroxydopamine treatment of adult rats was used to destroy nerve terminals of postganglionic fibers and to reduce retrograde transport of NGF to the ganglion *in vivo* (Johnson et al., 1979). Biochemical alterations produced by chemical axotomy with 6-hydroxydopamine *in vivo* were compared in the presence and absence of NGF administered systemically. The direct influence of NGF on muscarinic binding and enzyme activities was also examined on explants of adult superior cervical ganglion maintained in serum-free medium. The results suggest that whereas amounts of muscarinic binding in the ganglion are influenced by NGF, regulation of the activities of the two pentose phosphate pathway enzymes may involve additional factors.

Materials and Methods

Preparation of tissue. Adult male Sprague-Dawley rats (Charles River Breeding Laboratory, Wilmington, MA) weighing 125 to 200 gm were used in all experiments. Animals were anesthetized with chloral hydrate (400 mg/kg) and superior cervical ganglia were decentralized as described previously (Burt, 1978). Explant cultures of superior cervical ganglia were prepared in serum-free media according to the method reported earlier (Dombrowski and Kauffman, 1981) except that cultures were maintained in plastic culture wells (16 mm diameter, clusters of 24; Costar, Cambridge, MA) and 7 S NGF was used in all experiments. Only the four central sections from each ganglion were explanted for culture.

For biochemical analyses, animals were killed by decapitation and the ganglia were removed, desheathed in ice-cold saline, frozen on dry ice, and stored at -80°C . Explants of ganglia maintained *in vitro*, except those used for microscopic histochemistry, were rinsed in 50 ml of ice-cold saline, blotted, and frozen and stored at -80°C . Freeze-thawing had no effect on any of the biochemical determinations.

6-Hydroxydopamine and NGF treatment. The solutions of 6-hydroxydopamine (30 mg/ml) were prepared in cold saline containing 0.2 mg/ml of ascorbate directly before use. Injections of the drug (100 mg/kg) were either intraperitoneal or intravenous. The two routes of administration are equally effective in reducing retrograde transport of NGF (Johnson et al., 1979).

The 7 S complex of NGF was used in all studies and was prepared according to the method of Burton et al. (1978). The 7 S complex, which eluted as a single well-defined peak (peak II) from the Sephadex G-100 column was pooled, concentrated using Millipore CX-30 ultrafiltration units, and stored in small aliquots at -80°C . Typically, 350 to 400 μg of protein were obtained in this fraction per mouse. Analysis of the pooled fraction by isoelectric focusing (O'Farrell, 1975) in 6.8% polyacrylamide gels having a pH 4 to 8.5 gradient indicated the presence of only three bands that were identical to the subunits of the 7 S complex (Burton et al., 1978). Injections of NGF (2.5 to 3.8 mg/kg, s.c.) were in 0.025 M sodium phosphate, pH 6.8.

Biochemical analysis. Whole ganglia used for the determination of enzyme and binding activities were homogenized in 75 μl of 0.04 M Tris-HCl, pH 7.4, containing 100 μM NADP⁺. Explants (2) used for enzyme determinations were homogenized in 30 μl of the same medium. Explants (4) used for binding assays were homogenized in 45 μl of 0.02 M sodium phosphate, pH 7.4.

Tyrosine hydroxylase activity was measured using the assay system described by Coyle (1972). L-[side chain-2,3- ^3H]Tyrosine (Amersham; 15 Ci/mmol) was purified as described except the effluent from the alumina column was acidified with an equal volume of 2.0 N HCl, lyophilized at -40°C , resuspended in ethanol (about 0.14 mCi/ μl), and stored at -80°C . Unlabeled L-tyrosine was standardized spectrophotometrically (ϵ 279.5 nm = 1340 in 0.1 N HCl). A 5- μl aliquot of the explant homogenate or a 7- μl aliquot of whole ganglion homogenate was added to 20 or 43 μl , respectively, of 0.05 M Tris-HCl, pH 7.4, containing 0.02% Triton X. Incubations for tyrosine hydroxylase were started with the addition of an equal volume of reagent A containing: 0.04 M KH_2PO_4 adjusted to pH 5.5, 100 μM L-tyrosine, 0.5 $\mu\text{g}/\mu\text{l}$ of catalase, 40 $\mu\text{l}/\text{ml}$ of dihydropteridine reductase, 1.0 mM NADH⁺, 1 mM 6-methyltetrahydropteridine, 1 mM FeSO_4 , and 25,000 cpm [^3H]tyrosine/ μl . Reactions were carried out at 37°C , terminated with 500 μl of 0.4 M HClO_4 containing 6 μg of 3,4-dihydroxyphenylalanine (L-DOPA), and centrifuged at $3000 \times g$ for 5 min. A 500- μl aliquot was added to 6 ml of reagent B containing: 1.0 M Tris, 0.04 M KH_2PO_4 , 1% EDTA, and 15 mg of sodium bisulfite adjusted to pH 8.6 with HCl. Sodium bisulfite (15 mg) was added to the reagent directly before use. This mixture was immediately applied to 200 mg of acidified alumina in a column rinsed with 6 ml of reagent B. After washing twice with 10 ml of deionized water, the adsorbed L-DOPA was eluted with 3 ml of 0.3 N HCl. Hydromix (15 ml; Yorktown Research, Hackensack, NJ) was added to the samples which were then assayed for radioactivity in a liquid scintillation counter with a counting efficiency of about 33%. The assay was linear with time for at least 30 min with protein ranging up to 0.75 $\mu\text{g}/\mu\text{l}$ of reaction mixture. The blanks contained less

than 1% of the total radioactivity present and enzyme activities were typically 5 to 6 times the assay blanks.

Muscarinic receptor binding was measured with N -[^3H]methylscopolamine (New England Nuclear, 83.5 Ci/mmol) as the ligand (Birdsall et al., 1978). A 7- μl aliquot of the ganglion or explant homogenate was added to 45 μl of 0.02 M sodium phosphate, pH 7.4, containing 1.5 nM N -[^3H]methylscopolamine and incubated at 37°C for 30 min. Nonspecific binding was determined in the presence of 1.0 μM atropine. The reaction mixture tubes were rinsed three times with 2.5 ml of ice-cold saline onto 2.4-cm Whatman GF/B filters at negative pressure. The filters were washed with three 2.5-ml rinses of cold saline, drained of excess fluid, and counted in 4 ml of Hydromix. For homogenates of whole ganglia, triplicate measurements of total and duplicate measurements of nonspecific binding were made. Duplicate measurements of both total and nonspecific binding were done for homogenates of pooled explants. Total binding in control samples represented less than 5% of the available radioactivity. In assays of whole ganglia, the total binding was 3 times the nonspecific background, whereas in assays of pooled explants, the total binding was twice the nonspecific binding. Doubling the concentration of ligand did not increase specific binding. A preliminary study of N -[^3H]methylscopolamine binding in ganglion homogenates indicated a dissociation constant of 0.27 nM, which is comparable to the value reported for this ligand in brain homogenates and slices (Birdsall et al., 1978; Wamsley et al., 1980). Counting efficiency averaged 44%.

Glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase were assayed as described previously (Harkonen and Kauffman, 1974) using 1 μl of either ganglion or explant homogenates and 50 μl of the appropriate reagent. Protein was determined by the method of Lowry et al (1951).

Enzyme histochemistry. Glucose-6-phosphate and 6-phosphogluconate dehydrogenase activities in fresh frozen sections of ganglion explants were localized by the reduction of nitroblue tetrazolium at alkaline pH using the method described previously by Harkonen and Kauffman (1974) for whole ganglia. The explants were mounted on a cryostat chuck in brain paste stained with blue dextran and frozen in an acetone-dry ice bath. The 10- μm transverse sections were incubated in reaction mixtures buffered with 0.05 M 2-amino-2-methyl-1-propanol hydrochloride, pH 8.8.

Reagents. 6-Hydroxydopamine as the HCl or HBr salt and 6-methyltetrahydropteridine were obtained from Sigma Chemical Co., St. Louis, MO. Catalase was purchased from Boehringer Mannheim. N -[^3H]Methylscopolamine was a gift from the laboratory of Dr. M. Kuhar, and the dihydropteridine reductase was generously provided by Dr. J. Coyle.

Results

Effects of 6-hydroxydopamine and NGF on muscarinic receptor concentration and enzyme activities in vivo. The treatment of adult male rats with three consecutive daily injections of 6-hydroxydopamine (100 mg/kg) resulted in a 60% decrease in the specific binding of N -[^3H]methylscopolamine in the superior cervical ganglion at 24 hr after the last injection (Table I). Increases in the

specific activities of both glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase of about 28% also occurred over the same time period (Table II). These results are comparable to those noted after surgical transection of the postganglionic fibers of the superior cervical ganglion (Sinicropi et al., 1979).

The administration of three daily injections of NGF (3.8 mg/kg) along with 6-hydroxydopamine diminished the decrease in muscarinic binding seen with 6-hydroxydopamine alone (Table I). Furthermore, the induction of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase seen with chemical axotomy was prevented by NGF (Table II); however, activities of both oxidative enzymes of the pentose phosphate pathway in the superior cervical ganglion remained unchanged following treatment with NGF alone. The activity of glucose-6-phosphate dehydrogenase was significantly lower in ganglia of animals that had received 6-hydroxydopa-

TABLE I

The effect of 6-hydroxydopamine and NGF on N -[^3H]methylscopolamine binding and protein in vivo

Values are the mean $\pm$ SEM of the number of ganglia indicated in parentheses. Experimental animals were treated for 3 consecutive days with either 6-hydroxydopamine, NGF, or both. Control animals were injected with saline containing ascorbate, phosphate buffer, or both, respectively. The Student's t test was used for comparisons.

Treatment	Specific N -[^3H]- Methylscopolamine Binding	Protein
	<i>fmol/mg protein</i>	<i>$\mu\text{g/ganglion}$</i>
Control	171 $\pm$ 10 (25)	102 $\pm$ 4 (29)
6-Hydroxydopamine, 100 mg/kg	65.2 $\pm$ 7.1 (10) ^a	121 $\pm$ 9 (13) ^b
6-Hydroxydopamine, 100 mg/kg, and NGF, 3.8 mg/kg	112 $\pm$ 10 (11) ^{c,d}	98.2 $\pm$ 5.8 (11)
NGF, 2.5 mg/kg	186 $\pm$ 14 (10)	88.4 $\pm$ 3.4 (10)

^a $p < 0.001$, compared to controls.

^b $p < 0.05$, compared to controls.

^c $p < 0.01$, compared to controls.

^d $p < 0.01$, compared to 6-hydroxydopamine treated.

TABLE II

The effect of 6-hydroxydopamine and NGF on the specific activities of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase

Values are the mean $\pm$ SEM of the number of ganglia indicated in parentheses. Experimental animals were treated for 3 consecutive days with either 6-hydroxydopamine, NGF, or both. Controls were injected with vehicle alone. Analysis of variance was used to compare differences between groups.

Treatment	Glucose-6-phosphate Dehydrogenase	6-Phosphogluconate Dehydrogenase
	<i>$\mu\text{mol/mg protein/hr}$</i>	
Control	2.32 $\pm$ 0.17 (19)	2.62 $\pm$ 0.13 (17)
6-Hydroxydopamine, 100 mg/kg	2.97 $\pm$ 0.15 (10) ^a	3.48 $\pm$ 0.22 (13) ^b
6-Hydroxydopamine, 100 mg/kg, and NGF, 2.5 mg/kg	2.41 $\pm$ 0.19 (11) ^c	2.83 $\pm$ 0.17 (6)
NGF, 2.5 mg/kg	2.37 $\pm$ 0.14 (6)	2.92 $\pm$ 0.18 (6)

^a $p = 0.014$ compared to controls.

^b $p = 0.002$ compared to controls.

^c $p = 0.04$ compared to 6-hydroxydopamine.

Figure 1. The effect of NGF on enzyme activities and muscarinic ligand binding in explants of the superior cervical ganglion. Each point represents the mean $\pm$ SEM of three to five paired samples. Explants taken from the same ganglia as those cultured under the various conditions served as the matched controls. The tissue was cultured for 3 days. All values for glucose-6-phosphate (G-6-P) and 6-phosphogluconate (6-PG) dehydrogenase activity represent statistically significant changes from fresh control values noted in tissue. A matched pair *t* test was used for comparison of difference from control values. Data obtained in enzyme and binding

mine plus NFG than in ganglia from animals treated with 6-hydroxydopamine alone. Administration of NGF to animals treated with 6-hydroxydopamine also resulted in a marginally significant ($p = 0.06$) decrease in the activity of 6-phosphogluconate dehydrogenase.

Although there was a tendency ($0.05 < p < 0.1$) for NGF to reduce the increase in protein produced by 6-hydroxydopamine (Table I), the difference in muscarinic binding and enzyme activities between the two groups cannot be explained by the slight differences in ganglionic protein. Administration of three daily injections of a dose of NGF (2.5 mg/kg) alone had no effect on muscarinic binding in the superior cervical ganglion.

Effects of NGF on muscarinic receptors and enzyme activities *in vitro*. Changes in muscarinic receptor binding noted *in vivo* also occurred in explants of the superior cervical ganglion maintained in a serum-free culture medium for 3 days (Fig. 1). In the absence of added NGF or in the presence of NGF at concentrations less than 10 nM, muscarinic receptor binding decreased to values that averaged only 57% of that present prior to culture. Addition of higher concentrations of NGF (20 and 200 nM) prevented the decline in muscarinic binding in cultured tissue; i.e., muscarinic binding in these explants was the same as that observed in fresh explants. NGF had no effect on the total protein content of explants maintained *in vitro* (Table III).

Activities of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase increased 200 and 150%, respectively, during the first 3 days in culture in the absence of added NGF, while tyrosine hydroxylase decreased slightly but significantly (Fig. 1). Addition of NGF to the culture medium increased the activity of tyrosine hydroxylase and at 200 nM elevated the activity of this enzyme above control. In contrast, activities of the two oxidative enzymes of the pentose phosphate pathway were not affected by the addition of NGF to the culture medium.

The larger increases in activities of the two oxidative enzymes noted *in vitro* compared to changes observed in axotomized ganglia *in vivo* (Table II) may be explained, in part, by the influence of decentralization. Removal of presynaptic inputs to the ganglia by decentralization results in a significant enhancement of the induction of 6-phosphogluconate dehydrogenase produced by axotomy (Sinicropi et al., 1979). Decentralization alone has no effect on the activities of these enzymes, protein content, wet weight, or muscarinic binding in the ganglia (Burt, 1978; Sinicropi et al., 1979).

Localization of enzyme activities *in vitro*. Both oxidative enzymes were found to be localized mainly in the neurons of the explants maintained *in vitro* for 3 days. Figure 2 shows that deposition of reduced nitroblue tetrazolium resulting from glucose-6-phosphate dehydrogenase activity occurs mainly over nerve cell bodies with

TABLE III

The effect of NGF on protein content of explants after 3 days in vitro

Values are the mean $\pm$ SEM of the number of paired samples shown in parentheses. Explants taken from the same ganglia as those cultured under various conditions served as matched controls. Protein content of pooled (4) explant homogenates were compared. The average micrograms of protein per explant was 13.5 ± 0.9 (9) in the control group.

NGF Concentration	Protein
	% of control
None added	87.1 ± 11.7 (4)
<10 nM	92.0 ± 6.8 (11)
>10 nM	97.3 ± 9.5 (6)

much less staining of neuropil. Similar results were obtained for 6-phosphogluconate dehydrogenase.

Effects of glucocorticoids on muscarinic receptor and enzyme activities *in vitro*. It is known that NGF treatment elevates plasma glucocorticoids 7- to 8-fold in rats (Otten et al., 1979) and that glucocorticoids enhance the induction of tyrosine hydroxylase in rat superior cervical ganglia in organ culture (Otten and Thoenen, 1977). Thus, we examined the effects of these steroids on enzyme activities and muscarinic binding in explants maintained 3 days in culture.

Addition of corticosterone to the culture medium, at a concentration similar to that in plasma of NGF-treated rats ($0.57 \mu\text{M}$), did not prevent the induction of the two oxidative enzymes; i.e., activities were not significantly different from those noted in the presence of NGF alone. Activities of glucose-6-phosphate dehydrogenase in ganglion explants maintained in the presence of cortisone, NGF (200 nM) plus cortisone, or NGF alone were 4.33 ± 0.93 , 5.81 ± 1.23 , and $4.41 \pm 0.60 \mu\text{mol/mg}$ of protein/hr, respectively. Values for 6-phosphogluconate dehydrogenase in the three groups were 2.66 ± 0.21 , 3.67 ± 0.48 , and $3.43 \pm 0.50 \mu\text{mol/mg}$ of protein/hr, respectively. Addition of cortisol ($1.6 \mu\text{M}$), equivalent to 10 times the biological activity of corticosterone in plasma of NGF-treated rats, also failed to alter the induction of the oxidative enzyme in explants maintained in the NGF-containing medium. The specific binding of N -[^3H]methylscopolamine, which remains constant after 3 days in culture in the presence of 200 nM NGF, was also unaffected by the addition of the pharmacologic dose of cortisol.

Discussion

Changes noted in the rat superior cervical ganglion after 6-hydroxydopamine treatment closely resemble those seen after axotomy (Harkonen and Kauffman, 1974; Sinicropi et al., 1979). Although NGF is known to affect a number of biochemical properties of the nerve cell body after treatment with 6-hydroxydopamine, NGF does not prevent the destruction of the nerve terminals that occurs after administration of the drug (Levi-Mon-

assays of fresh explants were not significantly different from control values in assays of whole ganglia. The average activity of tyrosine hydroxylase in ganglia of saline-injected rats was 37.9 ± 1.5 (7) nmol/mg of protein/hr. Statistical differences for N -[^3H]methylscopolamine ($[^3\text{H}]/\text{NMS}$) and tyrosine hydroxylase from control values are indicated by asterisks: *, $p < 0.05$; **, $p < 0.01$.

Figure 2. Histochemical localization of glucose-6-phosphate dehydrogenase in explants of the superior cervical ganglion after 3 days in culture. Ganglion explants were maintained in medium containing 200 nM NGF. Note the relative lack of extraneuronal deposition of reduced tetrazolium. Magnification: A, $\times 130$; B, $\times 520$.

talcini et al., 1975). Destruction of nerve terminals by the drug is associated with a significant decrease in the retrograde transport of NGF to the superior cervical ganglion (Johnson et al., 1979). This may occur with NGF as well as other macromolecules and is apparently

dependent on the loss of specific binding sites in nerve endings that are necessary for their uptake and transport (Dumas et al., 1979). The finding that decreases in *N*-methylscopolamine binding that occur *in vivo* after exposure to 6-hydroxydopamine were markedly attenuated

by the administration of NGF suggested that these changes may be mediated by a loss of NGF supplied to the ganglion nerve cell bodies. The results of our studies *in vitro* give further support to this hypothesis.

Changes in muscarinic binding in the tissue elicited by NGF may involve interaction of NGF with more than a single class of receptors. Dumas et al. (1979) suggest a limited number of NGF receptors of two affinities at adrenergic nerve terminals *in vivo*. The work of Sutter et al. (1979), Olender and Stach (1980), and Landreth and Shooter (1980) on chick sympathetic and sensory neurons and pheochromocytoma cells in culture demonstrated that receptors for NGF have both high and low affinities for this peptide with dissociation constants on the order of 10 pM and 1 nM. Recently, Claude et al. (1982) described a single class of NGF receptors in dissociated neonatal rat sympathetic cell cultures. However, a second higher affinity class of receptors could not be ruled out. In our experiments *in vitro*, N -[^3H]methylscopolamine binding in the explants was only maintained in the presence of high concentrations of NGF, i.e., 20 or 200 nM. Good neurite outgrowth from the explants is achieved at 50 to 200 pM NGF. Thus, concentrations of NGF required for neurite outgrowth from the explants are considerably lower than concentrations needed to maintain muscarinic binding. Furthermore, tyrosine hydroxylase activity, which is known to increase in the whole ganglion cultured in the presence of high concentrations of NGF (Max et al., 1978), resembles muscarinic binding and was only elevated above control values with the highest concentration of NGF used, i.e., 200 nM. Earlier studies in adult mice also demonstrated that doses of NGF 3- to 4-fold greater than those used in our experiments over a 5-day period increased the content of protein and tyrosine hydroxylase activity in the ganglion *in vivo* (Thoenen et al., 1974). These data suggest that maintenance of binding sites as well as tyrosine hydroxylase in sympathetic neurons requires that a significant proportion of low affinity NGF receptor sites be occupied.

Recent studies employing PC12 cells maintained in a serum-containing medium also indicated that NGF increased muscarinic binding in this cell line *in vitro* (Jumblatt and Tischler, 1982). These data, along with our finding that NGF affects muscarinic binding in adult differentiated autonomic tissue both *in vivo* and *in vitro*, demonstrate that amounts of cholinergic receptor binding can be regulated by a substance unrelated structurally to acetylcholine. Results presented above suggesting that NGF increases muscarinic receptor content differ from those reported recently for vasoactive intestinal polypeptide, which appears to increase the affinity and rate of association of muscarinic ligands with receptors in cat submandibular salivary gland (Lundberg et al., 1982). Other factors influencing muscarinic receptor binding include cholinergic agonists and nonspecific physiological changes such as thyroidectomy and cell death induced by various lesions (for review see Westlind et al., 1981).

The influence of NGF on muscarinic binding may also involve an indirect action of this peptide at presynaptic sites in the superior cervical ganglion. Although NGF is not transferred transynaptically after retrograde transport (Schwab and Thoenen, 1977), exogenous NGF can

reverse the presynaptic degeneration seen following postsynaptic injury (Nja and Purves, 1978). Thoenen and Barde (1980) suggested that maintenance of the presynaptic terminal by NGF may occur via specific surface proteins on the postganglionic cells.

Although our earlier work had shown a coordinate and reciprocal action of surgical axotomy on muscarinic binding and 6-phosphogluconate dehydrogenase activity, the two oxidative enzymes of the pentose pathway examined failed to show a direct response to NGF *in vitro*. The results of the histochemical studies clearly indicate that the activities of these enzymes in the explants maintained in culture are primarily neuronal. The density of neurons as well as the pattern of staining is essentially the same as that observed in the intact ganglion *in vivo* (Harkonen and Kauffman, 1974). Thus, changes in enzyme activities and muscarinic binding observed *in vitro* probably do not reflect alterations in neuronal cell survival in the culture system used above. This conclusion is supported by previous findings indicating that the content of ATP (Dombrowski and Kauffman, 1981) and total DNA (Sinicropi et al., 1980) are essentially the same in explants of the ganglion maintained 7 days *in vitro* in the presence of 1 nM NGF as that noted in fresh ganglia.

Whereas injection of NGF and 6-hydroxydopamine concomitantly eliminated the induction of the oxidative enzymes seen with 6-hydroxydopamine alone *in vivo*, the addition of NGF to the medium or cultured explants did not reverse the increase in activities of these enzymes. Although NGF is known to elevate plasma glucocorticoids (Otten et al., 1979), changes in the oxidative enzymes do not appear to involve these steroids. Neither glucocorticoids alone nor glucocorticoids in combination with high concentrations of NGF prevented the induction of glucose-6-phosphate dehydrogenase or 6-phosphogluconate dehydrogenase after 3 days in culture. Thus, the action of NGF on the induction of the pentose phosphate pathway enzymes may involve participation of other hormones or trophic substances present only *in vivo*.

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