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Nerve Growth Factor Induces Volume Increase and Enhances Tyrosine Hydroxylase Synthesis in Chemically Axotomized Sympathetic Ganglia of Newborn Rats

(6-hydroxydopamine/dopa decarboxylase)

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ABSTRACT **Concomitant daily treatment of newborn rats for a 2-week to 1-month period with 10 μg/g of body weight of nerve growth factor and 100 μg/g of body weight of 6-hydroxydopamine produces in the cell bodies of adrenergic neurons the characteristic effects of the growth factor but in the nerve terminals the characteristic effects of 6-hydroxydopamine. The dual opposite effects result in a striking volume increase of sympathetic ganglia which far exceeds that produced by nerve growth factor alone. The selective induction of tyrosine hydroxylase [L-tyrosine, tetrahydropteridine:oxygen oxidoreductase (3-hydroxylating), EC 1.14.16.2] in these chemically axotomized adrenergic neurons is even more pronounced than that produced by nerve growth factor alone in intact neurons.**

The possibility of greatly enhancing growth and differentiative processes in immature sympathetic neurons by administration of nerve growth factor (NGF) to newborn mammals, and conversely producing cytotoxic lesions in the immature and fully differentiated sympathetic nerve cells by injecting 6-hydroxydopamine (6-OHdopamine), opened new avenues in the study of the growth control mechanisms of these cells. NGF not only brings about a marked size increase in the adrenergic neuron of newborn and adult rodents (1), but also enhances with high predilection the synthesis of tyrosine hydroxylase (TH) [L-tyrosine, tetrahydropteridine:oxygen oxidoreductase (3-hydroxylating), EC 1.14.16.2] and dopamine β-hydroxylase (2), the two key enzymes in the pathway of noradrenaline synthesis (3). On the other hand, 6-OHdopamine produces cytotoxic lesions of different severity and extent when injected into adult or newborn mammals (4). In adult animals the dopamine derivative causes a selective but reversible destruction of the sympathetic nerve endings, as shown by ultrastructural and biochemical studies (5, 6). The block of postganglionic sympathetic transmission which follows the destruction of the adrenergic nerve terminals disappears gradually within a 6- to 8-week period, the time needed for the regeneration of the destroyed terminal parts of the neuron (7). In newborn animals 6-OHdopamine produces, besides the above effects, widespread lesions in the cell body, leading to the destruction of a major part of the adrenergic neurons in sympathetic ganglia (8). This process became known as chemical sympathectomy.

In order to examine whether NGF could counteract the noxious effects of 6-OHdopamine in the immature sympathetic neurons, newborn rats were subjected to a dual concomitant treatment with NGF and 6-OHdopamine. As reported in previous articles (9, 10), NGF not only prevents the deleterious effects produced by 6-OHdopamine in the immature sympathetic nerve cells, but causes a paradoxic volume increase of sympathetic ganglia which far exceeds that produced by NGF alone.

It was the object of the present study to examine in detail this perplexing effect. Furthermore, it was of interest to see whether the hypertrophic and hyperplastic nerve cell population, which is prevented from establishing normal functional connections with the end organs by the destructive effect of 6-OHdopamine on the adrenergic nerve terminals, would continue to synthesize the enzymes involved in the formation of the physiological transmitter noradrenaline. This aspect is of particular interest since the adrenergic nerve terminals are normally the major site of transmitter synthesis, storage, and liberation.

The results reported below show that the chemically axotomized neurons preserve in full their ability to respond to NGF by undergoing a marked size increase and by synthesizing TH at an even greater rate than intact sympathetic nerve cells.

MATERIALS AND METHODS

NGF was prepared from mouse submaxillary glands according to the method of Bocchini and Angeletti (11). 6-OHdopamine hydrobromide (Roche Laboratories, Nutley, N.J.), kindly supplied by Dr. Scott, was dissolved just before use in distilled water containing 0.1 mg/ml of ascorbic acid to prevent oxidation. A total of 200 newborn rats were used. Pregnant rats were purchased from Zivic-Miller (Allison Parks, Pa.) and were housed in single cages upon arrival. As soon as delivered, each litter was reduced to eight pups which were injected daily as follows: two of the first group (I) were injected with 10 μg/g of body weight (b.w.) of NGF in the morning and 100 μg/g of b.w. of 6-OHdopamine in the evening; two of the second group (II) with 10 μg/g of b.w. of NGF in the morning, two of the third group (III) with 100 μg/g of b.w. of 6-OHdopamine in the evening, two of the fourth group (IV) with 0.1 ml of saline in the morning. Two pups from each group were sacrificed for inspection, histological, and ultrastructural studies every other day from the second day to the end of the first month. For light microscopic studies the animals were killed by cervical translocation and the superior cervical, stellate, and thoracic chain ganglia were dissected out under the stereomicroscope, fixed in alcoholic Bouin, and mounted *in toto*, or were embedded in Paraplast,

Abbreviations: NGF, nerve growth factor; 6-OHdopamine, 6-hydroxydopamine (3,4,6-trihydroxyphenethylamine); TH, tyrosine hydroxylase.

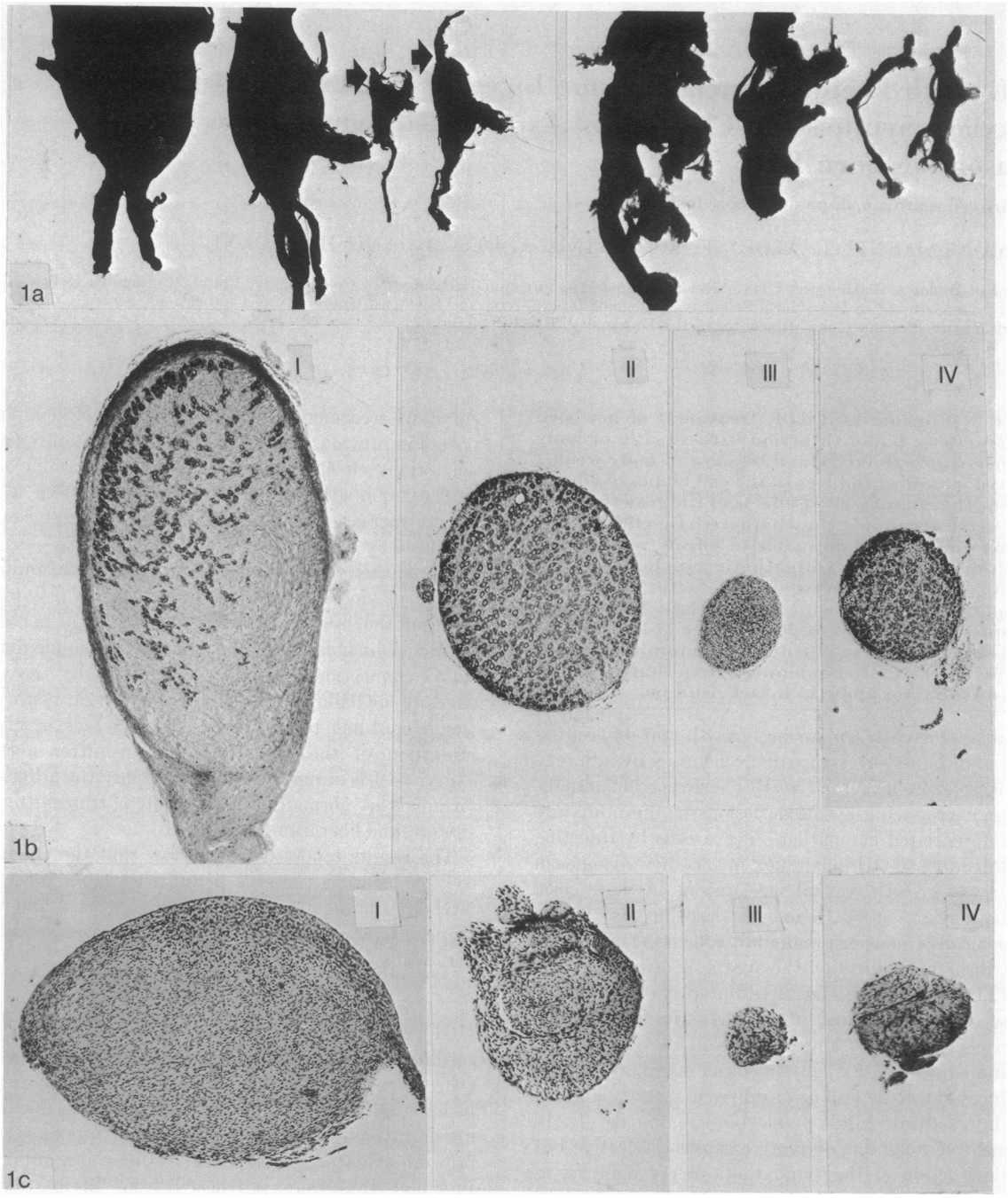

FIG. 1. (a) Comparison of whole mounts of superior cervical ganglia of 26-day (left) and stellate ganglia of 19-day (right) littermate rats treated with NGF and 6-OHdopamine (I), NGF (II), 6-OHdopamine (III), and saline (IV) (left: ×8.3, right ×5.8). (b) Cross sections of superior cervical ganglia of 19-day littermate rats treated with NGF and 6-OHdopamine (I), NGF (II), 6-OHdopamine (III), and saline (IV) (×33). (c) Cross sections of post-ganglionic roots indicated with arrows in (a) (×176).

sectioned into 8 μm thick slices, and stained in toluidine blue.

For electron microscopic studies, the animals were anesthetized with chloral hydrate and perfused with 2.5% glutaraldehyde and 1% paraformaldehyde in 0.1 M phosphate buffer, pH 7.3. Superior cervical ganglia, pre- and postganglionic nerves, and irides were then dissected out, washed, and postfixed in buffered 2% osmic acid and stained *en bloc* with 2% uranyl acetate. Some ganglia were fixed *in situ* either with 2% osmic acid in 0.1 M phosphate buffer, pH 7.3, or with 3% glutaraldehyde and postfixed in buffered 2% osmic acid (12). All samples were dehydrated with ethanol and propylene oxide and embedded in Epon. Ultrathin sections were double stained with uranyl acetate and lead citrate and examined with a Hitachi electron microscope.

For the determination of enzyme activities pairs of ganglia were homogenized in 0.5 ml of ice-cold 5 mM Tris·HCl (pH 7.4) containing 0.1% (w/v) Triton X-100. One gram of salivary glands was homogenized in 5 ml of the same mixture. The homogenates were centrifuged at 10,000 × *g* for 20 min and the supernatant fractions from ganglia were diluted 2-fold for controls, 4-fold after NGF-treatment, and 10-fold after combined treatment with 6-OHdopamine + NGF. The

supernatant fractions from the salivary glands were diluted 2-fold after NGF treatment. TH activities were then determined according to the method of Levitt *et al.* (13) with modifications described in detail by Mueller *et al.* (14), dopa decarboxylase (aromatic L-amino-acid carboxy-lyase, EC4.1.1.28) activities by the method of Håkanson and Owman (15) with modifications as described by Oesch *et al.* (16), and protein concentrations (ganglia only) by the method of Lowry *et al.* (17). The Student's *t*-test was used to establish the significance of differences between means (18). The measure of variation in this study is the SEM.

RESULTS

Rats injected with NGF and 6-OHdopamine or NGF or 6-OHdopamine alone were somewhat smaller than littermates injected with saline but otherwise vital and in good health as indicated by their behavior, which compared to that of controls. A mortality not exceeding 4–5% occurred during the early but not later stages of the treatment and was of the same extent in all three experimental groups. The low incidence of death shows that the combined NGF and 6-OHdopamine treatment as well as injections of NGF or 6-OHdopamine alone are well tolerated. Rats injected with 6-OHdopamine exhibited a complete ptosis well apparent at the middle of the third week and in all subsequent stages; a pronounced but less severe ptosis was also present in rats injected with NGF and 6-OHdopamine.

Fig. 1a compares whole mounts of superior cervical and stellate ganglia of pups of the same litters, sacrificed respectively at 26 (Fig. 1a, left) and 19 days (Fig. 1a, right) of age after daily treatment with NGF and 6-OHdopamine (I), NGF alone (II), 6-OHdopamine alone (III), and saline (IV). Sympathetic ganglia of the first group not only exceeded in volume those of the second group, but differed from these and from ganglia of other groups in their grossly altered profile and in the enormously enlarged postganglionic roots, which exhibited a beaded uneven contour. Volume determinations and cell counts performed in ganglia fixed between 4 and 26 days are given elsewhere (19). Here it suffices to mention that at the end of the third week of treatment the superior cervical ganglia of pups of the first group are about 30 times larger than those of the fourth group. Cell counts gave evidence for a nerve cell population in ganglia of the first group about twice as large as in controls, an increase only slightly inferior to that produced by NGF alone. In contrast, 6-OHdopamine treatment resulted at the end of the third week in the destruction of 90% of the same population. Thus, NGF not only counteracts the noxious and lethal effects of the dopamine derivative, but also brings about hyperplastic effects comparable to those produced by NGF alone. Hypertrophy of the cell bodies was also of the same extent in neurons of the first and second groups (9, 10).

Fig. 1b and c show the largest sections of superior cervical ganglia of 19-day-old littermates of the four groups (1b) and of their postganglionic roots (1c). The cross sections (1b) differ not only in their areas but also in the size of individual cells, in the pattern of nerve cell distribution within the ganglia, and in the ratio of nerve cell to non-neuronal tissue. Evidence presented in previous articles (9, 10) shows that the large spaces interposed between nerve cells, as well as the capsule-like structure which surrounds the ganglia in NGF and 6-OHdopamine-treated rats, consist of tightly packed

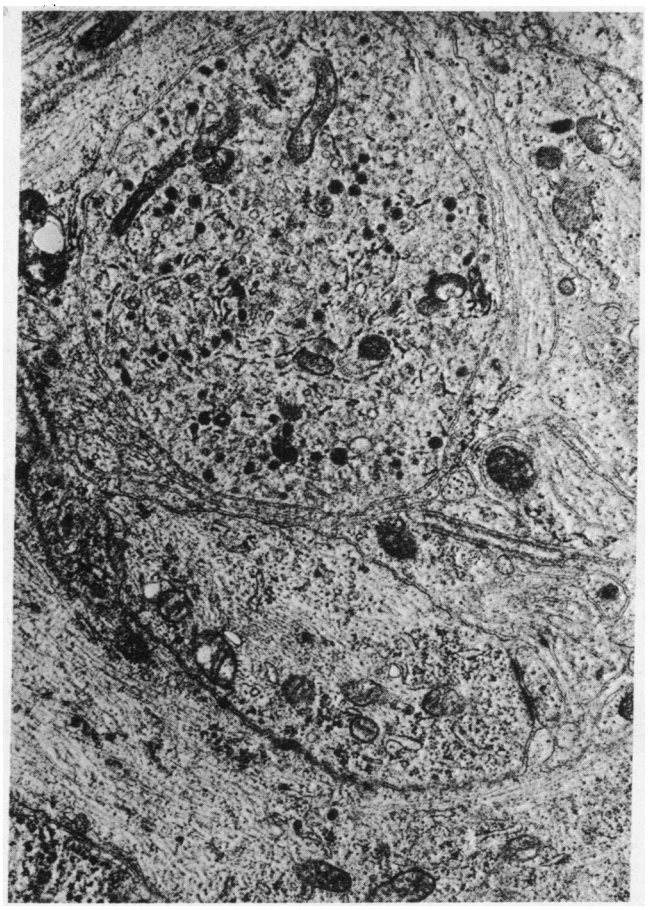

FIG. 2. Electron micrograph of axon in post-ganglionic root of superior cervical ganglion of 26-day-old rat treated with NGF and 6-OHdopamine. Note large number of dense core vesicles (×11,730).

axons, enormously increased in size and number compared to controls and also NGF-injected littermates.

Fig. 2 shows an electron microscope cross section of one axon from a NGF- and 6-OHdopamine-treated rat. One notices that the axon is replete with dense core vesicles. These organelles, which are a constant feature of the adrenergic nerve terminals and which have been identified as catecholamine storage organelles, are now seen along the entire length of postganglionic axons and also in increased number within the cell bodies of these neurons. The fine structure of the perikaryon compartment compares otherwise with that of nerve cells of group II, particularly with respect to increase in size and the presence of large numbers of microtubules and neurofilaments (Fig. 3). In no instances did we see evidence for degenerative processes in the perikaryon, such as disruption of the endoplasmic reticulum and cell lysis, features consistently found in sympathetic nerve cells of newborn rats treated with 6-OHdopamine alone (8). Cytotoxic lesions characteristic of 6-OHdopamine treatment are instead well apparent and in fact identical with those produced by 6-OHdopamine alone, in the nerve terminals of postganglionic adrenergic neurons of group I. Fig. 4 portrays two of these axons in different stages of degeneration in the iris of a 19-day-old rat treated with NGF and 6-OHdopamine.

As shown in Fig. 5, in accordance with the morphological studies the combined treatment with NGF and 6-OHdop-

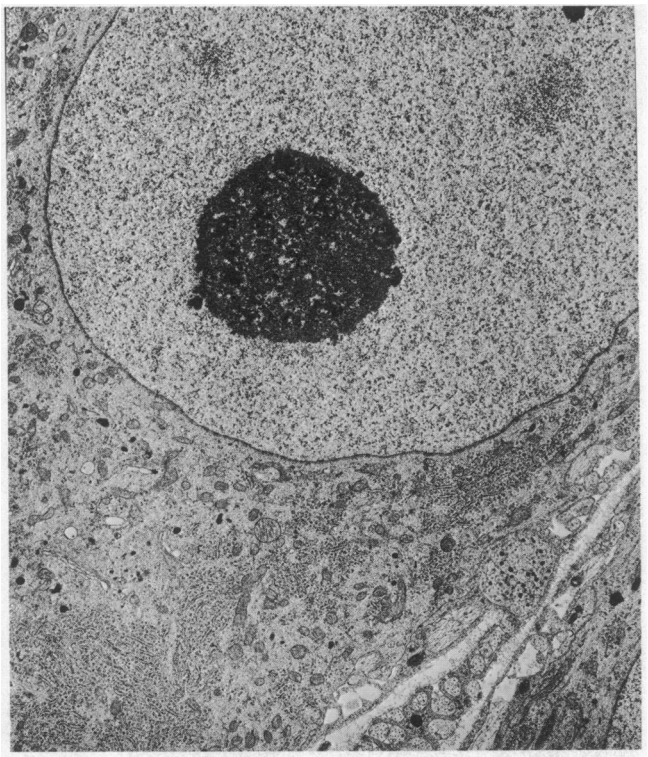

FIG. 3. Electron micrograph of neuron in superior cervical ganglion of 19-day-old rat treated with NGF and 6-OHdopamine. Dense core vesicles are scattered among neurofibrillar structures (×5,200).

amine produced a much larger increase in TH and dopa decarboxylase than NGF alone, providing biochemical evidence that the volume increase of sympathetic ganglia resulting from combined treatment was due to an increase in neuronal elements rather than satellite cells and fibroblasts. The fact that the combined treatment resulted not only in a further increase of total but also specific TH activity demonstrates that 6-OHdopamine potentiates the general growth-promoting action of NGF and also its selective effect on TH synthesis. The general growth-promoting effect, on the other hand, is characterized by the rise in total dopa decarboxylase activity. In sympathetic ganglia this enzyme is also exclusively located in adrenergic neurons (2). Thus, the slight increase in specific activity of dopa decarboxylase occurring both after treatment with NGF alone and after combined treatment with 6-OHdopamine and NGF may reflect a relative increase in neuronal versus nonneuronal elements in the ganglia.

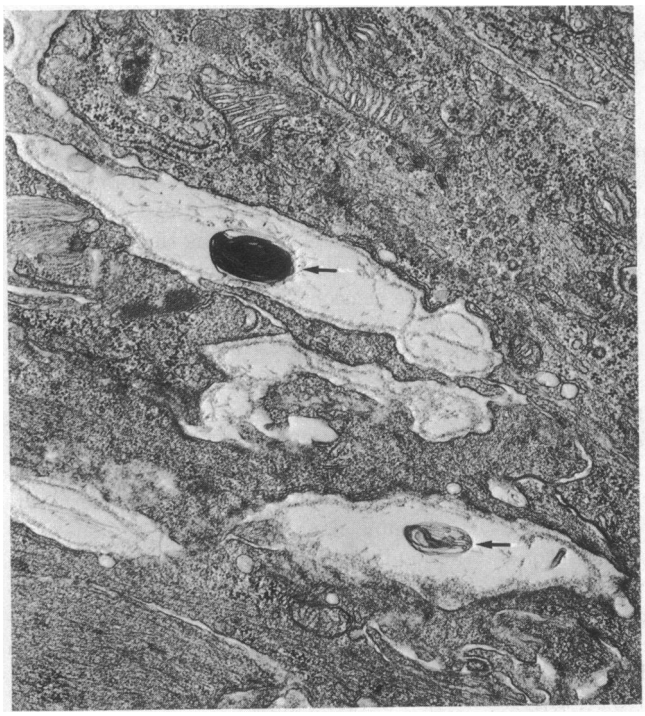

FIG. 4. Arrows point to two degenerated adrenergic endings in the iris of 19-day-old rats injected with NGF and 6-OHdopamine (×17,100).

TABLE 1. *Effect of treatment of newborn rats with 6-OHdopamine and/or NGF on TH in the salivary glands**

	Tyrosine hydroxylase	
	nmol of dopa/g of wet weight per hr	% of controls
Controls	1.41 ± 0.14	100 ± 10
6-OHdopamine	n.d.†	<10†
NGF	5.74 ± 0.50‡	407 ± 35‡
6-OHdopamine + NGF	n.d.†	<10†

* 6-OHdopamine (100 μg/g of body weight) and/or NGF (10 μg/g of body weight) were injected subcutaneously daily for 26 days. The animals were killed 1 day after the last treatment. Values represent means ± SEM of at least five determinations.

† n.d. = not detectable. Activities after treatment with 6-OHdopamine or with 6-OHdopamine + NGF were below the sensitivity of the assay procedure used, i.e. <10% as compared to controls.

‡ $P < 0.0005$.

Enzymatic studies were confined to TH in the salivary gland, where this enzyme is exclusively located in adrenergic nerve fibers. Thus, the level of this enzyme is a suitable biochemical measure for the density of the adrenergic innervation of this organ. As shown in Table 1, treatment with NGF alone produced a 4-fold increase in TH activity, demonstrating that the response to NGF is not confined to the adrenergic cell body but also involves the peripheral parts of the adrenergic neurons. In contrast to the superior cervical ganglion, the effect of combined treatment with NGF and 6-OHdopamine did not differ from that with 6-OHdopamine alone, i.e., the TH level was reduced to less than 10% in both cases. However, a small protective effect of NGF cannot be excluded with certainty, since TH levels below 10% of controls cannot be determined with sufficient accuracy.

DISCUSSION

The results reported above give evidence for the plurality and heterogeneity of the effects produced by the combined administration of NGF and 6-OHdopamine to newborn rats. NGF counteracts the noxious effects of 6-OHdopamine in the cell body, but it is apparently unable to prevent its action on the terminal parts of the adrenergic neuron, which undergo the same degenerative changes as after treatment with 6-OHdopamine alone. Nerve cells stimulated by NGF but at the same time prevented from establishing structural and functional connections with their end organs by 6-OHdopamine

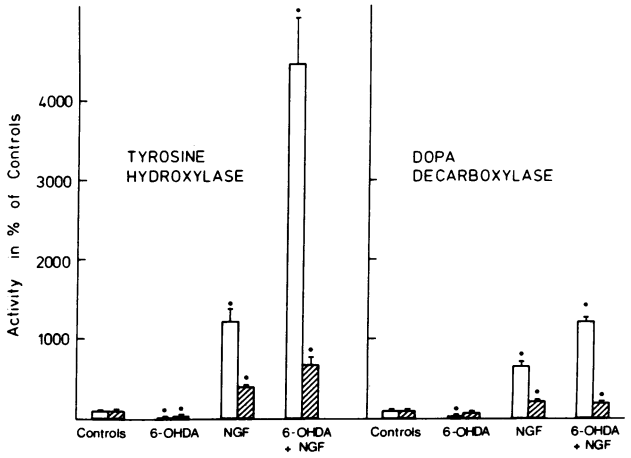

FIG. 5. Effect of 6-hydroxydopamine (6-OHDA) and/or NGF treatment on TH and dopa decarboxylase activity in the rat superior cervical ganglia. Newborn rats were injected subcutaneously daily for 26 days with 6-OHdopamine (100 μg/g of body weight) and/or NGF (10 μg/g of body weight). The animals were killed 1 day after the last treatment. Enzyme activities are expressed as percent of controls either in terms of total activities (open bars) or specific activities (hatched bars). Total activities in controls amounted to 0.564 $\pm$ 0.028 nmol of dopa per pair of ganglia per hr for TH and to 156 $\pm$ 17 nmol of dopamine per pair of ganglia per hr for dopa decarboxylase; specific activities amounted to 2.03 $\pm$ 0.17 nmol of dopa/mg of protein per hr for TH and to 536 $\pm$ 62 nmol of dopamine/mg of protein per hr for dopa decarboxylase. Values represent means $\pm$ SEM of at least four determinations.

* $P < 0.005$ as compared to controls.

exhibit an enhanced axonal production, which accounts to a large extent for the paradoxical volume increase of ganglia and of their post-ganglionic roots upon dual NGF and 6-OHdopamine treatment. These findings suggest that, under normal conditions, end organs do not only release growth-promoting factors of the same or similar nature as NGF (20, 21) but may also exert a restraining action on nerve fiber elongation, which holds even to some extent upon NGF treatment. This restraint would be relieved upon chemical axotomy produced by 6-OHdopamine. Disconnection by 6-OHdopamine of nerve fibers from their end organs likewise seems to be responsible for the appearance of a large number of dense core vesicles in the cell bodies and in the enlarged post-ganglionic axons: a finding reminiscent of morphological changes ensuing upon placement of a ligature constriction around sympathetic nerves (22, 23).

The striking increase of TH in the rat sympathetic ganglia after combined treatment with NGF and 6-OHdopamine, as opposed to the severe reduction of this enzyme in peripheral tissues, demonstrates that the selective effect of NGF on this enzyme in the cell body and axons is also increased by concomitant administration of 6-OHdopamine. However, the noxious effect of 6-OHdopamine on the adrenergic nerve terminals is not antagonized by NGF.

Finally, the finding of a pronounced but incomplete ptosis in rats submitted to NGF and 6-OHdopamine treatment calls for some comments, since it is difficult to reconcile with the ultrastructural evidence of a total degeneration of adrenergic nerve terminals and with the biochemical data, which show no difference between the reduction of TH in the end organ, whether the animals are treated with 6-OHdopamine alone or with NGF plus 6-OHdopamine. It is tempting to suggest that sympathetic nerve fibers replete with the neurotransmitter but chemically disconnected from their end organs are still able to release noradrenaline from the preterminal parts of the adrenergic neuron. Since it is known that a relatively small number of adrenergic nerve terminals can maintain basal adrenergic transmission (24), partially functioning (preterminal) amputation stumps could explain the observation that after combined treatment with NGF and 6-OHdopamine the ptosis was less complete than after 6-OHdopamine alone. The alternative hypothesis of an effect produced by circulating catecholamines on denervated supersensitive receptors seems unlikely, in view of the fact that ptosis is complete upon treatment with 6-OHdopamine alone, a condition which would likewise produce hypersensitivity of denervated effector organs.

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