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Differentiating effects of murine nerve growth factor in the peripheral and central nervous systems of *Xenopus laevis* tadpoles

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ABSTRACT **The present investigation was directed toward exploration of the spectrum of action of murine nerve growth factor (m-NGF) in peripheral cells and neurons in the central nervous system (CNS) of *Xenopus laevis* tadpoles. It was found that systemic m-NGF injections elicit growth and differentiative effects not only on sensory and sympathetic nerve cells but also on several populations in the CNS. The finding that aminergic and peptidergic neurons in brain centers are highly receptive to m-NGF activity provides evidence for the broad spectrum of action of this molecule in lower vertebrates and calls for a systematic search for these and other putative target cells in the CNS of higher vertebrates.**

Two of the most interesting areas in the field of hormone research are those concerned with the molecular events underlying their mechanisms of action and evolutionary history. Studies in this past decade have resulted in considerable progress in elucidating some of these mechanisms and in tracing back several vertebrate hormones to unicellular eukaryotic organisms and even to prokaryotes (1). In the class of hormone-like peptides, designated as specific growth factors because of their properties of enhancing proliferative or differentiative processes in their target cell lines, extensive work has been performed to gain information on their mode of action, but few attempts have been made to trace their origin and biological significance in lower vertebrates. Among the biologically active peptides listed in this class, nerve growth factor (NGF) holds a privileged position as the first to have been identified (2–4) and to have lent itself to extensive studies pursued in living organisms, in organ cultures, and on dissociated nerve cells (5). Surprisingly, very little is known about the response elicited by NGF in the nervous system of fishes and amphibians. Although early studies (6–9) described proliferative and differentiative effects on sensory and sympathetic cells of specimens of these lower vertebrates, these reports received only little attention. The belief that information on the NGF range of activity and spectrum of action in these simpler organisms would be of considerable interest prompted a more detailed study on its effects in tadpoles of the amphibian *Xenopus laevis*. Our results provide evidence that in this species NGF not only enhances differentiative processes of sensory and sympathetic nerve cells but also is an important factor for growth and differentiation of neuronal cell types in the central nervous system (CNS). This last finding, discussed previously (10), is the main focus of this presentation.

MATERIALS AND METHODS

Adult male and female *X. laevis* were treated with chorionic gonadotropin and induced artificially to mate following the method described by Gurdon (11). After removing the jelly coat, the fertilized eggs were transferred to a large jar containing charcoal-inactivated tap water thermoregulated at 23°C. During the first week, tadpoles were fed homogenized dried urtica or spinach and in later stages were fed Tubifex (Euraquarium, Bologna, Italy).

Age- and stage-matched tadpoles, varying from stage 40 to stage 52 according to the Nieuwkoop and Faber table (12), were divided prior to each experiment into pairs of 10–20 specimens and kept in separate water containers throughout the time of experimentation.

NGF was prepared from male mouse salivary glands (m-NGF) according to the method of Bocchini and Angeletti (13). NGF was iodinated following the procedure of the chloramine-T method (14). The reaction yields 95% ^{125}I-labeled NGF (^{125}I-NGF) precipitable with trichloroacetic acid, with a specific activity of 150,000–200,000 cpm/ng of protein. More than 600 tadpoles at stages between 40 and 52 were used in these studies. They were anesthetized by immersion for 2–3 min in ice-cold water and placed in Petri dishes containing moist filter paper. Under a dissecting stereomicroscope, tadpoles were injected with NGF or vehicle solution in the right periorbital area by means of a calibrated glass micropipette with a tip of about 10–15 μm in diameter. They were injected daily for periods of 3–7 days with 2 μg (stages 40–44), 4 μg (stages 45–48), and 10 μg (stages 49–52) of NGF in 1–2 μl of vehicle solution. Mortality in the NGF- and vehicle-injected tadpoles was about 20% and was slightly higher in the former than in the latter set.

Histological Studies. Age- and stage-matched tadpoles were fixed in alcoholic Bouin's fluid, embedded in Paraplast, sectioned serially (7 μm thick), and stained in hematoxylin/eosin or toluidine blue. Tadpoles of all premetamorphic stages were also stained by a silver impregnation technique (unpublished method) to better visualize cells and neurites of the peripheral and central nervous systems. Morphometric studies were carried out as described (6, 15).

Radioautographic Studies. For the *in vivo* binding assays, tadpoles were injected in the right periorbital area with 2 μl of ^{125}I-NGF with or without a 1000-fold excess of unlabeled NGF. Tadpoles were also injected with ^{125}I-labeled IgG (^{125}I-IgG). The bound labeled NGF or IgG was measured in isolated brains with a γ counter at various time intervals. Tadpoles were fixed in Bouin's fluid or 4% paraformaldehyde at time intervals for 15 min to 48 hr after injection. Tadpoles embedded in Paraplast were sectioned (5 μm thick), deparaffinized, dipped in K-2 Ilford emulsion, and 4 weeks later developed in D-19 Kodak solution. The sections were lightly stained with hematoxylin/eosin and examined with a Zeiss microscope.

Histofluorescence Studies. Saline- and NGF-treated tadpoles were exposed to the monoamine oxidase inhibitor

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Abbreviations: NGF, nerve growth factor; m-NGF, murine NGF; CNS, central nervous system; SP, substance P; Som, somatostatin.

iproniazide phosphate (Sigma) for 48 hr to better visualize the monoamine-containing cells. The specimens were then fixed in isopentane cooled by liquid nitrogen, freeze-dried for 3–4 days, and exposed to paraformaldehyde vapors at 70% relative humidity for 1 hr at 80°C (16). After embedding in pure Paraplast, tadpoles were serially sectioned (7 μm thick) and examined under a Zeiss standard fluorescence microscope equipped with the appropriate filters.

Immunofluorescence Studies. Tadpoles were fixed by immersion in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (pH 7.6) (P_i/NaCl) for 2 hr, washed several times in P_i/NaCl mounted on a tissue supporter, and frozen with dry ice. Serial sections (10–15μm) were cut with a cryostat at −20°C, collected on gelatin-coated glass slides, and processed for immunofluorescence according to the technique of Coons (17). Briefly, sections were treated for 12 hr with monoclonal antibodies against substance P (SP), enkephalin, or somatostatin (Som), rinsed in P_i/NaCl for 30 min, and incubated for 1 hr in fluorescein isothiocyanate-conjugated antibodies (Miles), diluted 1:20 at room temperature. After a brief rinse in P_i/NaCl, sections were mounted in glycerine and P_i/NaCl and examined with a Zeiss epi-fluorescence microscope. To test the specificity of the immunocytochemical reaction, alternative sections were incubated with antibodies previously preabsorbed for 60 min at 37°C with pure antigen.

***In Vitro* Studies.** Groups of 50 tadpoles ranging from stage 46 to stage 48 were washed several times with sterile distilled water, immersed in 0.1% H_2O_2 for 3 min, and immediately washed in Ca^{2+}/Mg^{2+}-free P_i/NaCl. Brains were then removed, cleaned, incubated for 30 min at 25°C in the same buffer containing 0.1% trypsin and 0.2% collagenase, washed in culture medium, and mechanically dissociated with a Pasteur pipette. Dispersed brain cells were plated in collagen-coated plastic dishes in a culture medium consisting of BEM/RPM, inactivated horse and fetal calf sera, hypoxanthine, and NGF or vehicle solution (to be reported elsewhere). Cultures were observed every 24 hr for 20 days under an inverted Leitz microscope.

RESULTS

m-NGF Effects on Sensory, Sympathetic, and Chromaffin Stem Cells. m-NGF daily injections in *Xenopus* tadpoles for a 7-day period at stages 42–46 produced a massive volume increase of sensory cephalic and dorsal root ganglia. The latter, which in stage-46 control specimens consisted of three pairs of small aggregates of 12–20 nerve cells innervating the cervicothoracic trunk muscles, underwent an 8-fold increase upon NGF daily administration as a result of a striking increase in size and number of individual cells. This effect is illustrated in Figs. 1 and 2 *A* and *B*. Hyperinnervation of trunk muscles was very evident in silver-stained preparations. A marked increase in synthesis of the peptidergic transmitter SP was detected by immunocytochemical studies with monoclonal antibodies. The response of sympathetic and chromaffin stem cells to NGF administration occurred according to the same pattern as in higher vertebrates (18): sympathetic nerve cells undergo precocious differentiation while chromaffin cell precursors are diverted toward the neuronal rather than the glandular phenotype upon NGF treatment. These effects are shown in Fig. 2 *C–F*.

m-NGF Effects in the CNS. Brain and spinal cord of tadpoles between stage 42 and stage 50 are very visible in living specimens due to the dense texture of the CNS that stands out against the background of soft, sponge-like surrounding tissue. Stereomicroscopic inspection of size-matched, stage-48 tadpoles submitted for a 5- to 7-day period to daily injection of m-NGF or vehicle solution showed that brain and rostral spinal segments are slightly but consistently larger in the experimental than control specimens (not shown). Camera lucida drawings and microphotographs of the brain and neural tube in serially sectioned larvae provided additional strong evidence for this effect (Fig. 2 *A* and *B*).

In toluidine blue-stained preparations, cells such as

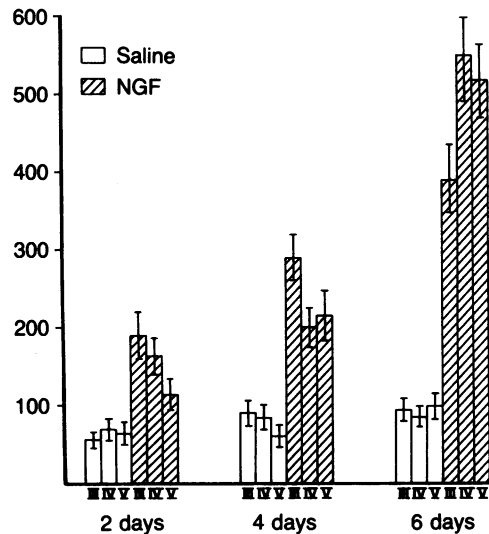

FIG. 1. Comparative volume (expressed in arbitrary units) of the third, fourth, and fifth spinal sensory ganglia of *Xenopus* tadpoles injected for 2, 4, and 6 days with saline (▭) or NGF (▭). Values are means ± SEM of four determinations.

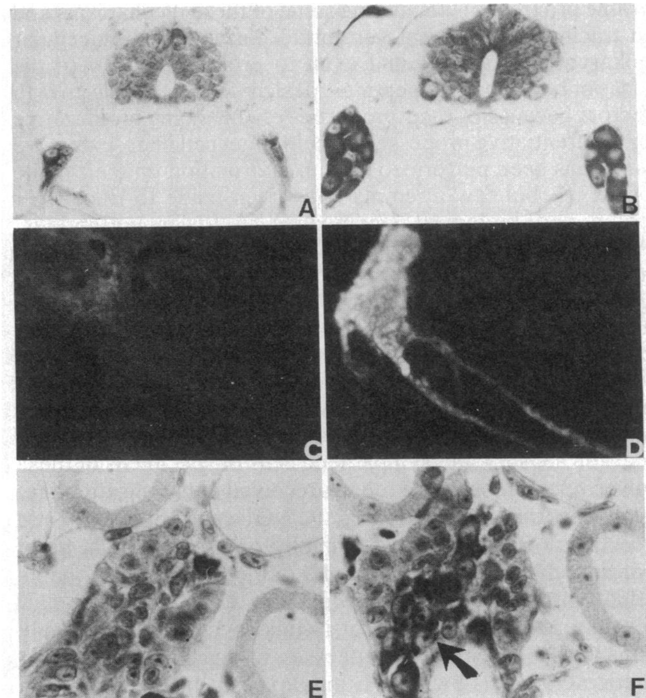

FIG. 2. (*A* and *B*) Comparative sections through the spinal cord of the third dorsal root ganglia of stage-48 tadpoles treated for 5 days with vehicle solution (*A*) and NGF (*B*). (Toluidine blue stain; ×125.) (*C* and *D*) Comparative sections at the level of the thoracic paravertebral ganglia in stage-50, vehicle-injected (*C*) and NGF-injected (*D*) tadpoles. The precocious differentiation of sympathetic ganglia in the NGF specimen (*D*) is very apparent. (Formaldehyde-induced fluorescence preparations; ×140.) (*E* and *F*) Comparative sections of the retroaortic area of stage-48 tadpoles treated for 6 days with vehicle solution (*E*) and NGF (*F*). Immature chromaffin cells are visible in *C*. In *F*, the arrow points to a group of sympathetic-like nerve cells. (Toluidine blue stain; ×185.)

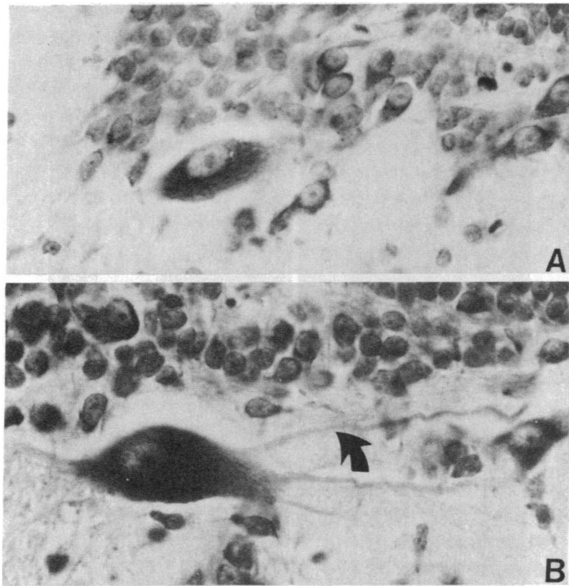

FIG. 3. Mauthner cells in stage-48 tadpoles injected for 6 days with vehicle (*A*) and NGF (*B*). The arrow in *B* points to well-developed dendritic processes. (Toluidine blue stain; ×320.)

Mauthner cells (Fig. 3 *A* and *B*), Miller pontotegmental neurons in the brainstem, motor neurons in the spinal cord, as well as associative and other still unidentified nerve cells exhibited a larger size and a more intense basophylia in NGF-injected than in vehicle-injected tadpoles. The hypothesis that these effects resulted from specific binding and interaction of m-NGF with a large number of nerve cells at an incipient stage of their differentiation received support from studies on ^{125}I-NGF uptake in the brains of stage-46 tadpoles. Fig. 4 compares the accumulation of radioactivity in brain tissue of specimens injected with ^{125}I-NGF or ^{125}I-IgG. More than 95% of the ^{125}I-NGF injected into the periorbital area and incorporated in the CNS was trichloroacetic acid-precipitable. Its localization in the spinal cord and brain sections of tadpoles fixed 2 hr after NGF injection is shown in microphotographs in Fig. 5. Most of the ^{125}I-NGF grains were concentrated on dorsal funiculi in the spinal cord (Fig. 5*A*), dorsolateral sensory fiber tracts originating from cephalic ganglia (Fig. 5*B*), and round-shaped cells lining the third ventricle (Fig. 5 *C–E*). Individual cells and cell clusters throughout the whole cerebrospinal axis and longitudinal fiber tracts running from the spinal cord to the rostral section of the diencephalon, illustrated in Fig. 5*G*, were covered with silver grains, although less densely than the above-mentioned fiber and cell systems. Injection of ^{125}I-NGF with an excess

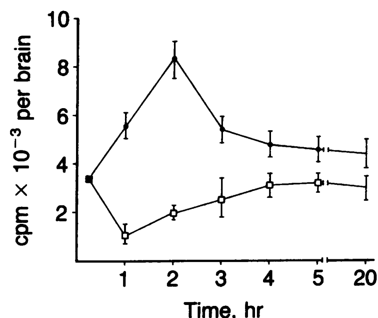

FIG. 4. Time-course accumulation of radioactive NGF in the isolated brain of *X. laevis* tadpoles at stage 46 injected in the right periorbital area with 2 μl of ^{125}I-NGF (●) or 2 μl of ^{125}I-IgG (□). Values are means ± SEM of four determinations.

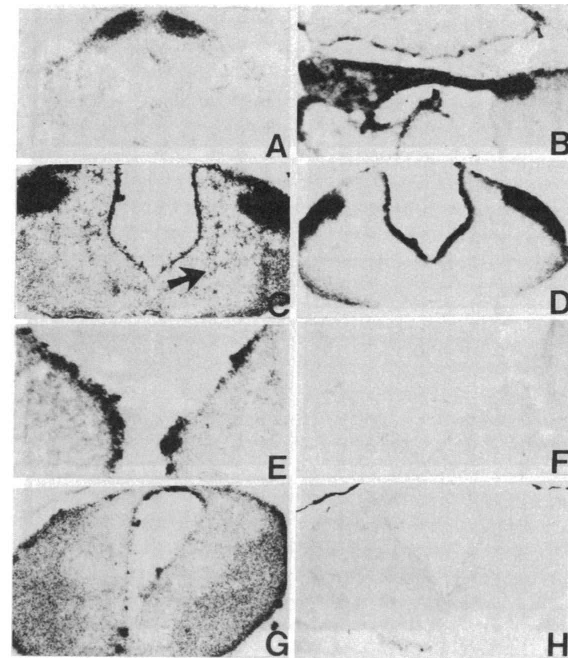

FIG. 5. Radioautographic histological sections of the CNS of *X. laevis* tadpoles at stage 46 following injection with ^{125}I-NGF (50 ng per tadpole) alone or with an excess of unlabeled NGF; fixation was 2 hr later. (*A*) Transverse section through the lower spinal cord showing the dorsal funicular area heavily labeled. (×140.) (*B*) Transverse section through the caudal medulla oblongata showing the intense labeling of the postotic ganglion, its nerve, and the afferent dorsal fiber bundles projecting into the medulla. (×140.) (*C* and *D*) Caudal and rostral brainstem sections showing heavily labeled fiber tracts afferent to the brainstem from the pre- and postotic ganglia. In *C*, intrinsic fiber tracts (arrow) and scattered cell clusters are also labeled. (*C*, ×180; *D*, ×140.) (*E*) Round-shaped heavily labeled cells bordering the third ventricle. (×106.) (*F*) Comparative histological section injected with ^{125}I-NGF and an excess of unlabeled NGF. (×106.) (*G* and *H*) Sections through rostral diencephalon injected with ^{125}I-NGF alone (*G*) and an excess of unlabeled NGF (*H*). Intrinsic fiber bundles in the ventrolateral area are also covered with iodinated NGF. (×73.)

of unlabeled NGF prevented completely the labeling of intracerebral nerve cells and fibers, as shown in Fig. 5 *F* and *H*.

Catechol/Indoleaminergic Systems. In stage-48 tadpoles, aminergic neurons are at the incipient phase of their differentiation. In histofluorescent transverse-sectioned preparations, small green fluorescent cell clusters—identified as belonging to the preoptic recess organ, the paraventricular organ, and the nucleus infundibularis dorsalis—are assembled around or at a short distance from (as in the case of the nucleus infundibularis dorsalis) the third ventricle. Upon m-NGF treatment, the same centers consist of markedly more fluorescent nerve cells that cover a larger area than in untreated specimens (Fig. 6 *A–F*). The number and cell size of individual units in NGF-injected tadpoles are markedly increased in pontomesencephalic reticular cells as compared to those of vehicle-injected matched specimens (Fig. 6 *G* and *H*).

Preliminary Neuropeptide Immunofluorescence Studies. Serial sections of tadpoles in stages 46–50 submitted for a 5-day period to NGF or vehicle solution injections were processed for immunoreactivity to enkephalin, SP, and Som. The weak histofluorescence reactivity to enkephalin monoclonal antibodies did not permit an evaluation of its distribution in brain sections of experimental and control specimens. Instead, immunoreactivity was quite evident in neuronal somata and

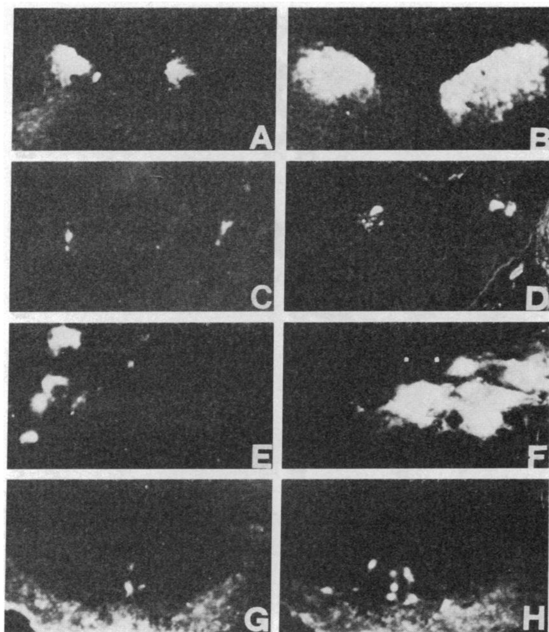

FIG. 6. Formaldehyde-induced fluorescence of tadpole brains at stage 48 injected for 6 days with vehicle (*A, C, E, G*) or NGF (*B, D, F, H*). (*A* and *B*) Comparative brain sections showing aminergic neurons in the paraventricular organs. (×50.) (*C* and *D*) Comparative brain sections showing the aminergic cells of the nucleus infundibularis dorsalis. (×15.) (*E* and *F*) Cells of nuclei infundibularis dorsalis at higher magnification. (×122.) (*G* and *H*) Comparative brain sections showing fluorescent nerve cells and fiber tracts in the pontomesencephalic reticular area. (×43.)

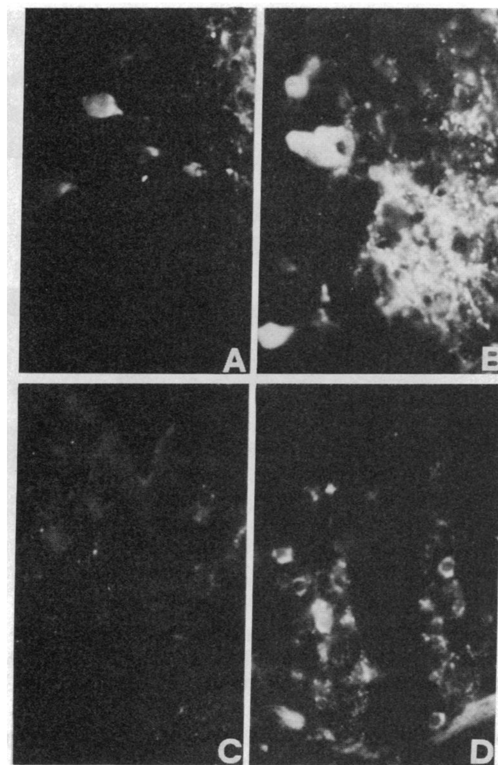

FIG. 7. (*A* and *B*) SP immunoreactive cells and fiber tracts in the mesencephalic area of tadpole brain at stage 48 injected for 6 days with vehicle (*A*) or NGF (*B*). (×86.) (*C* and *D*) Som immunoreactive nerve cells in the pontomesencephalic area of tadpole brains injected for 6 days with vehicle (*C*) or NGF (*D*). (×75.)

fiber network in diencephalic and pontomesencephalic segments processed to visualize SP and Som. Although in these preliminary studies no attempts were made to investigate the respective regional distributions of these two neuropeptides, the reactivity to SP was stronger in diencephalic than in pontomesencephalic centers, whereas the opposite was true for immunoreactivity to Som. In both experimental series, immunohistofluorescence was more pronounced in nerve cells, their dendrites, and axonal processes in sections of NGF-injected specimens than in control specimens. The more vivid fluorescence, larger somata size, and denser fibrillar network around cells immunoreactive to SP and Som is illustrated in Fig. 7 *A–D*.

***In Vitro* Studies.** Nerve cells dissociated from brain tissue of tadpoles between stage 46 and stage 48 were cultured for a 2- to 3-week period in the presence or absence of 10–50 ng of NGF per ml. The finding that these cells survived and produced axonal processes only upon addition of NGF to the culture medium provided evidence for its vital trophic role. Autoradiographic studies showed that the biological effect was mediated by NGF binding to specific receptors present in a fairly large number of cells; labeling of nerve cell somata in the presence of ^{125}I-NGF did not, in fact, materialize upon addition of excess unlabeled NGF to the incubation medium.

DISCUSSION

On the basis of previous reports (6–9), we anticipated that sensory and sympathetic amphibian nerve cells would be receptive to the growth and differentiation effects of m-NGF. The results, however, greatly exceeded our expectations with respect to the magnitude of the response elicited by NGF in early differentiated sensory neurons and to its range of action, which, in this species, also extends to several neuronal cell populations of the CNS. We shall here consider only the latter aspect of the biological activity of this protein molecule. In fact, with the exception of three reports on the enhanced regeneration elicited by m-NGF on surgically transected fish optic nerves (7–9), no attempts have been made to investigate whether other neuronal systems in lower vertebrates are receptive to the NGF differentiative effect. This effect is now experimentally demonstrated in several nerve centers in the amphibian CNS; the list of these cells is likely to increase as these studies are extended to other neuronal systems. Favoring this hypothesis is the finding that NGF selectively binds to a large number of nerve cells in the CNS of *Xenopus* tadpoles.

These observations call for some comment. The strikingly intense labeling of dorsal funiculi in the spinal cord and the dorsolateral fiber bundle collecting the central processes of sensory neurons of cephalic ganglia could result from (*i*) an anterograde ^{125}I-NGF transport, (*ii*) a direct binding to nerve fibers, or (*iii*) a retrograde ^{125}I-NGF transport along these fiber bundles. It is not possible at present to assess which of these mechanisms is responsible for the ^{125}I-NGF distribution. Previous studies performed on the mammalian nervous system would favor the latter hypothesis (19–21), which is also supported by a recent report (22, 23) showing that ^{125}I-NGF injected into the spinal cord of neonatal rats undergoes retrograde transport from the nerve endings to the cell compartments of sensory neurons in the dorsal root ganglia. The recent demonstration of a surprisingly high level of NGF mRNA in several mammalian tissues, including the brain, shows that the CNS shares a common property of most organs and vertebrate cell lines—synthesis of NGF (24). Also in favor of this hypothesis is a report based on immunohistochemical studies in goldfish brain, indicating that ependymal cells or astrocytes are the putative source of NGF in the CNS of these lower vertebrates (25). A local NGF production does not, however, rule out the possibility that

during early ontogenetic stages, when nerve cells are in their most active growth and differentiative phase, other extracerebral NGF sources supply additional amounts of the growth factor.

The results reported here lend support to this view. Exogenous NGF injected into the periorbital area gains access to the CNS, as shown from the high uptake of labeled NGF in brain tissue. Autoradiographic studies with ^{125}I-NGF gave evidence of the extraordinarily dense accumulation of silver grains in round-shaped cells lining the third ventricle and cerebral aqueduct. They were identified as cerebrospinal fluid contacting neurons, although an alternative hypothesis that cannot at present be definitely discarded is that they are specialized ependymal cells or tanycytes. Both cell types belong to the complex of midline structures designated as the circumventricular organs, which have recently aroused great interest in view of the very high content of the peptides thyrotropin-releasing hormone, luteinizing hormone-releasing hormone, and Som in some of these cellular aggregates and in the cerebrospinal fluid (26). Lack of the blood–brain barrier in most of these circumventricular organs allows the free circulation through these structures, viewed as "windows in the brain" (27), of these and other peptides as well as of steroid hormones, which would, according to some authors, impart a functional organization upon neurons in the hypothalamus (28). The present finding of the strikingly high uptake of NGF in cells lining the third ventricle suggests the possibility that, in these lower vertebrates, NGF may be endowed with an important physiological role in brain organization and function, at least during these premetamorphic stages.

After the present study was completed, the widespread expression of NGF receptors in neuronal and nonneuronal cell populations in chicken embryos and their marked decrease in late developmental stages was reported (29).

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