Proc. Natl. Acad. Sci. USA
Vol. 81, pp. 1580–1584, March 1984
Neurobiology

Biochemical and anatomical effects of antibodies against nerve growth factor on developing rat sensory ganglia

(dorsal root fibers/substance P/somatostatin/fluoride-resistant acid phosphatase/familial dysautonomia)

M. GOEDERT†, U. OTTEN‡, S. P. HUNT†, A. BOND†, D. CHAPMAN†, M. SCHLUMPF§, AND W. LICHTENSTEIGER§

†Medical Research Council Neurochemical Pharmacology Unit, Medical Research Council Centre, Medical School, Hills Road, Cambridge, U.K.; ‡Department of Pharmacology, Biocentre of the University, Basel, Switzerland; and §Department of Pharmacology, University of Zürich, Zürich, Switzerland

Communicated by Tadeus Reichstein, November 7, 1983

ABSTRACT **The importance of nerve growth factor (NGF) for the development of sensory ganglia was investigated by injecting rat fetuses (16.50 days of gestation) with a single dose of anti-NGF antiserum. Four months later the treated animals showed a very large decrease in substance P- and somatostatin-like immunoreactivities in dorsal root ganglia and skin with a lesser decrease in trigeminal ganglia. Fluoride-resistant acid phosphatase, substance P-, and somatostatin-like immunoreactivities were greatly decreased in the dorsal horn of the spinal cord. No change in neurotensin- and [Met]enkephalin-like immunoreactivities was observed. The anti-NGF antiserum treatment produced a >90% decrease in the number of unmyelinated dorsal root fibers and a 35% decrease in the total number of myelinated fibers. The loss in myelinated fibers was restricted to small-diameter fibers with no change in large-diameter fibers. No change in taste bud morphology was noted, thereby refuting the proposal that anti-NGF antiserum treatment may represent an animal model for familial dysautonomia. The present results indicate that NGF is a necessary requirement for the normal development of a significant population of prenatal rat dorsal root ganglion cells.**

The protein nerve growth factor (NGF) is required for the normal development and maintenance of function of the peripheral sympathetic nervous system (1). The main evidence for its physiological importance stems from the observation that the administration of anti-NGF antibodies to newborn animals leads to an irreversible destruction of their sympathetic ganglia (2). This effect of anti-NGF antibodies results from the neutralization of endogenous NGF rather than from a complement-mediated cytotoxic mechanism (3, 4). In rat sympathetic ganglia, anti-NGF antibodies produce an irreversible effect if administered before postnatal day 12; if administered after day 12, a full recovery is observed (5).

The effects of anti-NGF antibodies on neonatal sensory ganglia are less dramatic, although a substantial but reversible decrease in substance P-like immunoreactivity (SPLI) is observed in dorsal root ganglia, skin, and spinal cord (6, 7). One major difference between the development of sympathetic and sensory ganglia is that sensory ganglia mature much earlier than their sympathetic counterparts (8, 9). Therefore, an irreversible effect on sensory ganglia could be expected if they were exposed to anti-NGF antibodies prenatally.

Two different approaches have been used to study this question. The first involved the active immunization of female animals against mouse submandibular gland NGF and the subsequent placental transfer of anti-NGF antibodies (10). Using this approach, a marked decrease in total dorsal root ganglion cell numbers was obtained (11). This effect was accompanied by a decrease in SPLI, whereas somatostatin-like immunoreactivity (SRIFLI) remained unchanged (12). The second approach consisted of a direct intrauterine injection of anti-NGF antibodies into rat fetuses. This experimental paradigm also produced a degeneration of dorsal root ganglion cells (13). However, no attempt was made to quantify the losses or to relate them to biochemical changes.

In the present study, we have used the intrauterine injection of anti-NGF antiserum to investigate the importance of NGF for developing rat sensory ganglia and their terminal fields in the skin and spinal cord. SPLI, SRIFLI, and fluoride-resistant acid phosphatase (FRAP) were used as biochemical markers for nonoverlapping subpopulations of small sensory ganglion cells (14, 15). In addition, the effects were quantified by counting the number of myelinated and unmyelinated dorsal root fibers. Because the prenatal administration of anti-NGF antibodies has been proposed to represent an animal model for the human disease familial dysautonomia (11, 13, 16), and because one of the features of the disease is the absence or atrophy of taste buds in the tongue (16), we investigated the morphology of fungiform and circumvallate papillae in animals treated with anti-NGF antiserum. We report that the anti-NGF antiserum produced an irreversible decrease in the biochemical markers studied in dorsal root ganglia, trigeminal ganglia, spinal cord, and skin, extending preliminary observations (17). This effect was accompanied by a nearly complete loss of unmyelinated nerve fibers with a smaller decrease in the number of myelinated fibers. No change in taste bud morphology was noted.

MATERIALS AND METHODS

Preparation of NGF Antiserum. The 2.5S NGF was prepared from submandibular glands of adult male mice according to Bocchini and Angeletti (18) with the modifications described by Suda *et al.* (19). The biological activity, which amounted to 250 units per μg of protein, was determined according to Fenton (20). Antisera against 2.5S NGF were raised in sheep as described (4).

Surgical Procedures. Pregnant Sprague–Dawley rats (Süddeutsche Versuchstierfarm, Tuttlingen, F.R.G.) at day 16.50 of gestation were anesthetized with barbiturates, an abdominal incision was made under sterile conditions, and the uterine horns were exposed under constant illumination with a fiber optic light source. Each fetus was injected subcutaneously with 10 μl of anti-NGF antiserum and the wound was closed. Control animals received the same volume of preimmune serum. At 22 days of gestation the fetuses were removed by caesarean section and given to a lactating foster mother.

Radioimmunoassays. Controls and animals injected with anti-NGF antiserum were killed at the age of four months.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: NGF, nerve growth factor; SPLI, substance P-like immunoreactivity; SRIFLI, somatostatin-like immunoreactivity; FRAP, fluoride-resistant acid phosphatase.

Lumbar dorsal root ganglia, trigeminal ganglia, lumbar spinal cord, and a skin sample taken from the hind limb were dissected, weighed, and transferred onto dry ice. They were extracted using boiling 1 M acetic acid, homogenized using glass Teflon homogenizers, centrifuged, and freeze dried. The lyophilized samples were antisera directed against the carboxyl-terminal end of substance P, somatostatin, or neurotensin. A detailed characterization of the radioimmunoassays has been published (21–23).

Histochemistry. Controls and treated animals were placed under deep anesthesia using a saturated chloral hydrate solution and were perfused intracardially with 100 ml of 0.1 M sodium phosphate buffer, pH 7.40/800 units of heparin per liter/400 ml of 4% paraformaldehyde/0.1 M phosphate buffer containing lysine at 3.42 g/liter and sodium periodate at 0.55 g/liter. The lumbar spinal cord and the tongue were removed, fixed for 2 hr in the same fixative, and placed in 0.1 M phosphate buffer containing 30% (wt/vol) sucrose. Sections (25 μm) were obtained using a freezing microtome and were collected in 0.1 M phosphate buffer for peptide immunohistochemistry and in 0.1 M acetate buffer (pH 5.0) for FRAP histochemistry. For peptide immunohistochemistry, free floating sections were incubated in either substance P antiserum (dilution, 1:500), somatostatin antiserum (dilution, 1:500) or [Met]enkephalin antiserum (dilution, 1:1000) and processed according to Sternberger (24). The substance P and [Met]enkephalin antisera have been described (25) and the somatostatin antiserum was obtained from a commercial source (Immuno Nuclear, Stillwater, MN). All antisera were diluted in 0.1 M phosphate buffer/0.95% sodium chloride/0.3% Triton X-100/1% sheep serum. After the primary incubation for 48 hr at 4°C with agitation, the sections were washed in 0.1 M phosphate buffer and incubated with sheep anti-rabbit antiserum (Miles) diluted 1:20 for 30 min at 37°C. Then, the sections were washed, incubated with rabbit peroxidase-antiperoxidase (Miles) at a dilution of 1:100 for 1 hr, and washed again. The tissue then was incubated for 5–10 min in phosphate buffer containing 0.09% 3,3-diaminobenzidine (Sigma) and 0.005% hydrogen peroxide and subsequently washed in phosphate buffer. Histochemistry for FRAP was conducted as described (15). Tissue sections were incubated overnight at room temperature in 25 mM sodium acetate buffer (pH 5.0), incubated for 2 min at room temperature in 0.2 mM acetic acid containing 90 mM ammonium sulfide, and rinsed with several changes of sodium acetate buffer.

Quantification of Dorsal Root Fibers. Controls and treated animals were anesthetized with chloral hydrate and perfused intracardially with 100 ml of 0.1 M sodium phosphate buffer (pH 7.40) containing 800 units of heparin per liter followed by 400 ml of 1.25% glutaraldehyde/1% paraformaldehyde in 0.1 M phosphate buffer followed by 50 ml of 5% glutaraldehyde/4% paraformaldehyde in the same buffer. The third lumbar dorsal root (L3) was dissected out and fixed overnight in the same fixative. After washing in 0.1 M phosphate buffer containing 7.0% (wt/vol) sucrose, the roots were stained for 2 hr in 1% osmium tetroxide phosphate buffer containing 1.5% (wt/vol) potassium ferrocyanide, dehydrated, and embedded in epoxy resin. The myelinated and unmyelinated fibers in the L3 dorsal roots of four controls and of four animals treated with anti-NFG antiserum were analyzed. Semi-thin (0.5 μm) transverse sections of the roots were stained with toluidine blue. Unmyelinated fiber counts were established from an area of 5400 μm^2. Myelinated fiber diameters were determined on a Reichert Videoplan image analysis system and histograms were made using 139 fibers for most roots, one root comprising 103 and another comprising 113 fibers. The fibers were grouped according to diameter in 0.5-μm increments, and the total number of fibers in each category was calculated by extrapolation to the total root size.

Staining of Taste Buds. The tongues from the animals used for dorsal root fiber counts were removed and the tongue surface was stained with Ponceau S as described (26). Taste pores were counted under the light microscope. Sections (25 μm) of the tongue were cut on the microtome and stained with hematoxylin and eosin.

RESULTS

Biochemical Effects of Anti-NGF Antiserum Treatment. The prenatal injection of anti-NGF antiserum produced a 70% decrease in the wet weight of lumbar dorsal root ganglia in animals examined 4 months later (1.56 ± 0.12 mg per ganglion for control versus 0.47 ± 0.01 mg per ganglion for treated animals). This was accompanied by a large decrease in the specific content of SPLI and SRIFLI in dorsal root ganglia (86% for SPLI and 74% for SRIFLI) (Fig. 1*a*) with a smaller decrease in trigeminal ganglia (48% for SPLI and 47% for SRIFLI) (Table 1). The terminal fields in the skin showed an 83% decrease in SPLI and an 86% decrease in SRIFLI, (Fig. 1*c*), whereas some SP-positive fibers remained in the peripheral terminals in the tongue (see Fig. 5*B*). SPLI was decreased by 28% and SRIFLI by 13% in whole lumbar spinal cord (Fig. 1*b*). Because dorsal rhizotomy leads to a 50% decrease in SPLI and a 20% decrease in SRIFLI in the dorsal half of the spinal cord (27), the present

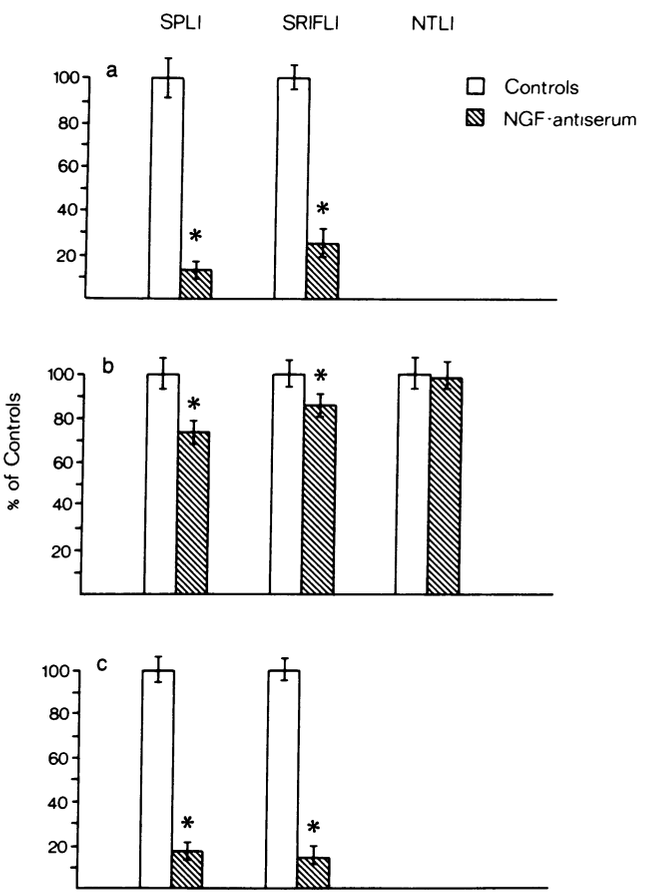

FIG. 1. Effects of the prenatal administration of anti-NGF antiserum on SPLI, SRIFLI, and neurotensin-like immunoreactivity (NTLI) in dorsal root ganglia (*a*), spinal cord (*b*), and skin (*c*). Each value is expressed as the percentage mean ± SEM (n = 8). The control values for SPLI were 13.45 ± 1.24 pmol/g in dorsal root ganglia, 208.74 ± 14.88 pmol/g in the spinal cord, and 1.56 ± 0.1 pmol/g in the skin. For SRIFLI the control values were 9.73 ± 0.54 pmol/g in dorsal root ganglia, 225.81 ± 12.44 pmol/g in the spinal cord, and 1.75 ± 0.09 pmol/g in the skin. The spinal cord NTLI amounted to 26.42 ± 1.13 pmol/g. *$P < 0.01$.

Table 1. Effect of prenatal anti-NGF-antiserum treatment on SPLI and SRIFLI in the trigeminal ganglion

	SPLI	SRIFLI
Control	64.20 ± 3.45	18.15 ± 0.94
Anti-NGF antiserum	34.90 ± 2.26*	9.56 ± 0.84*

SPLI and SRIFLI are expressed as pmol per g of tissue. Each value represents the mean ± SEM (n = 8).
*$P < 0.001$.

results suggest a very large loss in dorsal horn SPLI and SRIFLI of primary afferent origin. Immunohistochemistry showed an extensive decrease in SPLI, SRIFLI, and FRAP in the dorsal horn of the spinal cord from treated animals (Fig. 2 *A–F*). Some differences in the magnitude of the FRAP depletion between treated animals were observed, notably in those animals with the smallest loss of unmyelinated fibers, suggesting a possible sparing after the initial lesion. The peptides neurotensin and [Met]enkephalin, which are present in neurons intrinsic to the spinal cord (25), were not significantly depleted after the treatment with anti-NGF antiserum (Figs. 1*b* and 2 *G* and *H*).

Morphological Effects of Anti-NGF Antiserum Treatment. A single injection of anti-NGF antiserum into rat fetuses at 16.50 days of gestation had produced a 91% loss in the total number of unmyelinated dorsal root fibers when the animals were killed at the age of four months (8233 ± 661 for control animals and 719 ± 269* for animals treated with anti-NGF antiserum, *$P < 0.001$) (Fig. 3). The loss in myelinated fibers was less marked, with a 35% decrease in the total fiber number (2314 ± 223 for control animals and 1515 ± 69* for animals treated with anti-NGF antiserum, *$P < 0.001$). When the myelinated fibers were grouped according to increasing fiber diameters, the changes were restricted to the small-diameter fibers in the 1.0- to 1.5-μm range (745 ± 78 for control animals and 337 ± 49* for animals treated with anti-NGF antiserum, *$P < 0.001$). No statistically significant change was observed in large-diameter myelinated fibers (Fig. 4).

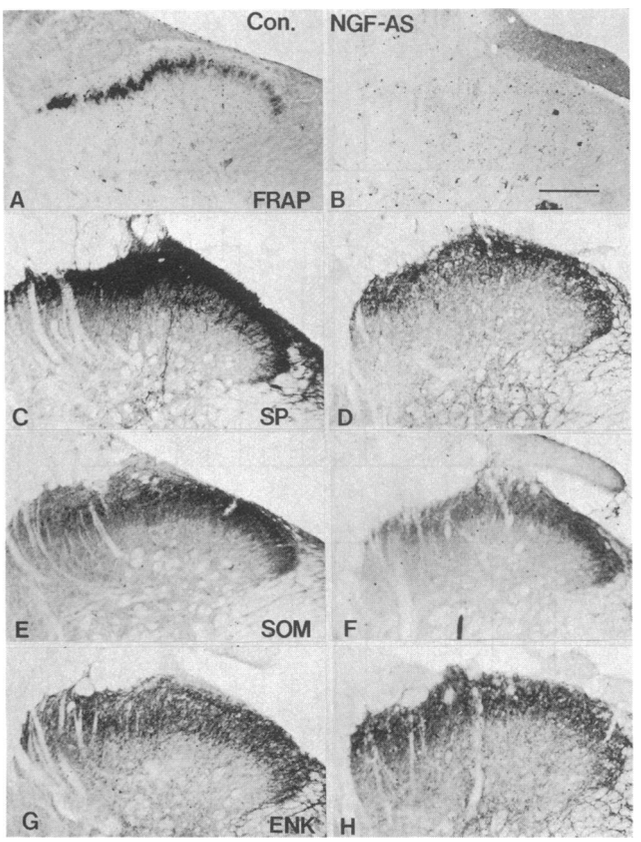

FIG. 2. The effects of prenatal anti-NGF antiserum (NGF-AS) treatment on the histochemical localization of FRAP (*A* and *B*) and the immunohistochemical localization of substance P-like immunoreactivity (SP) (*C* and *D*), somatostatin-like immunoreactivity (SOM) (*E* and *F*), and [Met]enkephalin-like immunoreactivity (ENK) (*G* and *H*) in the dorsal horn of the spinal cord. Bright-field photomicrographs. (Bar = 50 μm.)

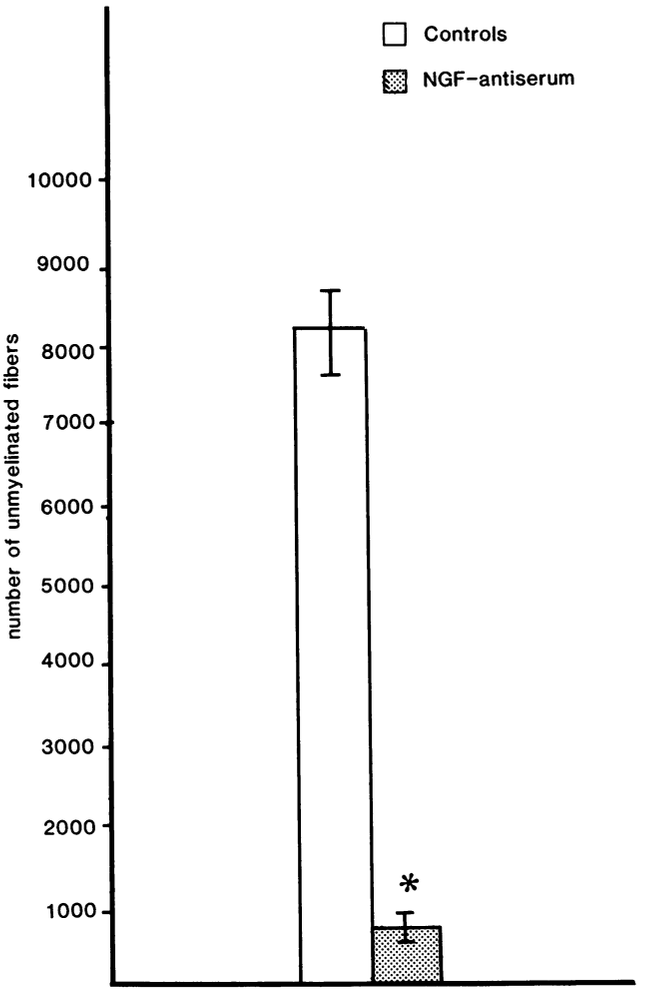

FIG. 3. The effect of prenatal NGF administration on the number of unmyelinated fibers in the third lumbar dorsal root. Each value represents the mean ± SEM of determinations from the roots of four animals. *$P < 0.001$.

Effects of Anti-NGF Antiserum on Taste Bud Morphology. The prenatal injection of anti-NGF antiserum had no effect on the number (controls, 21 ± 0.3; anti-NGF antiserum, 22 ± 1.2; n = 3) or the morphology of fungiform papillae (Fig. 5*A*) and the overall morphology of the circumvallate papilla was unchanged (Fig. 5*C*).

DISCUSSION

The present results confirm and extend previous studies showing that NGF is required for the normal development of mammalian sensory ganglia (11, 13, 17). A single administration of anti-NGF antiserum to 16.50-day-old rat fetuses produced an irreversible decrease in SPLI and SRIFLI in dorsal root and trigeminal ganglia and in the skin. A marked decrease in SPLI, SRIFLI, and FRAP with no change in neurotensin- and [Met]enkephalin-like immunoreactivities was observed in the dorsal horn of the spinal cord. This indicates that the spinal cord changes were a direct consequence of the effects of the anti-NGF antiserum on dorsal root ganglia

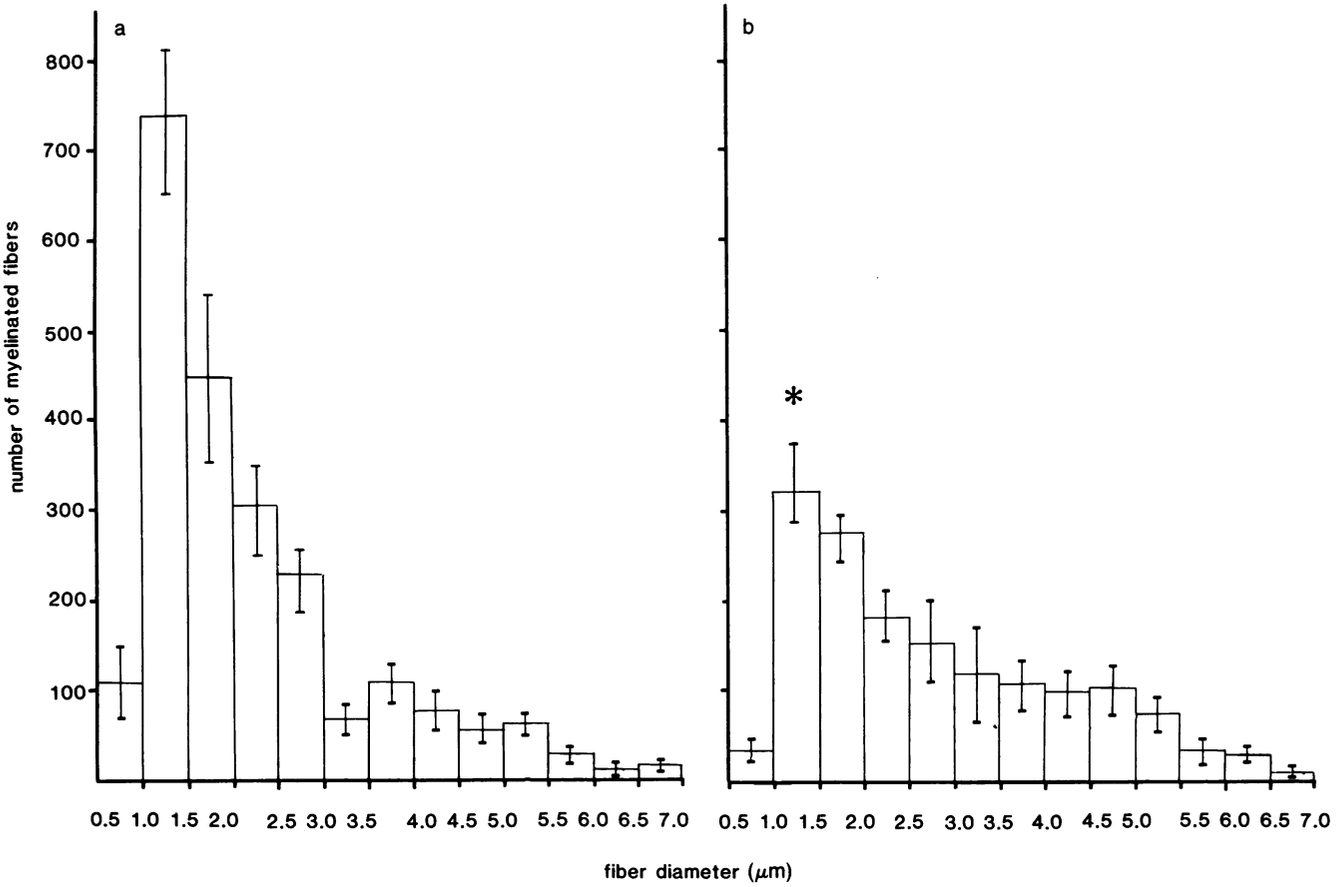

FIG. 4. Histogram of the axon diameter frequency distribution of myelinated fibers in the third lumbar dorsal root of control rats (*a*) and of rats treated prenatally with anti-NGF antiserum (*b*). Each histogram bar represents the mean ± SEM of determinations from the roots of four animals. $*P < 0.001$.

and argues against a role for NGF in the maturation of spinal cord neurons (28). A previous study has reported a decrease in SPLI but no effect on SRIFLI in dorsal root ganglia, skin, and spinal cord after the active immunization of female rats against NGF with subsequent placental transfer of anti-NGF antibodies (12). The reason for the discrepancy with the present study in which the biochemical markers SPLI, SRIFLI, and FRAP were all depleted may reside in an incomplete lesion using the active immunization model. This interpretation is supported by the fact that the decrease in SPLI observed by Ross *et al.* did not exceed 60% in dorsal root ganglia (12), whereas it amounted to 86% in the present study. In addition, the direct intrauterine injection of anti-NGF antiserum has the advantage of precise timing and close control of the amount of anti-NGF antibodies administered.

In the present study, trigeminal ganglia were less affected than dorsal root ganglia. This may be due to their dual embryological origin from both the neural crest and the placodal ectoderm (29, 30), whereas dorsal root ganglia are pure neural crest derivatives (30). The morphological analysis of dorsal root fibers revealed that the anti-NGF antiserum produced a >90% loss in unmyelinated nerve fibers with a less dramatic decrease in small myelinated fibers, whereas large myelinated fibers remained unaffected. This indicates that some but not all dorsal root ganglion neurons need NGF for normal development during the late gestation phase. Because small sensory ganglion cells give rise to unmyelinated

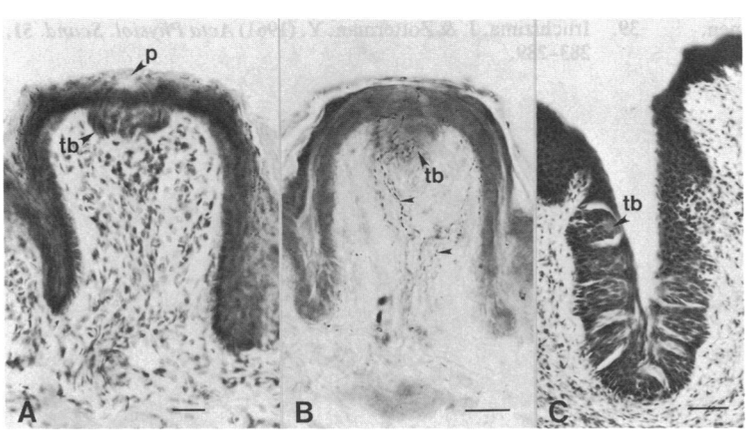

FIG. 5. Haematoxylin and eosin stain of the tongue from an animal treated prenatally with anti-NGF antiserum showing a normal fungiform (*A*) and circumvallate (*C*) papilla. (*B*) Fungiform papilla stained for SPLI from treated animal. Note the SP-positive fibers (arrowheads). tb, Taste buds; p, taste pore. (Bars = 15 μm in *A* and *B*, and 50 μm in *C*.)

nerve fibers and large cells give rise to myelinated ones (30, 31), NGF seems to be required for the development of virtually all small cells and for only a relatively small percentage of large cells. It is possible that the neurons resistant to anti-NGF antibodies at day 16.50 of gestation pass through a NGF-sensitive phase earlier in development. Alternatively, they may require a growth factor different from NGF. Studies using dissociated chicken dorsal root ganglia have presented evidence for a factor immunologically distinct from NGF with the ability to support the survival of sensory cells (32, 33). During recent years, it has been shown that the administration of high doses of the neurotoxic agent capsaicin to newborn rats produces an irreversible decrease in the number of unmyelinated nerve fibers and small myelinated fibers and in SPLI and SRIFLI levels with no effect on large myelinated fibers (27, 31, 34–36). Both the qualitative and quantitative effects of capsaicin and of anti-NGF antiserum strongly suggest that they affect the same population of dorsal root ganglion cells. In addition, it has been shown that NGF can prevent the neurotoxic effects of capsaicin (37, 38). However, capsaicin is able to destroy postnatal dorsal root ganglia, whereas the irreversible effect of anti-NGF antibodies is limited to the prenatal period.

The prenatal administration of anti-NGF antibodies has been proposed as an animal model for the human disease familial dysautonomia (11, 13, 16). Because one of the features of the human disease is the virtual absence of fungiform taste buds and the atrophy of circumvallate papillae, we investigated the presence of taste buds in the tongue from animals treated with anti-NGF antiserum. We failed to observe a change in the total number of fungiform taste papillae or in the overall morphology of fungiform and circumvallate papillae. The present results are in line with data indicating that the trophic effect exerted by sensory nerves on taste bud morphology is provided by large myelinated fibers (39), but they do not support the proposal that the prenatal exposure to anti-NGF antibodies may provide a model for familial dysautonomia.

In summary, the present study indicates that NGF is critically required for the normal development of a large population of rat dorsal ganglion cells. Whereas anti-NGF antibodies produce an irreversible destruction of sympathetic ganglia up to postnatal day 12, an irreversible effect on dorsal root ganglia is limited to the prenatal period.

The present study was supported by the Swiss National Foundation for Scientific Research (Grants 3077-081 and 3344-083) and by E. Barell Stiftung, F. Hoffmann-LaRoche, Basel.

1. Yankner, B. A. & Shooter, E. M. (1982) *Annu. Rev. Biochem.* **51,** 845–868.
2. Levi-Montalcini, R. & Booker, B. (1960) *Proc. Natl. Acad. Sci. USA* **42,** 384–391.
3. Ennis, M., Pearce, F. L. & Vernon, C. A. (1979) *Neuroscience* **4,** 1391–1398.
4. Goedert, M., Otten, U., Schäfer, T., Schwab, M. & Thoenen, H. (1980) *Brain Res.* **201,** 399–409.
5. Goedert, M., Otten, U. & Thoenen, H. (1978) *Brain Res.* **148,** 264–268.
6. Otten, U., Goedert, M., Mayer, N. & Lembeck, F. (1980) *Nature (London)* **287,** 158–159.
7. Mayer, N., Lembeck, F., Goedert, M. & Otten, U. (1982) *Neurosci. Lett.* **29,** 47–52.
8. Hendry, I. A. (1977) *J. Neurocytol.* **6,** 299–309.
9. Lawson, S. N. & Biscoe, T. J. (1979) *J. Neurocytol.* **8,** 265–274.
10. Gorin, P. D. & Johnson, E. M. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 5382–5386.
11. Johnson, E. M., Gorin, P. D., Brandeis, L. D. & Pearson, J. (1980) *Science* **210,** 916–918.
12. Ross, M., Löfstrandh, S., Gorin, P. D., Johnson, E. M. & Schwartz, J. P. (1981) *J. Neurosci.* **1,** 1304–1311.
13. Aloe, L., Cozzari, C., Calissano, P. & Levi-Montalcini, R. (1981) *Nature (London)* **291,** 413–415.
14. Hökfelt, T., Elde, R., Johansson, O., Luft, R., Nilsson, G. & Arimura, A. (1976) *Neuroscience* **1,** 131–136.
15. Nagy, J. I. & Hunt, S. P. (1982) *Neuroscience* **7,** 89–97.
16. Pearson, J. (1979) *J. Auton. Nerv. Syst.* **1,** 119–126.
17. Otten, U., Peck, M., Businger, F., Schlumpf, M. & Lichtensteiger, W. (1983) *Monogr. Neural Sci.* **10,** 29–35.
18. Bocchini, F. & Angeletti, P. U. (1969) *Proc. Natl. Acad. Sci. USA* **64,** 787–794.
19. Suda, K., Barde, Y. A. & Thoenen, H. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 4042–4046.
20. Fenton, E. L. (1970) *Exp. Cell Res.* **59,** 383–392.
21. Kanazawa, I. & Jessell, T. (1976) *Brain Res.* **117,** 362–367.
22. Emson, P. C., Rossor, M. & Lee, C. M. (1981) *Neurosci. Lett.* **22,** 319–324.
23. Emson, P. C., Goedert, M., Horsfield, P., Rioux, F. & St. Pierre, S. (1982) *J. Neurochem.* **38,** 192–199.
24. Sternberger, L. A. (1979) *Immunocytochemistry* (Wiley, New York).
25. Hunt, S. P., Kelly, J. S., Emson, P. C., Kimmel, J. R., Miller, R. J. & Wu, J. I. (1981) *Neuroscience* **6,** 1883–1898.
26. Nagy, J. I., Goedert, M., Hunt, S. P. & Bond, A. (1982) *Neuroscience* **7,** 3137–3151.
27. Nagy, J. I., Iversen, L. L., Goedert, M., Chapman, D. & Hunt, S. P. (1983) *J. Neurosci.* **3,** 399–406.
28. Kessler, J. A. & Black, I. B. (1981) *Brain Res.* **208,** 135–145.
29. Hamburger, V. (1961) *J. Exp. Zool.* **148,** 91–117.
30. Le Douarin, N. (1982) *The Neural Crest* (Cambridge University, London).
31. Lawson, S. N. & Nickels, M. (1980) *J. Physiol. (London)* **303,** 12P.
32. Barde, Y. A., Edgar, D. & Thoenen, H. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 1199–1203.
33. Barde, Y. A., Edgar, D. & Thoenen, H. (1982) *EMBO J.* **1,** 549–553.
34. Jancsó, G., Király, E. & Jancsó-Gábor, A. (1977) *Nature (London)* **270,** 741–743.
35. Jancsó, G. & Király, E. (1980) *J. Comp. Neurol.* **190,** 781–792.
36. Kessler, J. A. & Black, I. B. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 4644–4647.
37. Miller, M. S., Buck, S. H., Spies, I. G., Yamamura, H. I. & Burks, T. F. (1982) *Brain Res.* **250,** 193–196.
38. Otten, U., Lorez, H. P. & Businger, F. (1983) *Nature (London)* **301,** 515–517.
39. Iruchizima, J. & Zotterman, Y. (1961) *Acta Physiol. Scand.* **51,** 283–289.