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TARGET CELLS, BIOLOGICAL EFFECTS, AND MECHANISM OF ACTION OF NERVE GROWTH FACTOR AND ITS ANTIBODIES

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INTRODUCTION

Nerve growth factor (NGF) (1–5) has been detected at high concentrations in mouse submaxillary glands (1, 2); in the prostate glands and seminal plasmas of the guinea pig, rabbit, bull, sheep, and goat (4, 6, 7); and in snake venoms (1, 8). The administration of NGF[1] causes impressive morphological and biochemical changes in responsive target cells, all of which are derived from the neural crest. However, these "pharmacological" actions of NGF by no means imply a physiological function for NGF. Indeed, the high levels of NGF in these potent sources do not seem to play an essential physiological role in the development and maintenance of function of the responsive neurons in these species. This can be deduced from the following evidence: (*a*) Neurons in species without rich sources of NGF (e. g. rat) do not differ in their biochemistry or morphology, or in their responses to administered NGF or to NGF antibodies, from neurons in the mouse, a species with a known potent source of NGF. (*b*) Neurons innervating potent sources of NGF do not differ in any of the above ways from other neurons in the same species innervating effector organs devoid of detectable NGF. (*c*) The regulation by androgens of NGF production in the mouse

[1]NGF, for the purposes of this review, is defined as a group of macromolecules, each having the biological activities of the NGFs listed, usually mouse NGF, and whose activity is inhibited by antibodies to these NGFs (4).

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submaxillary glands (1), and in the prostate glands of other species (6, 7), is not accompanied by any detectable sex difference in the responsive neurons in these species (1). (*d*) Removal of the NGF sources has no general deleterious effect on the responsive neurons of the animal (1, 9, 10), other than the direct consequences for the neurons innervating the source tissue (see section on deprivation of endogenous nerve growth factor). These observations demonstrate that the high quantities of NGF detected in these sources are normally neither released into the circulation (4, 5, 11), nor directly transferred to their innervating neurons (see section on deprivation of endogenous nerve growth factor), but are exclusively released into the saliva (12), semen (4), or venom (8). The possible physiological functions of NGF in these glands and their secretions remain to be established, but they can be ignored in the following discussion of the physiological relevance of NGF for neurons.

However, there is a considerable body of indirect evidence that implies that effector organs innervated by responsive sympathetic and sensory neurons produce very low levels of NGF, and that it is *this* endogenous NGF which regulates neuronal development and the maintenance of function of differentiated neurons (see section on deprivation of endogenous nerve growth factor). Current assays, though able to detect nanogram quantities of NGF (1, 4, 5, 11, 13), are not sensitive enough to detect the small amounts produced by effector organs (4, 5, 11, 13). Thus the direct verification of this mechanism is not yet possible. The most persuasive indirect evidence is the ability of antibodies against NGF to interfere with the normal development of target cells, and with the maintenance of function in fully differentiated neurons.

This review therefore summarizes the effects of exogenous (administered) NGF on its target cells (for more details, see 1, 2, 5, 11), though only as far as is necessary for an analysis of the actions of NGF antibodies, which are discussed in more detail as evidence for a corresponding physiological role for *endogenous* NGF.

PRODUCTION AND CHARACTERIZATION OF ANTIBODIES TO NERVE GROWTH FACTOR

NGF appears to be a relatively potent immunogen, and antisera have been raised in many species to NGFs purified from the mouse submaxillary gland (1, 14–17), guinea pig prostate gland (18), and several snake venoms (8, 10, 17). The potency of an antiserum (1, 8, 15, 19) is normally determined in the standard biological assay for NGF (1, 4, 5, 11), in which 1 biological unit (BU) of NGF (usually 5–10 ng purified mouse NGF) per milliliter culture medium induces optimal outgrowth of nerve fibers from explanted

chick sensory ganglia (see section on sensory neurons). The antiserum titer is defined as the maximal dilution of the antiserum (in the final culture medium) that inhibits this optimal fiber outgrowth (1, 8, 15, 19); titers of the order of 2,000–15,000 are usual. Unfortunately, as the potency of NGF preparations and the exact conditions of the biological assay differ quite extensively between laboratories (1), it is sometimes difficult to assess the stated potencies of different antisera (19). NGF antibodies can be isolated from whole antisera by affinity chromatography (16), which enables experiments to be made under more defined conditions. The resolution of the poor comparability of results using antibodies from different laboratories (19) can soon be expected as monoclonal antibodies of constant, defined quality become available from stable clones of hybridoma cells.

Although the quantitative data depend on the particular antisera being investigated, very much higher concentrations of antisera to snake NGFs are required to inhibit the fiber outgrowth induced by mouse NGF in the biological assay than are needed to inhibit the effects of the homologous snake NGFs (8, 15). Similarly, antisera to mouse NGF are only very poorly effective in inhibiting the fiber outgrowth due to snake NGFs (8, 15). In some cases, the cross-reactivity is too low to be measured (15), and the specificity of the inhibitory effect must be questioned when very high concentrations of whole antisera are required in the biological assay. In contrast, antisera to mouse NGF are approximately equally potent against all the mammalian NGFs (4, 6, 7), and the antisera to various snake NGFs also exhibit substantial cross-reactivity (8, 15). Immunochemical studies (immunodiffusion, complement fixation, comparative radioimmunoassay) further demonstrate the substantial immunological differences between mammalian and snake NGFs (8, 20). Furthermore, their greater resolution reveals appreciable immunological divergence *within* the mammalian group of NGF molecules (4, 6, 7). In spite of these immunological differences, the biologically active site of all the NGFs must be the same or very similar, because they have, as far as has been investigated, the same biological properties (4, 6, 7, 8, 17, 20). Moreover, snake venom and mouse NGFs can largely (80%) displace each other from the NGF receptor on dorsal root ganglia of chick embryos (20) (see section on sensory neurons).

BIOLOGICAL EFFECTS OF NERVE GROWTH FACTOR AND ITS ANTIBODIES ON THEIR TARGET CELLS

Sympathetic Neurons

The biological effects of NGF have been most thoroughly characterized in sympathetic neurons. NGF administration to neonatal mice and rats has a

general growth-promoting effect on these neurons, reflected biochemically by an increase in the protein and RNA content (1, 2, 5, 21, 22), and morphologically by a rearrangement of the packed stacks of rough endoplasmic reticulum over a larger area of the cytoplasm and by an enhanced formation of Golgi cisternae (23–25). This general growth effect is preceded by a marked induction of ornithine decarboxylase (ODC) (11, 26), the rate-limiting enzyme in polyamine synthesis, whose induction is frequently associated with rapid growth and regeneration. The general growth effect becomes less marked, but does not disappear, in older animals (23, 27, 28), which is consistent with a substantially smaller induction of ODC by NGF in adult animals (11).

In addition to this general growth effect, NGF accelerates the differentiation of sympathetic neuroblasts into mature neurons. This is evident morphologically in the formation of large amounts of cytoskeletal constituents (21, 23), followed by an accelerated outgrowth of nerve fibers (1, 21, 24, 27, 29–31). Moreover, NGF exerts a marked chemotactic influence on the outgrowth of nerve fibers in vivo (1, 32) and in vitro (33). Once established, NGF-induced fiber outgrowth appears to remain fairly constant throughout life. Thus, NGF stimulates axonal outgrowth in vitro from the sympathetic ganglia of 14.5 day mouse embryos (34), and an enhanced axonal outgrowth is still evident in vivo in adult mice (27, 35) and adult guinea pigs (28). The apparent decline in fiber outgrowth from mouse sympathetic ganglia in vitro after 10 days postpartum (30) probably reflects poor accessibility of NGF to the relatively large ganglion, and an inhibition of fiber outgrowth by the increasingly tough connective tissue capsule (see 1, 36).

Biochemically, the enhanced differentiation stimulated by NGF is also shown by a selective induction of tyrosine hydroxylase (TH) and dopamine β-hydroxylase (DBH) (21, 25, 37–40, 132), enzymes involved in the synthesis of the neurotransmitter (norepinephrine) that is the key marker of differentiated function in these neurons. Consequently, catecholamine levels increase in both sympathetic neurons (1, 29, 41, 42) and their innervated effector organs (1, 27, 29, 35, 43, 44), though part of the latter effect is due to increased fiber outgrowth. The third enzyme involved in catecholamine synthesis (dopa decarboxylase, DDC) is increased only in proportion to the overall increase in protein content (21, 38, 39). In contrast to the induction of ODC, which reflects the general growth-promoting effect of NGF, and which decreases with age (11), the induction of TH and DBH remains essentially the same from birth to adulthood (11, 45, 46).

NGF is also essential for the survival of sympathetic neurons in vivo. Normally in the late embryonic and early postnatal development of mammals, "excess" neurons in the sympathetic ganglia die (47–49). NGF administration allows these "excess" neurons to survive, causing an apparent neuronal hyperplasia (21, 43, 47, 48, 50). If NGF treatment is stopped, at

least some of the extra cells die (47; see also 22). The importance of NGF for the regulation of sympathetic neuronal survival is also evident in vitro: Sympathetic neurons, dissociated from the ganglia of neonatal mice and rats (2, 41, 42, 51) or young embryonic chicks (1, 2, 31), and cultured in the absence of non-neuronal cells, die very rapidly unless NGF is added to the medium. Non-neuronal cells produce NGF and other factors that can support neuronal survival in mixed cultures or intact ganglia (2, 41). As the age of the animal from which the ganglia are taken increases, this in vitro dependence of the isolated sympathetic neurons on NGF diminishes (D. Edgar, unpublished results). Eventually, sympathetic neurons can survive in vitro in the absence of NGF, provided they are supplied with other neurotrophic agents (D. Edgar, unpublished results). Moreover, this "maturation" can also partially occur in vitro (42, 51).

As has been demonstrated for many biologically active polypeptides, NGF has been shown to act on its target cells via specific membrane receptors (52). Recently, two classes of NGF receptor ($K_d \simeq 10^{-11}$ and $\simeq 10^{-9}$M) have been detected on intact dissociated neurons from embryonic chick sympathetic ganglia (53). There is also indirect evidence for two classes of receptor on the nerve terminal membranes of adult rat sympathetic neurons in vivo (54). The effects of NGF on nerve fiber outgrowth and neuronal survival require much lower concentrations of NGF (ng/ml) than do those on TH induction and the stimulation of catecholamine production (31, 41, 45). This might indicate that the former effects are mediated via the higher affinity receptors, and the latter via the lower affinity receptors (see also section on sensory neurons).

The concept that NGF acts as a retrograde messenger between effector organs and innervating neurons is discussed in the section on deprivation of endogenous nerve growth factor (see also 4, 5, 11). It has been demonstrated that exogenous NGF is taken up at adrenergic nerve terminals, following a highly specific and saturable binding to the specific membrane receptors present on these terminals (54). The NGF is internalized and transported retrogradely up the axon (54–61), within membrane-bound vesicles (59, 60), to the nerve cell bodies, where it exerts its characteristic effects; that is, it induces TH (56, 58) and causes neuronal hypertrophy (58). As there is no evidence that the NGF accumulated in the cell body is released as such into the cytoplasm, nucleus, or extracellular space (59, 60), but rather that the NGF passes within its vesicles to secondary lysosomes for degradation (59, 60), it is likely that as yet unknown second messengers mediate the effects of the transported NGF in the neuronal cell body (see 5, 11).

All these studies have delineated the "pharmacological" effects of exogenous NGF; the physiological significance of these responses has been indicated by the actions of NGF antibodies during development and in adult

animals. Thus, the involvement of endogenous NGF in the regulation of the survival of sympathetic neurons is demonstrated by the irreversible degeneration of virtually all the neurons in the pre- and paravertebral sympathetic ganglia caused by treatment of neonatal mice or rats with NGF antibodies (1, 17, 19, 49, 62–64). The destruction of the adrenergic neurons, termed immunosympathectomy (1), is also evident in the *permanent* and substantial decreases in the levels of the neurotransmitter (norepinephrine) and of the corresponding enzymes (TH, DBH, and DDC) in both the ganglia (14, 37, 49, 65–67) and, subsequently (49), in the denervated effector organs [49; for reviews, see (14, 68)].

Several studies have been made on the effects of NGF antibodies during prenatal life, by injecting pregnant mothers with antibodies (passive immunization) (see 19), or with NGF (active immunization) (67, 69), but the interpretation of these investigations is difficult because it is not known how effective is the transfer of antibodies across the placenta to the embryo (see 19). For example, transplacental transfer of antibodies does not affect the adrenal medulla (69), but direct injection of the embryos reveals a potent action of the antibodies on these cells (70) (see section on adrenal chromaffin cells). Direct injection of the embryos must therefore be the method of choice for meaningful prenatal studies; unfortunately these studies are only now beginning to be made. Treatment of rats at gestational days 16–17 causes effects in sympathetic ganglia that are similar but more pronounced than those induced neonatally (71). NGF antibodies administered in utero are already able to decrease the TH levels permanently in mouse sympathetic neurons from the twelfth gestational day, but the effects are proportionally less than at later embryonic stages (50). The dependence of these neurons on endogenous NGF therefore seems to *increase* during prenatal development. This is even more apparent in vitro (34): NGF antibodies reduce the nerve fiber outgrowth and the TH content of ganglia from 18-day-old embryonic mice, but are *without* effect in ganglia from 14-day-old embryos, though these ganglia respond to *exogenous* NGF (34). The apparently reduced dependence on NGF of the younger neurons in vitro, compared to in vivo, may be due to an enhanced involvement in culture of other neurotrophic agents, e. g. from ganglionic non-neuronal cells (2).

Postnatally, the destructive effects of antiserum treatment *decline* very rapidly as the initiation of treatment is delayed (64, 66, 72, 73), and this is consistent with the decreasing dependence of older sympathetic neurons on exogenous NGF in vitro. Treatment of adults with single or multiple doses of NGF antibodies causes temporary decreases in neuronal size (72, 73; see 74), and ultrastructural changes indicative of decreased protein synthesis (72, 74), but no degenerating neurons can be seen (28, 64, 72, 73, 74). Biochemically, the NGF antibodies cause *temporary* decreases in TH and

DBH in the adult ganglia (66, see 65, 74), and hence *temporary* decreases in the catecholamine content of the neurons (73) and their effector organs (72, 73). Active immunization of adult rats with NGF, which induces the production of a continuously high concentration of circulating antibodies (74, 75), eventually produces degeneration of a limited number of sympathetic neurons (75), but these effects are incomparably smaller than those readily seen in neonatal mammals following only a single injection of NGF antibodies (see also 74).

Immunosympathectomy also demonstrates species-specificity, as would be expected from the immunochemical data (see section on production and characterization of antibodies to nerve growth factor). Thus, some antisera to mouse NGF seem to be less effective in rats than in mice (19), presumably as there is only partial immunological cross-reactivity between mouse and rat NGFs. However, immunosympathectomy is possible, using antibodies to mouse NGF, in rats, kittens, rabbits, and monkeys (19), and in hamsters and gerbils (13). Neonatal guinea pigs are already resistant to immunosympathectomy (with antimouse NGF) (19, 28), probably because of their greater developmental maturity at birth. However, transplacental transfer of NGF antibodies destroys sympathetic neurons in embryonic guinea pigs (67). Antisera to mouse NGF do not cause immunosympathectomy in birds, reptiles, and amphibia (19); preliminary reports suggest that anti-snake NGF is effective in these species (19) (but see section on central nervous system). Conversely, antisera to snake NGFs do not cause immunosympathectomy in mice (17, 19).

"Short" Adrenergic Neurons

In contrast to the classical pattern of the sympathetic innervation of peripheral effector organs, i.e. by "long" axons from sympathetic ganglia in the pre- and paravertebral chains, a system of "short" adrenergic axons from nerve cell bodies adjacent to or within the organs themselves has been described in some male (76, 77) and female (78) sex organs, the detailed pattern of innervation differing between species.

Early studies detected no morphological changes in these "short" adrenergic neurons, or in the innervation of their target tissues, following treatment with NGF (1, 19, 29) or its antibodies (1, 19, 43, 79). Moreover, biochemical studies showed that NGF-antiserum administration has no effect on the catecholamine content of male sex organs (14, 68) but partially reduces it in the mouse and rat uterus (14, 68), as a result of the loss of the "long" adrenergic innervation of the vasculature (78).

More recently, however, Goedert et al (46) have demonstrated that these "short" adrenergic neurons *do* respond to exogenous NGF; thus the levels of TH and DBH, but not DDC, are selectively increased in the vas deferens

of the neonatal and adult rat. NGF antibodies were again found to be without effect (46). The resistance of these neurons to NGF antibodies, in spite of an albeit reduced response to exogenous NGF, is discussed further in the section on deprivation of endogenous nerve growth factor.

Bjerre & Rosengren (77) found that a sensitivity of these neurons to NGF antibodies could be revealed, but only during nerve regeneration. NGF antibodies markedly inhibit the reinnervation of the male sex organs in adult mice, following destruction of the nerve terminals with 6-hydroxydopamine (6OHDA). The catecholamine content of the vas deferens and the "short" neurons is also decreased by the NGF antibodies, and the nerve cell bodies are slightly reduced in size. Conversely, exogenous NGF very slightly enhances the nerve regeneration, and this treatment induces a weak hypertrophic response in the nerve cell bodies (77). The effects of both NGF and NGF antibodies on the regeneration of these "short" neurons (77) are, however, substantially less than those on the regeneration of "long" adrenergic neurons (35, 80) (see section on deprivation of endogenous nerve growth factor).

Adrenal Chromaffin Cells

The sensitivity to NGF and its antibodies of adrenal chromaffin cells, embryologically derived from the neural crest and hence related to sympathetic neurons, has been the subject of considerable debate (37, 39, 43, 46, 70, 81). Recently, it has become clear that these cells exhibit the responses evident in sympathetic neurons, but that both the extent of the responses, and the developmental ages at which they are evident, are different.

The administration of repeated large doses of NGF to neonatal animals does not cause in the adrenal medulla (37) the substantial hypertrophy seen in sympathetic ganglia (23, 25, 27, 29, 37, 43, 47, 50, 58). However, NGF treatment of both neonatal and adult rats causes selective increases in the levels of TH and DBH, but not DDC, in the adrenal medulla (37, 39, 46); this response is followed by increases in catecholamine content and fluorescence (43). The effects on TH and DBH are smaller than in sympathetic ganglia (37, 39, 46), and, in contrast to the case in the ganglia, multiple injections of NGF do not further increase the enzyme levels in the medulla (39). The presence of cell membrane receptors for NGF on adult rat chromaffin cells is indicated by their selective ability to take up exogenous NGF (39).

Neonatal treatment with NGF antibodies causes no changes in medullary morphology and no death of chromaffin cells (37). However, the destruction of the sympathetic nervous system leads to a compensatory activation of the adrenal medulla (68), which is further enhanced as a result of the stress induced by the injections (37, 38). These effects explain the slight increases

in medullary TH and DBH (37, 46), followed by maintained or increased catecholamine content and turnover (14, 68), induced by the administration of NGF antibodies to neonatal (37, 46) and adult (46, 74) rats.

Thus, postnatal medullary cells respond to exogenous NGF, but do not depend on the endogenous factor for survival or for maintenance of normal function.

Prenatally, however, adrenal chromaffin cells both respond to exogenous NGF, and depend on endogenous NGF. Administration of NGF to rats from 17 days gestation causes a massive transformation of chromaffin cell precursors into cells with the morphology of adrenergic neurons, producing long nerve fibers (70). Colonies of chromaffin cells outside the medulla, which normally degenerate just before birth, are maintained and similarly transformed into neurons. If NGF treatment is stopped, these transformed cells die (cf "excess" sympathetic neurons), while direct administration of NGF antibodies to the fetus from 17 days gestation causes a permanent destruction of virtually all the adrenal medullary cells (70) (cf immunosympathectomy).

The physiological environments of adrenal medullary cells and sympathetic neurons differ significantly in that the former are continually exposed to very high concentrations of glucocorticoids from the surrounding adrenal cortex (see 81). The influence of these glucocorticoids on postnatal medullary cells can be removed by putting the cells into culture, where they survive in the absence of exogenous NGF (81) or in the presence of NGF antibodies (K. Naujoks, unpublished results), which is consistent with their loss of dependence on endogenous NGF in vivo. The medullary cells then reveal additional ("intrinsic") responses to exogenous NGF (81). Treatment with NGF thus induces the production of long nerve fibers, and the ultrastructure of the chromaffin cells becomes more like that of adrenergic neurons. NGF also induces TH in these cultured cells, the induction being modulated by glucocorticoids (81), as in sympathetic neurons (see 5, 81). The addition of glucocorticoids (in concentrations similar to those bathing the medullary cells in vivo) restores the chromaffin cell characteristics; thus nerve fiber outgrowth in the presence of NGF is abolished, and the ultrastructural appearance of normal medullary cells is restored (81). Consistently, glucocorticoids also partially inhibit the nerve fiber outgrowth induced by NGF from dissociated rat sympathetic neurons (81).

Preganglionic Sympathetic Neurons

Choline acetyltransferase (CAT) is a specific enzyme marker for cholinergic neurons, including, therefore, the preganglionic nerve terminals in sympathetic ganglia (see 38). Prolonged treatment with high doses of NGF leads to increases in the levels of CAT in neonatal rat superior cervical ganglia

(38, 40, 82). Morphologically, the NGF treatment increases the number of synapses (24, 40), and the number of preganglionic nerve fibers (40). The neonatal administration of NGF antibodies impairs the normal postnatal developmental increase in CAT in these ganglia in mice and rats (65, 66). With increasing age of administration, NGF antibodies become less effective, and in adult rats they cause only a temporary decrease in ganglionic CAT (66, see 65, 74). These biochemical effects have their morphological counterpart: NGF-antiserum treatment of neonatal rats causes a substantial decrease in the number of axons in the preganglionic nerve trunk (24, 83). This is consistent with the reduction in the compound action potential along the preganglionic nerve to the superior cervical ganglion of the neonatal mouse (30). NGF antibodies also cause a substantial loss of ganglionic transmission in the superior cervical ganglia of neonatal and young adult guinea pigs, as a result of a loss of synapses (28). These morphological and biochemical effects of NGF antibodies in preganglionic neurons can be mimicked by other treatments causing adrenergic neuronal loss (in neonates) or functional impairment (in adults) (see section on deprivation of endogenous nerve growth factor), e. g. postganglionic axotomy (28, 82–84), end-organ removal (9, 85), or treatment with 6OHDA (65, 83), colchicine (84, 86), or guanethidine (87). In the lattermost case, the corresponding loss of presynaptic neurons in the spinal cord has been demonstrated (87). The effects of postganglionic axotomy on ganglionic transmission and synapse number (28), and on CAT (82), can be prevented by the simultaneous administration of exogenous NGF (see section on deprivation of endogenous nerve growth factor).

The following evidence, however, indicates that these effects on the preganglionic sympathetic neurons do *not* result from a direct sensitivity to NGF but are *indirect* consequences of changes in the adrenergic postsynaptic neurons: (*a*) CAT is increased only where the cholinergic synapses end on NGF-sensitive adrenergic neurons, and not where they innervate other effector cells, e.g. heart muscle cells (38, see also 65). Interestingly, where cholinergic synapses end on adrenal medullary cells responding by selective enzyme induction, but not by hypertrophy, to NGF, there is no indirect stimulation of CAT by NGF (38). (*b*) Autoradiographic localization of NGF administered to adult rats detects no NGF in the preganglionic cholinergic neurons, and also excludes a transsynaptic transfer of NGF transported retrogradely up the postganglionic adrenergic neurons (59). So far, no clearly identified direct target cell for NGF has failed to demonstrate specific uptake of the factor.

These indirect effects of NGF therefore provide a further example of the well-established phenomenon by which the development and function of a neuronal center is regulated by the extent of its peripheral effector field.

Parasympathetic Neurons

NGF and its antibodies have no effects on the morphology or survival of cholinergic parasympathetic neurons in vivo or those maintained in vitro by other neurotrophic agents (88–90). However, mouse NGF binds to nerve terminals and is retrogradely transported up the axons of at least some of the cell bodies in the ciliary ganglion of the 1–2-day-old postnatal chick and the adult rat (91). No such effect is evident in the parasympathetic submandibular ganglion of the rat (91), and the experimental data for this phenomenon are too sparse to allow an assessment of the significance of the effects, but they might suggest that NGF plays an as yet unknown role in some parasympathetic neurons.

Some cholinergic neurons of the adult rat CNS also exhibit retrograde axonal transport of NGF (92); this requires NGF receptors (54), which have been detected in the brain (93, 94) (see section on central nervous system).

Sensory Neurons

NGF has been known to act on sensory neurons from the earliest investigations (1). As in sympathetic neurons, NGF is involved in the regulation of the survival, general growth, and differentiation of sensory neurons. The characterization of the biochemical manifestations of differentiation has been hampered by the absence of specific markers, such as are available for sympathetic neurons (TH and DBH). Recently, substance P has been suggested as a specific marker for sensory neurons (see 95, 96). Its usefulness has still to be evaluated, as it is neither exclusively located in sensory neurons nor present in *all* sensory neurons (see 95, 96).

In contrast to the sparse information on specific biochemical effects, a series of more general membrane effects of NGF have been investigated most thoroughly in chick sensory neurons in vitro. The addition of NGF almost immediately enhances the transfer of glucose, amino acids and RNA precursors into the chick sensory neurons (2, 97). These uptake mechanisms depend on the presence of sodium ions, the extrusion of which from the cell is enhanced by NGF (98). These general effects of NGF on the enhanced transfer of protein and RNA precursors, and of an essential substrate for energy metabolism, may represent an explanation for the general growth-promoting (see 99), and possibly also the survival, effects of NGF, but it is difficult to imagine how these general membrane effects could also be responsible for the more specific actions, such as the selective regulation of substance P levels and the regulation of fiber outgrowth (see below).

As in sympathetic neurons, NGF enhances the formation of nerve fibers in sensory neurons (1, 90, 99, 100). The direction in which the nerve fibers grow is also directed by concentration gradients of NGF in vitro (101–103)

and in vivo (1). NGF-induced neurite outgrowth in vitro is evident in chick sensory ganglia from about embryonic day 5 (90, 94), and has been reported in sensory ganglia from mice and rats at 13–14 days gestation (104). Subsequently, from around day 13 in embryonic chicks (36, 90), and between embryonic day 15 and birth in mammals (104), the fiber outgrowth induced by NGF from cultured intact ganglia gradually disappears. This is probably for reasons similar to those discussed for intact sympathetic ganglia (36, 100) (see section on sympathetic neurons), because *dissociated* neurons from neonatal rodents still respond to NGF by fiber outgrowth (2). However, NGF does not stimulate fiber outgrowth from, or cellular hypertrophy in, neonatal mammalian sensory neurons in vivo (1, 2, 57). The difference between the in vivo and in vitro responses of the neonatal rodent neurons might be explained as a further example of an extended responsiveness to NGF or its antibodies in regenerating neurons (as in "short" adrenergic neurons, qv) as placing these neurons in culture inevitably involves axotomy.

Although postnatal rat sensory neurons do not respond morphologically to NGF in vivo, they continue to respond biochemically, by selective increases in the levels of substance P (95, see also 96).

NGF seems to be an essential requirement for the survival of sensory neurons in vivo, although detailed quantitative studies are lacking. Thus, the administration of exogenous NGF to chick embryos maintains "excess" sensory neurons that otherwise degenerate as a physiological developmental process (1; cf 47, 48). The regulation of sensory neuronal survival by NGF is also apparent in vitro: Neurons dissociated from the ganglia of neonatal mice and rats (2) or young embryonic chicks (1, 97, 100, 105), and cultured in the absence of non-neuronal cells, degenerate in the absence of NGF. Non-neuronal cells in intact or dissociated ganglia can provide factors that support the survival of the neurons (2, 100). The dependence of the neurons on NGF diminishes with increasing age in vivo (100, 105) and in vitro (100), and eventually disappears as the neurons become dependent on other neurotrophic factors (105). The similarities to the regulation of survival of sympathetic neurons (see section on sympathetic neurons) are obvious.

Two classes of cell-membrane receptors for NGF have been characterized on dissociated sensory neurons from embryonic chicks (106) ($K_d = 2.3 \times 10^{-11}$ M; 1.7×10^{-9} M). As for sympathetic neurons, both neuronal survival and neurite outgrowth seem to be mediated only by the higher affinity receptors (94, 100, 106). The two receptors also seem to be differently regulated developmentally, as the selective induction by NGF of substance P (95) continues into later developmental ages than the stimulation of fiber outgrowth or of cellular survival. A similar phenomenon has been noted for the induction by NGF of TH and DBH in "short" adrenergic

neurons and adrenal chromaffin cells. Herrup & Shooter (36) have reported that NGF receptors disappear from the cell bodies of dissociated chick sensory neurons from about embryonic day 14. However, the continued existence, at least at the nerve terminals, of cell-membrane receptors for NGF is shown in mammals by the presence of the specific retrograde axonal transport of NGF in the dorsal root sensory neurons of the neonatal and adult rat (57).

These studies indicate that sensory neurons respond to *exogenous* NGF in ways broadly similar to the responses of sympathetic neurons. There are also recent indications of a prenatal dependence on *endogenous* NGF. In embryonic rats, transplacental transfer (67, 69) or direct embryonic injection (71) of NGF antibodies destroys the majority of the sensory neurons. However, the administration of NGF antibodies to *neonatal* mice and rats causes no degeneration of sensory neurons (67, 69, 71), and NGF antibodies do not reduce ganglionic substance P postnatally in the rat (95). This may indicate that endogenous NGF plays no role in the *postnatal* development and maintenance of function of these neurons, and that by the criterion of susceptibility to NGF antibodies, mammalian sensory neurons are regulated by endogenous NGF at a significantly earlier developmental age than are sympathetic neurons (cf adrenal chromaffin cells).

The only evidence for a physiological role for endogenous NGF in avian sensory neurons is indirect: That exogenous NGF maintains "excess" neurons during development (see above) *implies* that NGF antibodies should be able to enhance the physiological cell death occurring at this time, by analogy with the sympathetic neurons. However, antibodies to mouse NGF do not destroy embryonic chick sensory neurons (Y. A. Barde, unpublished results), probably as a result of limited cross-reactivity with avian NGF (see 15, 17). Antibodies against snake NGFs should be more effective in birds (see 19).

Non-Neuronal Cells in Sensory and Sympathetic Ganglia

Early studies on the effects of NGF described an increase in the mitotic index in sensory and sympathetic ganglia (1); this was thought to indicate a mitogenic action of NGF on responsive neurons (1). More recently, quantitative investigations showed that the direct effect of NGF is only to support the survival of "excess" neurons that normally degenerate (47, 48) (see section on sympathetic neurons). The enhanced mitosis is exclusively limited to non-neuronal cells (47, 48). However, NGF fails to induce mitosis in pure cultures of non-neuronal cells (107). The presence of neurons in these cultures causes mitosis of the non-neuronal cells (107, 108); the effect is mediated by cell-to-cell contact and is thus limited to the non-neuronal cells touching neurons (107, 108). Membrane fragments of neurons (109),

but not neuronally conditioned medium (107, 108), can mimic the effect. The addition of NGF to the mixed cultures enhances the neuronally induced mitosis of the non-neuronal cells (107). The effects of NGF on non-neuronal cells are therefore only indirect, and are mediated by interactions with NGF-responsive neurons. The mechanism of this effect is unknown, but it may be an example of the phenomenon demonstrated in several systems, by which the number of non-neuronal cells varies so as to maintain a constant number per unit area of neuronal cell membrane (83, 110, 111).

A similar indirect response of non-neuronal cells to NGF has been described in the regenerating newt optic nerve (111).

Consistent with this indirect response, but awaiting quantitative confirmation, is the reported eventual decline in the number of non-neuronal cells as a secondary consequence of the destruction of the sympathetic neurons in immunosympathectomized neonatal animals (1, 19, see 83).

Central Nervous System

Studies on the influence of NGF or its antibodies on the central nervous system (CNS) following systemic administration can be discounted, because neither NGF (112) nor antibodies in general cross the blood-brain barrier. Furthermore, recent investigations have shown that many of the reported effects of intracerebral injections of NGF are in fact due to the presence of renin activity in standard preparations of mouse NGF (113, 114).

The majority of the remaining studies indicate an absence of responsiveness in catecholaminergic CNS neurons to NGF and its antibodies, indicative of fundamental differences between central and peripheral adrenergic neurons. Thus, no selective retrograde transport of NGF can be detected, e. g. from the caudate nucleus to the dopaminergic neurons of the substantia nigra, or from the hippocampus to the noradrenergic neurons of the locus coeruleus (92), indicating that the NGF receptors detected in the brain (93, 94) are probably not on adrenergic neurons (see section on parasympathetic neurons). Furthermore, these adrenergic neurons fail to respond to NGF administration by changes in their TH levels (92). Moreover, intracerebral NGF (32) or NGF-antibody (115) administration has no effect, in neonatal mice and rats, on the catecholamine fluorescence of intact dorsal and medial forebrain bundles, or on the number, size, and catecholamine fluorescence of neurons in the locus coeruleus and other areas of the brain. These observations render difficult to rationalize the reports of temporary changes in catecholamine content and turnover following intracerebral NGF or NGF-antibody injection in neonatal or adult rats (115, 116). It is possible that these effects involve the activities of renin and angiotensin (see above).

Though these studies indicate the lack of a response of intact CNS

adrenergic neurons to NGF (and the absence of the corresponding receptors), a response to NGF (and so presumably the generation or unmasking of NGF receptors) has been reported during nerve regeneration (cf "short" adrenergic and sensory neurons). Thus, Bjerre and co-workers found that NGF (117) markedly stimulates and NGF antibodies (118) markedly inhibit the regrowth of transected norepinephrine-, dopamine-, and indoleamine-containing neurons (from the locus coeruleus, substantia nigra, and other CNS centers, respectively) into an iris transplanted across these dorsal and medial forebrain nerve bundles in the adult rat brain. Similarly, NGF significantly enhances (119, 120), and NGF antibodies inhibit (121, 122), the regeneration of axons in the newt optic nerve, with morphological changes in the retinal ganglion cells (120, 122) which are similar to those reported for peripheral sympathetic neurons (qv). The effectiveness of antibodies against mouse NGF in these amphibians is surprising, in view of the reported lack of immunosympathectomy in non-mammals treated with these antibodies (19).

On the basis of these experiments, a sensitivity to NGF and NGF antibodies would be expected under other conditions where nerve regeneration occurs, but (*a*) NGF has only an inhibitory effect on process formation from dopaminergic neurons of the substantia nigra in vitro (123), (*b*) neither NGF nor its antibodies influence the outgrowth of nerve fibers from the locus coeruleus in the anterior eye chamber or in vitro (124; see also 94), and (*c*) NGF and its antibodies are without influence on the depletion of catecholamines, and on the neural sprouting, caused by 6OHDA in the rat brain in vivo (115, 116). The reasons for these apparently discordant results are not yet clear.

MECHANISM OF ACTION OF NERVE GROWTH FACTOR AND ITS ANTIBODIES

At the appropriate developmental stages, NGF antibodies can destroy the target cells of NGF. Two mechanisms by which this might occur have been suggested: (*a*) fixation of complement, leading to lysis of NGF-responsive cells, and (*b*) deprivation of endogenous NGF, essential for neuronal survival and normal function. The experimental investigations of this phenomenon have been limited to sympathetic neurons (immunosympathectomy) but, in principle, sensory neurons and adrenal chromaffin cells are probably destroyed by similar mechanisms.

Evidence Against a Complement-Mediated Cell Lysis In Vivo

Complement fixation follows the binding of antibodies to the corresponding antigens on the cell membrane. As this can also occur if the antibodies are

linked to the membrane via a receptor ligand such as NGF (see 94), neurons binding exogenous NGF in vitro *can* be lysed in the presence of NGF antibodies and complement (62, 94). However, this need not be the mechanism of immunosympathectomy in vivo.

Perhaps the greatest difficulty in explaining the action of NGF antibodies in vivo by a complement-mediated mechanism is that it cannot explain the transition in the response (see section on biological effects of nerve growth factor and its antibodies on their target cells) from a destruction of the target cells at the appropriate stages of development, to a subsequent stage at which the antibodies either fail to influence the cells, or merely cause a temporary impairment of normal function, even though the cells continue to carry NGF receptors and therefore continue to respond to exogenous NGF. The ease with which the alternative mechanism (see section on deprivation of endogenous nerve growth factor) can rationalize this dual effect of the antibodies, and indeed the whole body of experimental observations, argues against a complement-mediated mechanism. Direct experimental evidence against a complement-mediated cell lysis is provided by the following observations: (*a*) NGF antibodies are still able to destroy sympathetic neurons in neonatal mice genetically deficient in the crucial complement component C5 (49, 125), and in mice partially deprived of complement component C3 by treatment with a cobra venom factor (125). (*b*) Neuronal cell death, though apparent in some cells very quickly (within 12 hr postinjection), occurs as a gradual and progressive phenomenon over many days after antiserum administration (19, 49, 62, 63). In contrast, complement-mediated cell lysis should affect all responsive cells more or less equally rapidly. (*c*) Most of the degenerative effects of the antibodies can be reversed for up to 48 hr by treatment with exogenous NGF (49), which is inconsistent with an irreversible lytic action.

Deprivation of Endogenous Nerve Growth Factor

The alternative mechanism by which NGF antibodies might act is as follows (5, 9, 11, 22, 33, 61, 73, 126, 127): Endogenous NGF is thought to be produced by effector organs, taken up by nerve terminals via the NGF receptors on the plasma membranes, and transported retrogradely to the neuronal cell bodies, as has been demonstrated for exogenous NGF. NGF antibodies act by sequestering this endogenous NGF during transfer between effector organ and nerve terminals. At the appropriate stages of development, loss of this vital supportive factor leads to death of the target cells, whereas subsequently their decreased dependence on NGF, as a result of their novel dependence on other trophic factors, means that the loss of endogenous NGF has at the most only temporary effects on normal func-

tion, and may be without detectable effect, though the cells continue to be sensitive to *exogenous* NGF.

This mechanism entirely fits the data presented in the previous section (on biological effects of nerve growth factor and its antibodies on their target cells), but the evidence in its favor remains indirect, namely, that various procedures that would interfere with the transfer of endogenous NGF from effector organs to neurons cause biochemical and morphological effects that exactly coincide with those caused by the administration of NGF antibodies (see section on biological effects of nerve growth factor and its antibodies on their target cells), and that these effects can be prevented by the simultaneous administration of exogenous NGF. Thus prevention of NGF transfer from effector organ to nerve cell bodies (55–57, 61, 112) by removal of the end-organ (85, 112), by destruction of the nerve terminals with 6OHDA (44, 65, 112, 129), by surgical axotomy (47, 55–57, 82, 126) or by blockade of axonal transport mechanisms with colchicine (55, 56, 127) or vinblastine (127), variously cause, in neonatal animals, an enhancement of the normally progressing sympathetic neuronal death (44, 47, 65, 85, 127, 129), reflected by *permanent* decreases in the levels of ganglionic TH and DDC (44, 47, 56, 65, 85, 126, 127, 128), and *permanent* decreases in the catecholamine content and uptake in peripheral effector organs (44) (see also section on preganglionic sympathetic neurons). The administration of sufficiently large amounts of NGF prevents the degenerative effects in the neuronal cell bodies (22, 44, 47, 82, 127), by acting on the NGF receptors also present on the membranes of the neuronal cell bodies themselves (52, 53). Consistently, the destruction of sympathetic neurons in neonatal mice by sequestering the endogenous NGF supplies with antibodies to mouse NGF can be prevented by the simultaneous administration of snake NGF (125). The snake NGF cannot directly inhibit the antibodies to mouse NGF because there is virtually no immunological cross-reactivity (15).

The progressive degeneration of increasing numbers of sympathetic neurons 12–72 hr after administration of NGF antibodies to neonatal rats (19, 49, 62), and the ability substantially to reverse the deleterious effects with exogenous NGF during the first 48 hr (49), speaks in favor of a deprivation of endogenous NGF by the antibodies which gradually causes the neurons to degenerate. The initial stages of this process are not irreparable, but prolonged NGF deprivation allows the deleterious process to progress beyond a critical stage, and cell death must follow.

The effects of interfering with supplies of endogenous NGF in adult animals can be divided into two classes. First, relatively brief interruptions of supply by (*a*) temporary destruction of the nerve terminals by 6OHDA (61, 129), (*b*) blockade of axonal transport by vinblastine (61, 127) or

colchicine (86, 129), or (*c*) treatment with NGF antibodies (19, 64, 66, 72, 73) produce *temporary* reductions in ganglionic TH, DBH, and DDC levels (66, 127), but no neuronal death occurs (19, 64, 72, 86, 127, 129). Second, longer-lasting treatments, i.e. (*a*) surgical axotomy (28, 84, 120, 126), (*b*) end-organ removal (9, 10, 128), or (*c*) prolonged sequestration of endogenous NGF supplies following active immunization against NGF (74, 75), can *eventually* cause degeneration of limited numbers of neurons (28, 75), with the corresponding biochemical consequences (9, 75, 84, 128). This indicates that the maintenance of the neurons by other neurotrophic agents is probably barely adequate. However, these effects are incomparably slower and smaller (10, 71, 120, 126) than the degenerative effects seen in neonates.

The only evidence for the outlined mechanism in sensory neurons is the observation that exogenous NGF prevents the decrease in the substance P content of neonatal rat sensory neurons caused by removal of the peripheral field (95).

It is possible to rationalize the behavior of "short" adrenergic neurons (see section on "short" adrenergic neurons) according to this mechanism. It is unlikely that these "short" neurons fail to respond neonatally to NGF antibodies because they depend on endogenous NGF at an earlier developmental age than do "long" sympathetic neurons (as is evident for adrenal chromaffin cells and sensory neurons) as their development parallels that of the "long" adrenergic neurons (130). However, the nerve terminal-effector cell distances in tissues innervated by "short" adrenergic neurons are particularly narrow (e. g. in the vas deferens 10–30 nm; cf in blood vessels innervated by "long" adrenergic neurons, 50–400 nm), with the terminals of the "short" neurons often being intimately ensheathed by the effector cells (131). This intimate contact may make the transfer of endogenous NGF to the nerve terminals particularly efficient and resistant to interference by NGF antibodies (46). Administered (exogenous) NGF can still elicit responses from these cells as it can reach NGF receptors on the membranes of the cell bodies via the circulation (46), though the inaccessibility of the additional receptors on terminal membranes, or an almost optimal neurotrophic supply from the effector cells, might render the effects of exogenous NGF less apparent than in "long" adrenergic neurons. 6OHDA can cause degeneration of these nerve terminals, as a result of a greater accessibility to this smaller molecule (77). Moreover, nerve-terminal destruction by 6OHDA abolishes the intimate passage of endogenous NGF from the effector cells, and so the regenerative ability of these neurons becomes subject to a greater interference by NGF and its antibodies (77; see section on "short" adrenergic neurons).

CONCLUSIONS

1. *Exogenous* NGF acts directly on sympathetic neurons, sensory neurons, and adrenal chromaffin cells; possible actions on parasympathetic neurons require further investigation. NGF indirectly affects preganglionic sympathetic neurons, and non-neuronal cells in sensory and sympathetic ganglia.

2. The ability of NGF antibodies to interfere with the normal development and function of sympathetic and sensory neurons, and of adrenal chromaffin cells, indicates that *endogenous* NGF plays a physiological role in these target cells. Endogenous NGF is essential for adrenal chromaffin cells and sensory neurons at significantly earlier developmental ages than it is for sympathetic neurons.

3. Endogenous NGF is thought to be produced by effector organs, and retrogradely transported to sympathetic and sensory nerve cell bodies. NGF antibodies cause their disruptive effects by sequestering this supply of endogenous NGF, and thereby inducing the effects of NGF deprivation in the target cells.

4. Central adrenergic neurons seem to differ from their peripheral counterparts in that they are generally unresponsive to NGF and its antibodies.

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