Annu. Rev. Pharmacol. Toxicol. 1991. 32:303–28

THEORETICAL BASIS FOR A PHARMACOLOGY OF NERVE GROWTH FACTOR BIOSYNTHESIS

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KEY WORDS: steroids, interleukin-1, β-adrenergic receptor, cAMP, Alzheimer's

INTRODUCTION

Neuronal plasticity is a term widely used to indicate a number of neuronal mechanisms that are coordinated and triggered in the peripheral and central nervous system (CNS) to overcome functional impairment due to damage evoked by disease processes and trauma. Though this term is mainly descriptive for various molecular processes whose operation and nature are still unclear, we now believe that neuronal plasticity is controlled by a variety of trophic factors produced by neuronal and glial cells. Nerve growth factor (NGF) could be one of these trophic agents. Indeed, several lines of evidence assign to NGF a role as trophic factor for specific populations of central cholinergic neurons. It is currently thought that NGF might be used to prevent the progression of neurodegenerative diseases. However, its inability to cross the blood brain barrier prevents NGF from being easily used in CNS disorder therapy. One alternative that should be explored is the possibility of enhancing NGF availability via a pharmacologically induced increase of NGF biosynthesis and release in the CNS.

At present, the mechanisms that regulate the synthesis and release of NGF are still unknown. These mechanisms must be characterized before drugs can be developed that will ameliorate the symptoms of neurodegenerative diseases

0362-1642/92/0415-0303$02.00

by enhancing brain NGF content. Hence, this review addresses in vitro and in vivo studies, showing that a variety of agents, such as interleukin-1, steroids and neurotransmitters, enhance the production of NGF in the CNS. The molecular mechanisms of the regulation of NGF biosynthesis are also discussed as a useful source of information for the development of pharmacological agents that may slow down the progression of neurodegenerative disease.

Functional Significance of NGF in the CNS

The functional significance of NGF in the brain and its mechanism of action have already been elegantly reviewed elsewhere (1–4). However, recapitulation of some of this information is needed as a background to the pharmacology of NGF, the main topic of this review.

In this review the term NGF refers to the β-NGF, the biologically active protein dimer generated from a cleavage of a larger biologically inactive precursor molecule (5–8). NGF was discovered and characterized as a trophic factor for the peripheral nervous system (PNS) (9). In recent years, evidence has been accumulated on the trophic activity of NGF in the CNS as well. Recombinant DNA techniques together with NGF and NGF receptor protein determinations have been used to demonstrate unequivocally that NGF and NGF receptor molecules are synthesized in the rat CNS. Interestingly, those regions of the brain and spinal cord containing cholinergic neurons or their fibers show high expression of NGF and/or NGF receptor genes (10–16). Moreover, ontogenetic studies have demonstrated a good correlation between the onset of NGF gene expression and the development of the cholinergic neurons of the rat basal forebrain (17, 18). These findings suggest that the differentiation, survival, and maintenance of a selective population of cholinergic neurons in the CNS may depend on NGF availability.

Cholinergic neurons of the rat basal forebrain innervate the hippocampal formation, the neocortex, the olfactory bulb, and other structures (19). NGF produced in these structures can be retrogradely transported to the basal forebrain cell bodies (20) to activate the expression of specific proteins (21) including choline acetyltransferase (ChAT) (22), the key enzyme regulating the synthesis of acetylcholine. Indeed, a considerable body of evidence demonstrates that, in rats, intracerebroventricular administration of exogenous NGF elicits a trophic effect on forebrain cholinergic neurons. For example, NGF infused for several days can reverse the atrophy and loss of ChAT in cholinergic septal neurons caused by lesions of the fimbria-fornix (23–27) and mediate cholinergic neuron hypertrophy in damaged striatum (28). Prolonged NGF treatments also cause hypertrophy and increase in ChAT activity in uninjured adult rat septal cholinergic neurons (29) and in developing rat basal forebrain neurons (30). These studies all provide suppor-

tive evidence for the hypothesis that pharmacological manipulations that elevate endogenous NGF levels will also induce physiological effects on both intact and injured forebrain cholinergic neuronal somata.

Studies on aged rats have provided evidence for the involvement of the forebrain cholinergic system in age-related learning impairments (31–33). For example, in behaviorally impaired animals, there is a reduction in ChAT activity in the basal forebrain (32, 34) and atrophy of cholinergic neurons as inferred from the loss of ChAT protein (32, 35). The forebrain of aged rats also exhibits a reduction in protein and mRNA levels of NGF (36) and NGF receptor (37), suggesting that restoration of optimal levels of NGF could prevent neuronal damage in aged-impaired rats. Indeed, continuous intracerebral infusion of NGF has been shown to partly reverse cholinergic cell body atrophy in striatum and nucleus basalis (38, 39) and ameliorate the spatial memory impairments in aged rats (38). These results suggest that a pharmacological treatment that elevates NGF levels in the aged brain might also attenuate the aged-related functional decline occurring in the cholinergic forebrain system. These and other considerations, which assign to NGF a neurotrophic action on specific central cholinergic neurons, also suggest that NGF could be used as a therapeutic tool both to slow down the progress of pathological conditions associated with cholinergic neuronal death and to reverse the progression of degenerative diseases.

Recent experiments have further indicated that the trophic action of NGF may not be limited to cholinergic neurons. NGF binding sites, and to some extent NGF receptor mRNA, have been detected in the cochlear nuclei, the prepositus hypoglossal nucleus, the supragenicular nucleus and the dorsal part of the lateral lemniscus (42–44), which are devoid of cholinergic neurons. Moreover, the presence of NGF receptor has been established in the gamma-aminobutyric acid (GABA) containing cerebellar Purkinje cells (45) and in GABAergic neurons of the basal forebrain (46). NGF receptors are not limited to neuronal populations: glial and meningeal cells also express NGF receptor mRNA and binding sites (43, 47, 48). These data, taken together, support the role of NGF as a trophic factor for various cell types in the CNS.

Use of NGF in the Therapy of Human Neurodegenerative Diseases

The possibility that NGF therapy may retard the progress of some neurodegenerative diseases in humans has been under discussion for many years, but has only recently been demonstrated. NGF infused into the brain of a patient with Alzheimer's disease has given promising indications of its beneficial effects on some cholinergic parameters and cognitive functions (49). These results justify the initiation of clinical trials with NGF and support the development of appropriate technologies to deliver NGF into human

brain. However, the determination of an appropriate route of administration (to circumvent the presence of a barrier preventing the access of NGF to brain tissue), dosage, and stability of human NGF in the delivery systems are matters of concern. Studies have already begun to develop devices for long-term delivery of NGF. One such controlled release implant for NGF made from a biocompatible polymer, ethylene-vinyl acetate copolymer, has in fact been developed recently (50). This implant stimulated neurite outgrowth in the PC12 cells by providing continuous release of NGF for almost two months (50). A similar implant has also been used in rats with a fimbria-fornix lesion to prevent the lesion-induced reduction in ChAT expression in basal forebrain neurons (51). These studies show that NGF retains its biological properties over a relatively long period of time and suggest that a similar approach could thus be used in humans. However, there is one important limitation to the use of NGF as a potential "drug" for the CNS: poor absorption across the blood-brain barrier prevents parental administration. Theoretically, this problem could be overcome by delivering NGF via a brain implanted cannula. Such a drastic approach, however, would raise serious ethical and medical concerns, not least the disruption of the blood-brain barrier. Alternative strategies are thus underway, the most promising of which seems to be the grafting of NGF-synthesizing cells into the brain. Pilot experiments in rats have demonstrated that genetically modified NGF-producing fibroblasts grafted on brain prevented cholinergic neurons degeneration and death following a fimbria-fornix lesion (52, 53). Although intracerebral grafting is becoming a valid tool for neurobiologists, many questions must still be resolved before such procedures are extended to the therapy of human neurodegenerative diseases. The grafting of NGF-producing cells shares some of the difficulties inherent in dopamine cell grafting to replace the loss of dopamine neurons characteristic of Parkinson's disease (54), e. g. the long-term survival of the transplanted cells. It was recently reported that fibroblasts, genetically altered to express tyrosine hydroxylase, survived implantation in rat brain and produced L-dopa for at least 10 weeks (55). Despite the encouragement of this finding, many technical problems must still be resolved before the promise of effective transplants to provide continuous, long-term NGF delivery can be realized. The problem of immunoresponses of the host to grafted cells is also of great concern.

One alternative to the infusion of NGF may be the use of pharmacological agents that enhance brain NGF biosynthesis. This approach has several advantages over infusion or grafting technologies. These include: (*a*) making NGF available to neurons without brain surgery and therefore without damaging the blood-brain barrier; (*b*) producing NGF in specific CNS cell populations by targeting selected transmitter receptor subtypes; (*c*) limiting side effects of drugs inducing NGF by more tightly controlling the dosing regimen.

TOWARD THE PHARMACOLOGICAL REGULATION OF NGF BIOSYNTHESIS IN THE CNS

Estimation of NGF Biosynthesis

Over the past few years technologies have been developed that are capable of estimating changes in NGF biosynthesis, using in vitro and in vivo models. Such estimation is not easy since NGF is generated by cleavage from a larger molecular weight precursor that is encoded by a specific mRNA (7, 56, 57). The measurement of NGF content at the steady state cannot be used to assess the rate of synthesis because it does not reflect the dynamic equilibrium between synthesis and utilization. However, changes in biosynthesis of NGF can be estimated by combining the determination of the content of the specific mRNA encoding for the precursor and the measurement of the biologically active peptide. The contribution of this procedure in the understanding of mechanisms underlying NGF synthesis regulation was elegantly demonstrated by using as a model the denervation of target organs of sympathetic neurons (where NGF is synthesized) by 6-hydroxy-dopamine (6-OHDA). 6-OHDA is a neurotoxin known to cause degeneration of sympathetic neurons by destruction of nerve terminals. It was found that 6-OHDA increases NGF content in sympathetically innervated organs and rapidly decreases the NGF content in sympathetic ganglia (58). This increase of NGF in the target organs seems to be due to the inhibition of the retrograde NGF axonal transport in sympathetic nerve terminals and not to changes in the rate of synthesis. In fact, the amount of NGF mRNA present in these target organs does not change after chemical sympathectomy (59). Thus, when NGF content increases without a parallel change in NGF mRNA, presumably NGF accumulates because utilization, catabolism, or axonal transport are decreased. It follows therefore that both specific mRNA and peptide content must be measured in order to evaluate the mechanisms whereby NGF content changes (60, 61).

This strategy was used to estimate whether NGF biosynthesis changed after nerve crush. It was discovered that the mechanical blockade of axonal transport caused by crushing the sciatic nerve leads to a rapid accumulation of NGF distal to the injury, reaching maximal values 12 hr post injury (62, 63). Quantitative measurements of the NGF mRNA show a transient but several fold rise in NGF mRNA content in both distal and proximal segments. Further analysis revealed that the non-neuronal cells surrounding the site of injury respond quickly by producing NGF and NGF receptor, presumably to reestablish trophic support for the injured axons (62, 63). These and other experiments led to the finding that interleukin-1 (IL-1), produced by the invading macrophages in the injured nerve, was responsible for regulating NGF gene expression in non-neuronal cells (63, 64). Interestingly, IL-1 appears to regulate NGF synthesis in the CNS in vivo (65) and in astrocyte cultures (65,

66). These data indicate that in the CNS, NGF gene expression can be regulated by stimuli similar to those operative in the PNS. Since NGF is the prototype of a family of growth factors known as neurotrophins (67, 68), elucidating how NGF synthesis is regulated will provide valuable information on the biosynthesis of the other neurotrophic factors that are important in fostering synaptic plasticity.

Regulation of NGF Biosynthesis: In Vitro Studies

NEUROTRANSMITTER RECEPTOR ACTIVATION INDUCES NGF BIOSYNTHESIS Glial cells might be an important source of neurotrophic factors including NGF. Regulation of synthesis and release of these factors may involve neuronal-glial cell interactions and may be triggered by stimulation of transmitter receptors located on glial cell membranes. Such interactions may promote neuronal survival, maintenance, and differentiation as well as axonal outgrowth.

A rat glioma cell line (C6) contains and releases NGF (69–71). In these cells, β-adrenergic receptor (BAR) stimulation increases the content and secretion of NGF (72). Northern blot hybridization analysis of mRNA from C6 glioma cells has shown that the BAR-agonist isoproterenol (ISO) increases NGF mRNA and NGF content within three hours (73–75). This increase is blocked by the preincubation of cells with BAR-antagonist (-) propranolol, but not by the alpha-adrenergic receptor antagonist phentolamine (73, 74), suggesting that BAR stimulation is the triggering event operative in the regulation of NGF mRNA expression. The rate of increase appears to rapidly reach a new steady state that is maintained for several hours (73). Similar data that have been reported in fibroblast cultures and in primary culture of astrocytes suggest that BAR stimulation initiates a chain of events leading to the increase of NGF in a number of cell types (76, 77).

An earlier report suggested that the increase of NGF mRNA content elicited by ISO is due to the chemical structure of ISO (catechol), and not to its property of being a BAR agonist (78). A series of synthetic analogs of catecholamines, such as 4-alkylcatechols, can increase the content of NGF mRNA and protein in fibroblasts and in cultured astrocytes, suggesting that the catechol ring might be essential to induce NGF biosynthesis (78). To examine this possibility, clenbuterol, a BAR agonist that does not possess the typical structure of catecholamines (79, 80), was tested in C6 glioma cells (2B clone) (81). Clenbuterol does indeed elicit an increase in NGF mRNA levels similar to that obtained by ISO (82, 83). This increase is blocked by (-) propranolol and not by phentolamine, corroborating the finding that BAR stimulation is the key event underlying the regulation of NGF biosynthesis (82). Moreover, the increase in NGF mRNA elicited by clenbuterol is blocked by the more selective β-2 receptor antagonist ICI 118,551 (84), suggesting

that in this glioma cell line NGF biosynthesis can be regulated by the stimulation of the BAR-2 subtypes (82, 83).

The observation that 4-alkylcatechols can induce NGF is of particular pharmacological interest. These studies represent the first attempt to generate synthetic molecules that specifically affect NGF levels in the CNS. As a result of this observation, other compounds have been studied. Molecules such as hydroquinone and related derivatives that are not considered catechols but that exhibit the two hydroxyl groups in para-configuration, and vitamin K3 increase NGF protein in cultured mouse astrocytes (85). These results suggest that various molecules can enhance NGF synthesis in cultured cells and point to the possibility of developing a variety of synthetic compounds able to enhance the production of endogenous NGF. Though still in its infancy, this work is of crucial importance since it allows the molecular mechanisms underlying the induction of neurotrophic factors to be studied.

TRANSCRIPTIONAL REGULATION OF NGF The BAR-mediated increase in NGF mRNA content could result from increased transcription as well as mRNA stabilization. Both have been shown to be the mechanism whereby IL-1 increases NGF mRNA in cultured rat fibroblasts (86).

The 3' untranslated region of the NGF gene contains a long AU-rich region with a conserved AUUUA pentamer sequence (3), which has been proposed to control mRNA stability (87). Hence, changes in mRNA stability become a critical issue in understanding the molecular mechanisms underlying the regulation of NGF mRNA translation. In C6-2B cells, the protein synthesis inhibitor cycloheximide increases NGF mRNA content within one hour (88), which suggests that this compound stabilizes NGF mRNA by blocking either NGF translation or activation of an inhibitory protein. When C6-2B cells were exposed to cycloheximide for 3 hr, the increase in NGF mRNA content elicited by ISO was blocked, although the effect of cycloheximide on the stabilization of NGF mRNA was still operative (88). This result prompted speculation that the delayed increase in NGF mRNA caused by ISO could be due to stimulation of NGF mRNA transcription rather than to mRNA stabilization. Nuclear run-on studies in C6-2B cells finally showed that the accumulation of NGF mRNA caused by BAR stimulation is indeed due to an increase in NGF gene transcription rate (82).

THE ACTIVATION OF ADENYLATE CYCLASE INDUCES NGF BIOSYNTHESIS BAR stimulation with specific agonists produces a dose- and time-dependent increase in the intracellular cAMP level by activating the catalytic subunit of adenylate cyclase (89). It was hypothesized that the increase of NGF gene expression induced by BAR agonists could be mediated via the activation of cAMP-dependent protein kinase A. To test this hypothesis, a cell-perme-

able cAMP analogue, dibutyryl cAMP (dbcAMP), was used. The addition of dbcAMP to C6-2B glioma cells for 3 hr increased NGF mRNA content to an extent similar to that obtained with ISO (73, 74).

The stimulation of prostaglandin E (PGE) receptors also leads to cAMP accumulation (74). The addition of PGE_1 to C6-2B cultures significantly increases NGF mRNA and NGF content, although less than that elicited by BAR stimulation (74). This finding is consistent with the smaller increase in cellular cAMP levels induced by PGE_1 than by ISO (74). The intensity of the cAMP increase may be related either to the relatively low density of PGE receptors present in these cells, as compared to the BAR density, or to a difference in the coupling efficiency of PGE receptors and BAR to adenylate cyclase. However, a greater increase in cAMP level is obtained by incubating the cells with PGE_1 and 3-isobutyl-1-methyl-xanthine (IBMX), a phosphodiesterase inhibitor. Under these conditions, the effect of PGE_1 on NGF mRNA content is potentiated (74).

The importance of cAMP as a regulatory signal in NGF biosynthesis is further demonstrated by the increase in NGF mRNA content elicited by forskolin (75, 82), a diterpene known to activate directly the catalytic subunit of adenylate cyclase (Table 1). However, the three- to fourfold increase in NGF mRNA content elicited by BAR activation appears to be the maximal effect observed among the various factors that increase cAMP content. When cells are incubated with ISO and IBMX, no significant increase in NGF mRNA content occurs over that elicited by ISO alone (90). These data support and confirm the initial hypothesis that BAR stimulation is one of the key events underlying the induction of NGF synthesis and release.

Propentofylline, a xanthine derivative, has been shown to induce NGF content in astroglial cells (91). This compound also protects hippocampal neurons against ischemic damage in Mongolian gerbils, which suggests that the induction of NGF or related molecules might participate in the neuroprotective action of this drug (92). Since certain xanthine derivatives prevent the degradation of intracellular cAMP by inhibiting phosphodiesterases activity, it should be of interest to ascertain whether propentofyllin increases NGF content by a cAMP-dependent mechanism.

PROTEIN KINASE C ACTIVATION The activation of a protein kinase A-dependent pathway enhances NGF biosynthesis and release in astroglial cells (72–77, 88). To evaluate the specificity of this regulation studies were conducted to examine whether the activation of protein kinase C could also enhance NGF gene expression. The phorbol ester, 12-O-tetradecanoyl phorbol-13-acetate (TPA), an activator of protein kinase C, is commonly used to study the chain of events triggered by protein kinase C activation. The fact that TPA induces morphological changes in glial cells that can be attributed to

Table 1 Stimuli and their possible mechanism of induction of NGF synthesis in cultured cells

Stimulus	Mechanisms	Cells*	Ref
Isoproterenol	Activation of neuro-transmitter receptors	G, A	72, 74–78
Prostaglandin E		G	74, 76
Catechols		G, A	79, 84
Steroids		G	131
dbcAMP	Activation of second messengers	G, A	74, 76, 78, 87
Forskolin		G	76, 88
TPA		F	93, 94
IL-1	Growth factors	A, F	65, 66
bFGF		A	109
aFGF		A	110

*G = rat C6 glioma; A = primary culture of astrocytes; F = fibroblasts

the induction of some growth factor, supports a possible interaction between protein kinase C and NGF synthesis. TPA was shown to transiently upregulate NGF gene expression in L929 fibroblast cells and mouse kidney primary fibroblasts (93, 94). Since the unesterified compound 4-alpha phorbol, which is devoid of kinase C activity, failed to change NGF mRNA, an involvement of protein kinase C in the regulation of NGF has been suggested. However, fibroblasts might retain an unique spectrum of response to TPA, since in PC12 cells, phorbol esters are insufficient to generate the neural differentiation response elicited by NGF (95). These preliminary results must be confirmed in cells of neural origin before extrapolating conclusions of whether NGF can also be induced by similar mechanisms in vivo.

EARLY INDUCIBLE GENES AS A "THIRD MESSENGER" The characterization of the molecular mechanisms underlying the induction of NGF gene is important in view of the possibility of designing specific compounds that affect cell growth via an increase of NGF content. Protein kinase A and C activation induces NGF gene expression, which indicates that the NGF gene itself might be a potential target for drug development. The NGF gene does not appear to contain cAMP-responsive elements (CREs). These are small DNA sequences, upstream from the transcriptional initiation site, that confer cAMP-responsiveness to a heterologous promoter (96). Furthermore, the NGF gene also appears to lack TPA-responsive sequences (TREs). Nevertheless, cAMP and TPA induce NGF gene expression in different cell cultures, suggesting that an intermediate process might be operative in the increase of NGF gene expression elicited by the activation of protein kinase A or C.

Two possibilities have been considered: after cAMP increases, the activated catalytic subunit of protein kinase A translocates to the nucleus and stimulates gene expression through the phosphorylation of a nuclear regulatory protein; protein kinase A phosphorylates a cytosolic transcriptional activating protein, which then translocates to the nucleus. In either case, it is possible that the phosphorylation of a protein, which binds to a specific DNA sequence within the promoter or enhancer region of NGF gene, increases the transcription rate for NGF gene. This hypothesis accords with the finding that cycloheximide blocks the induction of NGF elicited by ISO (88).

The stimulation of cell surface receptors rapidly induces the expression of *c-fos* and other early response genes (97, 98) that encode for nuclear proteins. These proteins, referred to as "third messengers", regulate the expression of a number of neuropeptide genes after the formation of heterodimers, which bind with high affinity to specific DNA regulatory motifs (99). BAR stimulation and phorbol esters (88, 97) induce *c-fos* expression within 20–40 min, preceding the appearance of the newly synthesized NGF mRNA. Since *c-Fos* synthesis is also blocked by cycloheximide (88), it was suggested that the activation of early inducible genes could be involved in the induction of NGF transcription (88). This suggestion was supported by the recent finding that lesion-induced increase in NGF mRNA is mediated by *c-Fos* (100). Indeed, a putative binding site for a transcription factor resembling the activator protein 1 (AP-1) site has been found in the first intron of the NGF gene (100), indicating that the Fos/Jun heterodimer could function as a regulatory transcriptional factor for the NGF gene. More experimental evidence on the regulatory elements of the NGF gene will provide insight into the cell-specific mechanisms inducing NGF gene expression.

REGULATORY ASPECTS OF NGF EXPRESSION In astroglial and fibroblast cells, IL-1, BAR stimulation, and protein kinase A and C activation enhance NGF content and release by increasing the biosynthesis of this peptide (Table 1). The biological and functional significance of NGF in cultured astroglial cells has been debated. It is hypothesized, based on data obtained in hippocampal and to a lesser extent in hypothalamic tissue, that, in vivo, NGF can be only synthesized in neurons (101–103). However, several reports have shown that cultured astroglial cells produce and release NGF (69–77, 104–106). Since these cultures are grown in the presence of serum, which enhances NGF synthesis (65, 106–108), it has been inferred that glial production of NGF might be an artifact of the cultured cells. This implies that the data obtained in these cultures cannot be used to generate relevant information for the development of drugs able to foster NGF biosynthesis in vivo. Nevertheless, IL-1 enhances NGF gene expression in astrocytes and in rat hippocampus (65, 66), suggesting that similar mechanisms might be operative

in neurons and glial cells in vivo. Moreover, the NGF mRNA and NGF content of astroglial cells can be increased by stimulating neurotransmitter receptors coupled to adenylate cyclase, even in the absence of serum (74).

It has been speculated that astrocytes in culture may reflect a functional state similar to the pathological condition following a disease or CNS trauma. In view of the importance of astroglial cells in supporting and stimulating changes in neuronal physiology during the early stage of brain injury, the production of NGF, as well as other neurotrophic factors in astroglial cells, may be enhanced to promote regenerative processes by mechanisms that operate only in pathological conditions. These could include the release of IL-1 from the invasive macrophages or agents that promote inflammatory activity, or the release of other growth factors, such as basic or acidic fibroblast growth factor from wounded cells. Indeed, these agents have been demonstrated to increase NGF content and mRNA in primary culture of astrocytes (65, 66, 109, 110), mixed hippocampal cultures (111), and in the hippocampus of 10-day-old rats (65).

In astrocytes, NGF biosynthesis is also induced by BAR stimulation. This response, unlike the events associated with brain injury or diseases, might be of physiological significance. The tacit assumption that noradrenaline could tonically regulate the production and release of NGF in normal brain can be now tested in vivo to verify the physiological relevance of such interaction. Hence, prospective pharmacological agents that increase NGF availability may act by accelerating the biosynthesis of endogenous NGF via the above mechanisms.

Regulation of NGF Gene Expression in the CNS: in Vivo Studies

DEVELOPMENTAL REGULATION OF NGF GENE: A CHOLINERGIC HYPOTHESIS The expression of NGF gene in the rat CNS is developmentally regulated and temporally correlated with the maturation of cholinergic neurons (17, 18). NGF mRNA is not detected prenatally in brain but it increases gradually, reaching the maximum level of expression around three weeks postnatally (17, 18). This finding suggests that the increase in synaptic connection and activity might regulate the availability of NGF and thus allow this trophic factor to support differentiation of cholinergic neurons. Because of the closely ontogenetic relationship of hippocampal NGF mRNA with cholinergic neurons of the basal forebrain, it has been hypothesized that a cholinergic denervation of the septo-hippocampal pathway by transection of the fimbria-fornix might alter NGF biosynthesis in hippocampus. Both in neonatal and adult rats, fimbria transection increased hippocampal NGF levels, whereas AChE and ChAT staining decreased. This increase occurred up to 14 days postlesion and NGF levels returned to control values 30 days

postlesion (112, 113). Interestingly, hippocampal NGF mRNA content only changed in neonatal and not in adult rats (18, 112, 113), which suggests that NGF synthesis might be under an age-related mechanism of transcriptional and translational regulation. In the adult, the lack of change in NGF mRNA content favors the hypothesis that NGF accumulates because the retrograde axonal transport system is interrupted (3). It is also possible that in the adult there is a storage pool of NGF that is not necessarily utilized and therefore not degraded. NGF levels increase only after a lesion of this cholinergic pathway since the ibotenic acid-induced lesion of the nucleus basalis or the aspiration of entorhinal cortex, which also partially deafferent the hippocampus, failed to change NGF content (113, 114).

A less invasive "denervation" of cholinergic transmission can be obtained by a prolonged blockade of muscarinic receptors by scopolamine. When administered in adult rats for 3 or 7 days, scopolamine, in contrast to the fimbria-fornix lesion, decreased NGF levels in the septum, but failed to change NGF mRNA content (115), suggesting that this drug affects NGF content by a mechanism other than decreased synthesis.

It is difficult to compare data obtained from these two models of impaired cholinergic transmission since, unlike scopolamine treatment, fimbria transection also denervates noradrenergic and serotonergic fibers. Moreover, 7-day infusion with the AChE inhibitor physostigmine, which increases brain acetylcholine content, decreased NGF levels but not NGF mRNA in hippocampus and cortex (115). It therefore appears that functional alteration of cholinergic transmission by either blockade or enhancement of receptor function reduces NGF availability by a mechanism that is still unclear. Because cholinergic agonists are ineffective in promoting NGF synthesis in adult rats, these drugs are unlikely to be therapeutically useful in increasing NGF production.

Hormonal Regulation of NGF Biosynthesis in the CNS

BEHAVIORAL STUDIES The nervous and endocrine systems retain an intense biological dialogue by sharing many chemical signals and thereby interacting at several levels to shield the organism against noxious agents or hostile environmental conditions. This view is supported by independent experimental evidence, including data showing that the hypothalamus, which controls the endocrine system, also expresses NGF protein and mRNA (116, 117). This finding opens new lines of investigation into whether NGF may also serve as a link in the interaction between brain and endocrine functions. The strategically important location of NGF in the hypothalamus raises the hypothesis that NGF synthesis in the hypothalamus could be regulated by hormones.

Little is known at present about regulation of NGF synthesis in the hypothalamus. In mice, intermale aggressive behavior elicited by social isolation for 6–8 weeks, induces an increase in NGF immunoreactivity and NGF mRNA content in the magnocellular preoptic and the ventrolateral nuclei of the hypothalamus (103). Moreover, the levels of NGF in adrenalectomized mice increased less than the hypothalamic levels of sham-operated fighting mice (116). Interestingly, the increase in NGF expression in this behavioral model is confined to the hypothalamus and thus suggests that NGF may be involved in the regulation of those hypothalamic functions that integrate aggression linked to sexuality with sex-related endocrine function. Since hypothalamic oligodendrocytes and neurons express NGF protein in vitro (117, 118), experiments can be designed to test whether specific hormones play a role in the hypothalamic regulation of NGF synthesis.

ADRENAL STEROIDS The role of steroids in hypothalamic neuroendocrine regulation during stress is well documented. Furthermore, steroids appear to regulate memory consolidation and learning (119) and, according to recent studies, might also modulate complex endocrine and behavioral interactions (119). The CNS contains both Type I (mineralcorticoid) and Type II (glucocorticoid) receptors for adrenal corticosteroids (120, 121). Moreover, glucocorticoids have been postulated to exert a trophic action on selected neuronal populations (122). For instance, the administration of corticosterone significantly reduces changes in both cell morphology and death of a large number of rat hippocampal neurons following adrenalectomy. This reduction suggests that hippocampal neurons require a continuous influx of glucocorticoids to maintain normal morphology and even survival (122). However, one outstanding question is whether glucocorticoids may exert such trophic action by acting on DNA transcription directly or via the induction of growth factors.

Glucocorticoids have been shown to control the transcription of several genes and thereby change specific mRNA and protein content (123). Thus glucocorticoids might also regulate NGF biosynthesis in selected neuronal populations. This hypothesis is now supported by several lines of evidence. Aloe (124) reported that in the hippocampus of young adult rats, adrenalectomy reduces NGF content by about 50%, suggesting a potential role of adrenal steroids in the modulation of NGF biosynthesis. These results are complemented by studies of Fabrazzo et al (125) that support a direct action of adrenal steroids on NGF biosynthesis in the CNS. The synthetic glucocorticoid dexamethasone was administered to 21-day old rats, at a dose that could approximate a plasma glucocorticoid level comparable to that occurring during stress (126, 127). Within 3 hr, dexamethasone elicited a two- to threefold increase in the amount of NGF mRNA in the cerebral cortex (125). A smaller, but significant, increase in NGF protein was apparent at 6 and 9 hr

after dexamethasone treatment (125). Since similar data could be obtained in adult (3-month old) rats (Mocchetti et al, unpublished observations), these findings suggest that glucocorticoids released during stress might induce NGF gene expression (Figure 1).

In the adrenalectomized rat, the content of cortical NGF mRNA is only reduced by about 20% (125), suggesting that adrenal steroids do not tonically regulate cortical NGF expression. It is possible that NGF expression can only be induced when the steroid plasma content exceeds physiological oscillations, e. g. during stress. A similar increase in NGF expression can be obtained with reserpine, a monoamine-depleting alkaloid that also increases plasma corticosteroid levels (126, 128). It is well-established that reserpine activates the hypothalamic/pituitary/adrenalcortical axis, which increases the adrenocorticotropic hormone (ACTH) release from the pituitary and, in turn, stimulates steroidogenesis and steroid secretion in adrenal cortex (Figure 1). Reserpine can therefore be used as a tool to induce high levels of adrenal

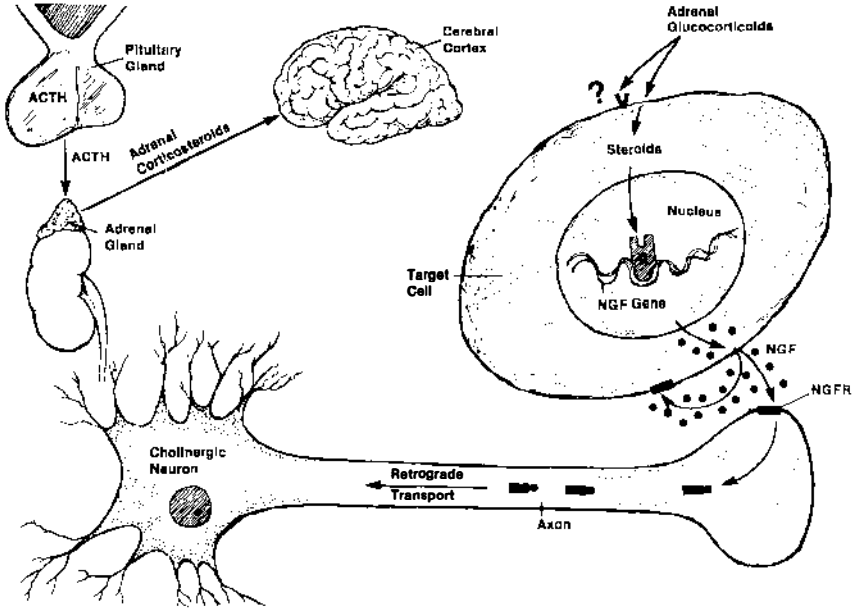

Figure 1 Schematic illustration of the cascade of events operative in the induction of NGF gene expression by steroids. ACTH released from pituitary (because of stress or pharmacological treatments) stimulates adrenal cortex to synthesize and secrete adrenal corticosteroids. These steroids, after binding to a specific intracellular brain steroid receptor, induce NGF gene expression and subsequently NGF biosynthesis in cerebral cortex. NGF released from target cells (either glia or neurons) binds to the high-affinity receptors that are presumably located on cholinergic neuron terminals.

steroids by a mechanism similar to that operative during stress. Reserpine induced a three- to fourfold increase in the content of cortical NGF mRNA 9 hr after injection (125). In adrenalectomized rats, reserpine failed to induce cortical NGF mRNA, which indicates that the presence of intact adrenocortical function is required (125). Since similar results were also obtained in adult (3-month old) rats (129), these data suggest that in addition to an action on DNA transcription, adrenal steroid could mediate synaptic plasticity by increasing NGF biosynthesis. It is interesting to note that both dexamethasone and reserpine induced NGF mRNA and NGF protein in the cerebral cortex only and not in other brain structures that also possess glucocorticoid receptors. The molecular mechanisms operative in this cell-specific induction of NGF are currently difficult to explain. We do not know whether this increase is occurring in glial cells or in neurons. It is now known that glial cells of various brain structures can be steroidogenic, but the mechanism that regulates steroidogenesis by extracellular signals is still unknown. However, a working hypothesis can be formulated that steroids, released from glial cells, may promote expression of a neuron-specific steroid receptor that, in turn, regulates NGF gene expression.

Other hormones are capable of inducing NGF expression. Thyroxine, administered to adult mice daily for 21 days, markedly increased NGF concentration in the cerebral cortex, brain stem, and cerebellum (130). Furthermore, it has been shown that in C6 glioma cells, synthesis and release of NGF are increased in the presence of 17β-estradiol (131). Because steroid and thyroid hormones affect brain growth and maturation, these hormones might possibly exert their effect on CNS homeostasis via the regulation of NGF production.

Glucocorticoids interact in a complex fashion with the AP-1 transcriptional factor. The result of this interaction can be either inhibition or stimulation of gene expression (132). The data presented by Aloe (124) and Fabazzo et al (125) suggest that glucocorticoids exert a positive regulation on NGF genes. However, this suggestion seems to be at variance with the finding that glucocorticoids decrease NGF production in L-929 fibroblasts (133) or in lesioned sciatic nerve (134). Given the assumption that DNA elements responsive to glucocorticoids but not transcriptional factors are similar in different cell types, it is likely that the enhancement of NGF gene expression is a complex phenomenon linked to a particular cell type. To this extent, it is important to recall the finding of Diamond et al (135) on the "composite", a glucocorticoid responsive element associated with the proliferin gene. This 25-base element confers positive and negative glucocorticoid response depending in part on the presence of other transcription factors that participate in the regulatory process. The expression of these other factors (such as *c-Fos* and *c-Jun*) can be cell specific and may vary depending upon different

functional states of the cell. Perhaps this element could be a missing stimulus in fibroblasts or in the PNS. Further experiments are needed to elucidate the mechanism whereby glucocorticoids induce NGF expression in neuronal cells.

Neurotransmitter Receptor Regulation of NGF Biosynthesis

LIMBIC SEIZURES The hippocampus contains the highest levels of NGF in the CNS. Neurons in the granule and pyramidal cell layers of the rat hippocampus express NGF mRNA (101, 102). ^{125}I-labeled NGF injected in the hippocampus can be retrogradely transported to the cholinergic cell bodies in the diagonal band of Broca, which suggests that NGF synthesized in the hippocampus can exert trophic action on the cholinergic neurons of the basal forebrain (20). GABA and glutamate provide the major inhibitory and excitatory input, respectively, to pyramidal neurons of the hippocampus. Thus, NGF might be synthesized and released in response to changes in GABAergic or glutamatergic activity. This hypothesis is supported by recent findings showing that NGF mRNA content is enhanced in neurons of hippocampal cultures exposed to kainic acid, a glutamatergic receptor agonist (136).

Increases in NGF gene expression have been observed in hippocampus in vivo, following experimental seizures in which it is presumed that excitatory amino acid transmission overtakes inhibitory GABAergic transmission. The seizure models include: electrolytic lesion of the dentate gyrus hilus (137); the injection of the glutamatergic receptor agonist kainic acid, either intracerebroventricularly (138) or intraperitoneally (136); the electrical stimulation in the ventral hippocampus (139); and the injection of bicuculline either focally in the area tempestas (140) or intravenously (141). The systemic injection of MK 801 (in doses up to 1 mg/kg), an antagonist of the N-methyl-D-aspartate (NMDA) sensitive glutamate receptor, failed to block the increase in NGF mRNA elicited by either the systemic injection of kainic acid (136) or by focal electrical stimulation (139). In contrast, 6-cyano-7-nitroquinoxaline-2,3- dione (CNQX), a non-NMDA receptor antagonist, prevented the increase in NGF mRNA elicited by electrical stimulation (139). These data, taken together, suggest that stimulation of non-NMDA receptors plays an important role in regulating NGF mRNA expression. Since these studies did not include experiments using NMDA receptor antagonists other then MK 801 (which by itself can cause neurotoxicity), the role of the NMDA receptor in the regulation of NGF mRNA expression remains to be determined.

The ability of intravenously administered bicuculline to induce seizures and increase NGF mRNA is blocked by diazepam (141). Diazepam also prevents the increase in NGF mRNA obtained after systemic kainic acid injection (136), which suggests that the balance between excitatory glutamatergic and

inhibitory GABAergic transmission may be a critical aspect of NGF regulation. Interestingly, during seizure activity in vivo, the mRNA content of one of the neurotrophin family member, brain-derived neurotrophic factor, is induced although with a different time course (136, 139, 142). Activity-dependent regulation may therefore be an inherent property of this class of neurotrophic factors.

Isackson et al (142) have suggested that the increased expression of NGF and brain-derived neurotrophic factor may be related to their role in maintaining neuronal viability and circuitry in brain. Thus, at physiological levels of synaptic transmission, NGF expression may be under a tonic inhibition that limits its trophic action. In conditions of excessive synaptic activity, such as following the blockade of an inhibitory neurotransmitter or the induced release of an excitatory neurotransmitter that produces convulsions, stimulation of NGF expression may occur to protect against the neuronal degeneration that might follow. NGF, along with the other neurotrophic factors, could act as a protective factor to control the spread of neuronal degeneration. Thus, it is tempting to speculate that some of the pathology of neurodegenerative disorders derives from insufficient neuronal trophic support in response to sustained abnormal synaptic transmission.

Stress induced as a consequence of convulsive activity may increase adrenal corticosteroid levels. Increases in NGF expression following seizures could therefore be due to the increased plasma corticosteroids. In adrenalectomized rats, a convulsant dose of intravenous bicuculline failed to increase NGF mRNA in the hippocampus (141). These preliminary results suggest that adrenal steroids might represent a common mechanism whereby NGF expression is induced in the CNS by a variety of drugs with different mechanisms of action.

BAR ACTIVATION Experiments have been performed on rats to obtain data that confirm and strengthen the hypothesis that BAR stimulation can enhance NGF biosynthesis in the brain. Because ISO crosses the blood-brain barrier poorly, clenbuterol, a lipophilic BAR agonist that penetrates the blood-brain barrier to interact with central BAR (79, 80), was used. Northern blot hybridization revealed that an acute administration of clenbuterol in 21-day old rats elicits a transient threefold increase in NGF mRNA in the cerebral cortex 5 hr after its administration (83). This effect is blocked by the concomitant injection of the BAR antagonist (-)propranolol. This result suggests that the effect of clenbuterol on NGF mRNA is mediated via the stimulation of BAR. Moreover, the induction of NGF mRNA is not due to a general increase in RNA, since the mRNA encoding for the structural and stable protein cyclophilin (83, 143) did not change.

That clenbuterol also increases NGF protein level suggests that the BAR

agonist induces NGF biosynthesis. These data allow the speculative hypothesis that noradrenaline, by interacting with the postsynaptic BAR, presumably located on the astroglial cells (based on the C6 cells and astrocytes data), induces the production of NGF.

Clenbuterol acts mainly as an agonist of the BAR-2 subtypes (79, 80), and thus might affect only those brain regions that contain these receptor subtypes. Interestingly, cerebral cortex and cerebellum possess more BAR-2 subtypes than other brain regions (80, 84). However, although cerebellum expresses NGF receptor, it does not seem to synthesize high levels of NGF even after BAR stimulation. Thus, in the cerebellum BAR may not be located on cells synthesizing NGF. Further studies are needed to determine whether other brain areas are sensitive to BAR activation and whether this activation can also induce the expression of NGF in the brain of adult rats.

These data, although preliminary, open up the prospect that NGF production can be enhanced in brain by stimulation of specific neurotransmitter receptor subtypes. A more careful pharmacological characterization, using BAR agonists and antagonists that can distinguish between BAR subtypes, should be carried out to test this hypothesis. Side effects of BAR-2 agonists in the peripheral sympathetic system can be blocked by specific antagonists that do not cross the blood brain barrier. Clearly, this is a wide open area of interest to pharmacologists and toxicologists.

SUMMARY AND CONSIDERATIONS FOR FUTURE STUDIES

A variety of stimuli regulate NGF biosynthesis in brain and in cultured cells, such as hormone and neurotransmitter receptor activation. The endocrine system appears to be involved in the regulation of NGF expression. Intermale aggressive behavior affects hypothalamic NGF (103, 116), whereas adrenal steroids induce NGF biosynthesis in the cerebral cortex and hippocampus (124, 125). The elevation of steroid levels in rats, either by reserpine or dexamethasone, induces NGF biosynthesis in the cerebral cortex. The findings that adrenal steroids, particularly glucocorticoids, might function as regulatory stimuli in the modulation of brain NGF expression support the possibility that NGF represents a link whereby the adrenal cortical system can exert trophic action on the CNS (144). This proposal should be viewed with caution since high levels of glucocorticoids have been reported to cause pronounced toxic effects in selected population of neurons (119). Small doses of glucocorticoids should perhaps be given in combination with BAR agonists or other pharmacological interventions to promote the efficiency of neuronal plasticity.

Neurotransmitter receptor activation increases NGF gene expression in specific brain structures. The increased synaptic activity during seizures induces NGF gene expression in the hippocampus and cerebral cortex by a mechanism that is apparently linked to the activation of non-NMDA receptor. BAR stimulation appears to enhance NGF mRNA and NGF content only in the cerebral cortex, suggesting that a primary neurotransmitter can regulate NGF gene expression in specific brain structures. Thus, different stimuli may regulate NGF gene expression in different brain areas, probably by activating specific signal transduction mechanisms. If evidence in support of these hypotheses can be provided, new possibilities may be opened up to develop therapeutic interventions in those pathological conditions where NGF is deficient. The identification and characterization of mechanism(s) controlling NGF biosynthesis are vital precursors to the development of drugs to enhance NGF availability in specific population of neurons. Although this review has focused on NGF, it is important to remember that NGF exerts its biological activity by binding to a high-affinity receptor on a cell surface (21). The stimulation of NGF biosynthesis should therefore be followed by an increased expression of NGF receptor since availability of this receptor regulates NGF biological activity. This consideration relies on the observations that higher levels of NGF in the brain, such as occur after a chronic infusion or as a result of spinal cord injury, enhanced gene expression of NGF receptor (145–147). Neurons apparently respond to high production and availability of NGF by increasing the expression of NGF receptor. This event may allow for a more efficient retrograde transport of NGF to the neuronal cell body where NGF can regulate the activity of a wide variety of proteins and, ultimately, modulate CNS plasticity and neuronal function.

We are at last close to obtaining pharmacological tools to enhance NGF in the CNS. These tools should simplify and accelerate the procedures needed to study NGF-responsive proteins. More research into the mechanisms of synaptic plasticity combined with an improved understanding of neurotrophic factor biosynthesis regulation should foster pharmacological prevention and perhaps facilitate the reversal of neuronal degeneration.

ACKNOWLEDGMENTS

The author is indebted to his colleagues Drs. G. Brooker, R. Dal Toso, M.A. De Bernardi, and M. Fabrazzo, who over the years have provided much of the conceptual background and experimental evidence on which this review was based. Special thanks to Dr. E. Costa for his generous support and guidance. Supported in part by NIH grant NS29664-01.

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