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NGF Regulatory Role in Stress and Coping of Rodents and Humans

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ALLEVA, E., S. PETRUZZI, F. CIRULLI AND L. ALOE. *NGF regulatory role in stress and coping of rodents and humans.* PHARMACOL BIOCHEM BEHAV **54**(1) 65-72, 1996.—Nerve growth factor (NGF) is a polypeptide growth factor which exerts trophic and differentiative effects on specific peripheral and central populations of neurons. Recent data showing that various cellular types of the endocrine and immune systems are able to synthesize and release NGF have suggested that this neurotrophic factor may also play an important role in vertebrate physiologic homeostasis. Previous studies using a mouse model of aggressive behavior have shown that NGF levels increase in both plasma and the CNS following intermale agonistic encounters. More recently, we have extended this research area to include other species: in particular, humans. The data now available indicate that labour and lactation, or the occurrence of a stressful event such as the very first jump with a parachute causes an increase in NGF plasma levels as well as changes in the distribution of NGF receptors on lymphocytes. This review aimed to outline the current understanding of NGF role in vertebrates in stress-related events.

NGF Stress Physiologic regulation Humans Rodents

EARLY studies on the role played by the target tissue in regulating the quality and amount of innervation led to the discovery of NGF (64). This neurotrophic factor is produced and released by the target tissue, subsequently taken up by the responsive neuron through a mechanism of receptor-mediated endocytosis, and finally transported retrogradely to the cell body (50), where it exerts its trophic/differentiative effects [for a review, see (65,68,77,100)].

In the peripheral nervous system (PNS), NGF plays an important role in survival, differentiation, and maintenance of neural crest-derived sensory as well as sympathetic neurons (46,67,69,71,100). Moreover, it has been shown that NGF and its receptors are present in the CNS, mainly in hippocampus, cortex, and olfactory bulb (60). Each of these regions is innervated by basal forebrain cholinergic neurons for which NGF acts as a trophic factor. In fact, in vivo administration of NGF increases the level of choline acetyltransferase (41,78) and rescues basal forebrain neurons from death following fimbria/fornix transection (56). NGF might also act as a trophic factor for other populations of CNS neurons such as Purkinje cells of the cerebellum and hypothalamic neurons (29,84). In this latter brain structure, NGF and its mRNA have been specifically localized by in situ hybridization techniques or immunohistochemistry in the preoptic area and the ventrolateral nucleus, two areas involved in neuroendocrine regulation (7,95).

ROLE FOR NGF OUTSIDE THE NERVOUS SYSTEM

In the past, studies investigating the physiologic role of NGF focussed mainly on its effects on the nervous system during development. More recent work has shown that NGF also plays an important role in the physiologic regulation of adult vertebrates (2,6,58,59,96). In addition, a growing body of evidence suggests a role for this neurotrophic factor outside the nervous system. In fact, a large amount of NGF is present in the submaxillary salivary glands (SSGs) of the mouse, from which it is released as a biologically active compound. Furthermore, a number of other cells outside the nervous system,

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including epithelial cells, fibroblasts, lymphocytes, and activated macrophages, synthesize NGF (17,45,72). The possible implications of these findings will be discussed later.

Submaxillary salivary glands represent the main source of NGF in the adult male mouse (19,71,65). These exocrine structures produce around 0.1% of total $mRNA^{NGF}$ in the male mouse (54), although the physiologic role of such a high concentration is not completely understood (9,65,100). NGF is also released by an endocrine mechanism (9,22,49,58,83), but the data regarding the presence of NGF in the circulation have been highly conflicting. Several studies claimed (9,27,105), whereas others denied (26,81,82), the presence of NGF in the bloodstream of mice. More recent observations indicate that NGF is in fact secreted directly into the peripheral circulation (6,66), suggesting a role for this growth factor in neuroendocrine and inflammatory events (75).

NGF and Aggressive Behavior

In the wild, adult male mice exhibit high levels of intraspecific aggressive behavior toward conspecific males. In laboratory conditions, the agonistic behavior of male mice can be elicited after 4–6 weeks of social isolation (103). Blood samples obtained from the retro-orbital plexus of isolated mice following fighting have been shown to contain about 100 ng/ml of NGF. Electron microscopic examination of the glandular tissue has also evidenced a marked depletion of the ductal granules which normally store NGF. These data suggest that SSGs are the main contributors to the increase in blood NGF levels following aggressive behavior (6). Other studies involving sialoadenectomized animals (i.e., upon surgical SSG removal) also found that aggression-induced release of NGF and EGF mostly occurs from SSGs tissue (58,68). Following sialoadenectomy, plasma NGF concentration first drops and then subsequently increases (6,49), suggesting the existence of alternative sources of NGF. A study by Lakshmanan (58) failed to show significant differences in basal serum NGF concentrations between sham-operated and sialoadenectomized mice. This discrepancy could possibly be due to differences in the specificity of antisera used in the respective RIA systems. Stephani and colleagues (96) were also unable to find changes in basal NGF levels following sialoadenectomy, although this surgical procedure eliminates the increase in NGF following an aggressive episode.

The frequency of fighting episodes is positively correlated with the increase in NGF serum concentration observed after an agonistic encounter. Fighting per se, rather than isolation, causes a peak in NGF concentration (300 ng/ml of serum) during the first 2–3 h following the aggressive encounter (6). In fact, high levels of plasma NGF have been detected in nonisolated male mice forced to fight with isolated ones (6). This clearly shows that the release of NGF is specifically induced by intraspecific fighting, a classical form of psychosocial stress (15).

It has been shown that α- and β-adrenergic agonists induce a release of both NGF and EGF from SSGs into the saliva (80). Adrenergic mechanisms, however, are not responsible for the release of NGF into the bloodstream. In fact, mice that have been subjected to immunosympathectomy by treatment with NGF antibodies at birth, or that have been chemically sympathectomized with 6-hydroxydopamine as adults, still show large amounts of blood NGF levels following an agonistic encounter (6). Therefore, the release of NGF from SSGs into the bloodstream is not apparently mediated by the activity of the adrenergic terminals innervating the tubular portion of these glands or, alternatively, by an enhancement of norepinephrine release from postganglionic nerves.

Social Status Affects Circulating NGF Levels

The increase in circulating NGF following intermale aggressive behavior does not represent an aspecific physiologic response to fighting (74). In fact, a correlation has been found between NGF plasma levels in fighting mice and their social status: Serum NGF levels of mice that repeatedly experienced defeat and submission show a twofold increase compared to those of dominant attacking mice. These findings also indicate that the NGF released following an agonistic encounter is not necessarily related to the actual display of aggressive behavior. Moreover, they are consistent with previous reports showing that mean NGF levels in the plasma of a fighting mouse pair are a function of the number of fighting episodes (6). Interestingly, the changes in NGF values of each pair were found to correlate with the amount of time spent freezing by the subordinate: The greater the amount of freezing shown by the subordinate animal, the greater the difference in NGF serum values of the two partners. It is conceivable that the subordinate animals perceive their situation as uncontrollable, because their efforts to assume submissive postures are not likely to prevent the attack of the opponent that becomes dominant (37). Such a lack of controllability could stimulate the release of NGF. A recent study conducted by our group also supported the finding of an association between increased NGF levels and the experience of a lack of control (4). It is known that crouching posture characterizes subordinate animals in rodent intermale aggressive behavior, and it has been interpreted as reflecting a high level of fear. In the study just mentioned, a positive correlation was evidenced between NGF levels and a pattern of aggression by lactating female mice encountering an unfamiliar male intruder. In particular, when confronting male intruders, lactating females displaying a greater amount of bites to vulnerable regions of the body, as well as crouching and escapes, also had higher levels of NGF in the bloodstream when sampled 3 h following the aggressive episode.

A subordinate-like profile can be induced by systemic NGF administration. In fact, male mice treated with NGF spend less time fighting than do controls and show a longer latency to the first attack episode (21). Moreover, NGF-treated mice display significantly low levels of aggressive grooming, an activity which consists of harsh grooming to the back of the fighting opponent and is indicative of a strong motivation to attack it.

Biological Targets of NGF Released Following Aggressive Behavior

An important question concerns the physiologic role played by the NGF released from the SSGs as a consequence of fighting behavior. Because the adrenal gland appears to be one of the biological targets of NGF, one way this growth factor could exert a physiologic role is through its action on this structure. Exogenous administration of NGF results in adrenal gland hypertrophy (6,21,110). NGF administered for 6–10 consecutive days causes a dose-dependent increase in adrenal weight and volume by enlarging both the adrenal medulla and the cortex. It should be noted, however, that such effect is more pronounced in the medulla than in the cortex (6,21). NGF has been shown to exert hypertrophic effects and to induce tyrosine hydroxylase activity in rodent adrenal chromaffine cells (102). At present, it is not clear whether adrenal

cortex hypertrophy following NGF administration in adult mice is the result of a direct action of this growth factor on adrenal cortex cells or whether it is mediated through its effects on ACTH release.

Changes in adrenal hormone secretion (e.g., increased corticosterone levels) resulting from an increase of NGF circulating levels could, in turn, influence aggressive behavior. Experimental studies indicate that in mice both ACTH and glucocorticoid hormones can act on agonistic behavior, but given the negative feedback relationship between these hormones, the effects are complex. Glucocorticoids have been shown to exert a facilitation of submissiveness; they also indirectly enhance aggressive behavior through negative feedback suppression of ACTH production. In fact, ACTH enhances submissiveness in mice (62,63). There is no evidence of direct NGF effects on neural substrates of aggressive behavior, and the high molecular weight of the NGF protein seems to exclude the possibility that it could cross the blood-brain barrier. However, a possible breakage of the NGF protein into fragments acting on the CNS can be postulated (98).

In summary, NGF released following male mice intraspecific fighting may exert an inhibitory feedback effect on aggressive behavior. The greater quantity of NGF released by subordinates (74) and their hypertrophic adrenals (53) points to a regulatory loop involving NGF-promoted adrenal hormone activation, which in turn enhances a subordination profile (62,63) [also see (21) for further details].

Changes in CNS NGF Levels Following Stress

High levels of $mRNA^{NGF}$ are present in the neocortex and hippocampus of several mammalian species (25,42,43,47,55, 60,88,94,99,106). From these structures, NGF is retrogradely transported (92,93) to the cell bodies of basal forebrain magnocellular cholinergic neurons, where it exerts a trophic action (41,48,52). Degeneration of basal forebrain neurons upon transection of the septohippocampal pathway is markedly reduced by the administration of exogenous NGF (47,57,107). The widespread occurrence of NGF and its mRNA throughout the CNS suggests a variety of additional functions (e.g., in the olfactory, visual, and acoustic systems) (104). The expression of the NGF gene is localized to neuronal cells, suggesting that neuronal activity may regulate NGF synthesis. Intraventricular injection of kainic acid into limbic structures markedly increases NGF mRNA in neurons of the hippocampus and neocortex (38,39). Moreover, under physiologic conditions, glutamate may exert an important role in regulating NGF gene expression, as activation of glutamate receptors in vivo increases NGF mRNA in the rat hippocampus and cortex (109). Neuropeptides or cytokines, such as IL-1β, may also be involved in the regulation of NGF synthesis (72).

Recent observations support the notion that NGF expression is regulated by physiologic activity. For example, in mice, intermale fighting leads to a marked increase in $mRNA^{NGF}$ in the hypothalamus (95). The increase in $mRNA^{NGF}$ appears after 30 min, peaks at 3 h, and declines to control levels between 6 and 12 h. This increase is not evident in other CNS or peripheral areas such as cerebral cortex, hippocampus, cerebellum, heart, and submaxillary glands. The lack of a change in the latter tissue indicates that the massive release of NGF into the bloodstream of fighting mice is not paralleled by a rapid NGF gene transcription in these glands. The large increase in $mRNA^{NGF}$ in the hypothalamus is also accompanied by an increase in biologically active NGF. NGF antibodies are able to prevent the neurite outgrowth induced in sensory and sympathetic ganglia by hypothalamic extracts from isolated fighting mice. The study also identified the cellular localization of NGF mRNA by in situ hybridization techniques. Labelled cells have been found in the magnocellular preoptic and ventrolateral nucleus of the hypothalamus. No specific labelling of glial cells was observed in this or other brain regions. Therefore, it seems that glia does not contribute to the increase in NGF expression in this region. Changes in hypothalamic NGF levels following intermale aggressive behavior are not abolished by sialoadenectomy, suggesting that the NGF found in the hypothalamus is not of salivary origin (6).

Although, as mentioned earlier, it is unlikely that NGF released following intraspecific fighting could reach the CNS and exert a physiologic role at that level, changes in adrenal hormones production that accompany aggressive behavior seem to affect brain NGF levels. Aloe (14) investigated the effects of adrenalectomy on hippocampal NGF levels and on the distribution of choline acetyltransferase immunoreactivity in forebrain cholinergic neurons of developing rats. Adrenalectomy results in a reduction of NGF levels and a concomitant change in the distribution of its receptor in the hippocampus, while decreasing choline acetyltransferase immunoreactivity in the lateral septum (14). This suggests an involvement of adrenal hormones in the regulation of NGF levels in the hippocampus and of NGF receptors in the septum.

A great body of evidence in both intact and injured brain structures indicates that NGF is expressed in the hypothalamus, although its role in this brain structure is not yet understood (20,24,95). It has also been shown that the administration of exogenous NGF facilitates recovery following specific hypothalamic damage (20,24). Recent studies have demonstrated that genes encoding NGF and its receptor are present in the hypothalamus (44,84) and that the NGF receptor protein is present in this brain region (13). All of these findings seem to support the hypothesis that hypothalamic NGF might be involved in neuroendocrine function.

No information is currently available about the possible role of NGF in the hypothalamus or the mechanism involved in its increase in this brain structure following intraspecific fighting. Alleva and Aloe (2) hypothesized that the rather rapid increase in brain NGF levels following a psychosocial stressful event could allow some phenomena of brain plasticity to take place. NGF could facilitate structural changes, such as the formation of dendritic spines or collateral sprouting, possibly modifying the neural connections of the mature brain (31,33). Moreover, it might affect levels of other peptides or hormones present in the hypothalamus (1,97). An interaction between NGF, thyroid hormones, and ACTH has been reported in the past (11,85,86,108). Recent findings show an increase in brain-derived neurotrophic factor (BDNF) mRNA in the paraventricular nucleus of the hypothalamus following osmotic stimulation (28). These data suggest that in addition to NGF, the expression of other growth factors could be modified following psychosocial stress.

A recent experiment by our group showed that stressful situations different from those occurring during intermale fighting can modify NGF levels in CNS areas other than the hypothalamus (13). Sixteen-day-old rats undergoing a 20-min session of cold water swimming stress (CWSS) for 8 consecutive days showed increased levels of NGF and NGF receptors in the hippocampus and cerebral cortex, and decreased levels in the hypothalamus. These data seem to indicate that NGF synthesis is differentially regulated in different brain regions.

This could be explained by taking into account the fact that the local concentration of neurotransmitters or hormones might ultimately be responsible for the changes in the expression of NGF and/or of its receptors. Moreover, these data indicate that different stimuli result in specific changes in brain NGF levels. In fact, the use of a CWSS paradigm leads to a decrease of hypothalamic NGF levels, while another stressful paradigm such as intermale aggressive behavior leads to an increase in hypothalamic NGF levels. These apparently discrepant data can be reconciled by taking into account methodologic differences between experiments. First, the use of a repeated stress paradigm as in the case of CWSS might result in a long-term downregulation of NGF levels. In addition, this study employed young subjects (21 days old). Further, compared to a subordinate animal, which probably perceives its situation as uncontrollable, a rat in a water tank is more likely to exert some control on the situation by swimming and attempting to escape. However, the levels of glucocorticoids (GCs) or ACTH have not been measured in these animals, so we cannot draw any final conclusion. Interestingly, in another recent experiment, our group found that haloperidol, an antipsychotic drug known to reduce behavioral arousal, can reduce hypothalamic NGF levels when administered to adult mice (5). Taken together, these data suggest that hypothalamic NGF levels are modified following stressful experiences (see Table 1). Because the hypothalamus is involved in the maintenance of physiologic homeostasis, it could be hypothesized that NGF, in concert with hormones and/or neurotransmitters present in this structure, might ultimately contribute to integrate behavioral and neuroendocrine responses. Data showing changes in BDNF levels in the paraventricular nucleus following osmotic stimulation support this hypothesis (28).

HUMAN STUDIES

In another set of experiments we investigated whether stress might affect NGF levels in human subjects. In our species, pregnancy, parturition, and lactation are characterized by dramatic changes in neuroendocrine physiology. Ovarian steroids and oxytocin are the main hormones contributing to the physiologic changes occurring during reproduction. We tested NGF levels in plasma of young women during the third trimester of pregnancy, labour, and lactation, and found that plasma NGF levels did not change during pregnancy (73). However, the plasma concentration of this neurotrophin reached a peak of about 20 pg/ml (i.e., a fivefold increase compared to baseline values) during labour and was maintained at this high level during lactation. These data suggest that NGF might play a physiologic role in neuroendocrine changes occurring during human labour and lactation, possibly in cooperation with oxytocin. In fact, female virgin rats injected with oxytocin show an increase in hypothalamic NGF content (73). Moreover, animal models of maternal behavior suggest that changes in brain plasticity occur concurrently with the onset of maternal behavior (79).

Subsequent studies investigated possible alterations of plasma NGF levels in human subjects with neurobehavioral alterations and in psychiatric patients treated with electroshock (ECT) (12). The results indicated that plasma NGF levels of subjects affected by paranoid schizophrenia were double the amount found in healthy subjects. In ECT-treated subjects, there was an increase in plasma NGF level which was maximal following a 30-min treatment. At this time, NGF levels of ECT patients were comparable to those of the schizophrenic group. Interestingly, depressed patients did not show any significant difference in plasma NGF levels compared to healthy controls. Thus, although a correlation between neurobehavioral state and plasma NGF seems to exist, the levels of this growth factor are not modified in all neurobehavioral disturbances.

A well-characterized stress model (the first parachute jump from an aeroplane by young soldiers) was also used by our group to assess the effects of stress on plasma NGF levels in human subjects (8,34,91). Dependent measures were plasma corticosterone, ACTH, and NGF levels, as well as the distribution of NGF receptors in peripheral lymphocytes. Blood samples of soldiers at their first jump were collected both the evening before and 20 min after landing. Plasma NGF levels increased to 84 and 107% of control levels, respectively, the evening before and 20 min after the jump. Parachute jumping caused an activation of the hypothalamic-pituitary-adrenal axis, which resulted in increased plasma cortisol and ACTH levels. However, the concentration of these hormones was not significantly different from control values the evening before the jump. Thus, the alterations in NGF plasma levels, possibly due to the anticipation of the jump, occurred at a time when no changes in the activity of the HPA axis were seen. Immunofluorescence analysis showed that both high ($p140^{trkA}$) and low (p75) affinity NGF receptors, present on peripheral blood lymphocytes of the soldiers, were increased. This change was clearly evident in samples collected following landing, although it was already detectable the evening before the jump.

No clear inferences can be drawn about the physiologic role of the NGF released following parachute jumping or other stressful events. However, in light of the findings that human natural killer cells increase following parachute jump-

TABLE 1

STRESS-RELATED FACTORS KNOWN TO AFFECT NGF LEVELS

Type of Stress	Tissue	Species
Aggressive behavior	Blood, hypothalamus	Mouse (74, 95)
Cold water swim stress (CWSS)	Hypothalamus, hippocampus	Rat (13)
Seizures	Brain	Rat (38, 39)
Paranoid schizophrenia	Blood	Human (12)
ECT	Blood	Human (12)
Delivery and lactation	Blood	Human (73)
Delivery and lactation	Hypothalamus	Rat (73)
Parachute jumping	Blood	Human (8)

ing (89) and that NGF induces the expression of interleukin-2 receptors in those cells (101), it could be hypothesized that NGF might act on peripheral targets (e.g., enhancing the function of the immune system). High levels of endogenous NGF in the plasma of parachutists might therefore be involved in the activation of this system. Previous studies have shown that NGF may act on the cells belonging to the immune system (23,35,40,68). In vivo administration of NGF to neonatal rodents increases the number and size of peritoneal mast cells (MCs) in several peripheral tissues (10). Furthermore, it has been found that the rise in plasma NGF after intermale aggressive encounters in mice increases the number of peritoneal MCs and causes them to degranulate, with a consequent increase in peritoneal histamine (32). As a whole, these data suggest that NGF released in response to psychosocial stress is involved in potentiating the immune responses of an organism, when facing a potential threat.

CONCLUSIONS

The results of our studies raise the question of the source and storage of endogenous NGF in human subjects, given that human SSGs do not produce and store significant amounts of this neurotrophin. Recent findings indicate that MCs, basophils, and lymphocytes can synthesize and release NGF, thus suggesting that these cells might contribute, at least in part, to both the basal and stress-induced levels of NGF in the peripheral circulation (61). This hypothesis is strengthened by in vitro data showing that spleen lymphocytes synthesize and release a factor with NGF-like properties and express NGF mRNA (45). Several peripheral nonneuronal cell populations (such as Schwann cells and perineural fibroblasts) other than those belonging to the immune system can also be induced to synthesize and release NGF (51). Germ cells in the adult testis express NGF (16), whereas supporting Sertoli cells express the NGF receptor. This expression is under hormonal control (87).

Another important question concerns the mechanism(s) responsible for the rapid expression of NGF at the CNS level. Recent data from our laboratory suggest an involvement of hypothalamic peptides in the regulation of NGF secretion (73). In fact, female virgin rats injected with oxytocin show a significant increase in hypothalamic NGF content compared to controls. In addition, interleukins or other cytokines may play a functional role in promoting NGF expression or release (72). Similarly, hormones released as a consequence of stress might interact with NGF or other neurotrophins and affect their rate of synthesis. Adrenal steroids have been found to regulate the levels of mRNA of a number of different neurotrophic factors in the hippocampus and other brain areas (14,18,36). Increased levels of neurotrophic factors, and in particular NGF, during stressful events could in turn reduce the neuronal damage and loss caused by the hypersecretion of GCs (76,90). Changes in NGF levels [e.g., following CWSS (see above)] may support this hypothesis.

A hypothesis worthy of consideration is that changes in NGF levels, as a result of the organism's exposure to stress, could provide a link between the emotional status caused by a psychosocial stressor and the need for the organism to "remember" the events leading to appropriate (or inappropriate) coping with the stressor itself (2,3). It can be argued that the effects of NGF on the adrenal gland previously described could alter the hormonal set point of the organism so that future responses to stressors might also be modified as a result of previous stressful experiences. Moreover, because peripheral innervation has been found to influence human immune responses and cooperate in the development of the so-called "immunological memory" (30), and as NGF exerts a potent stimulatory effect on both peripheral innervation (65,67) and immunocytes of young and adult rats (10,23,35,40,68), this neurotrophin could be associated with mechanisms regulating these events. Although some reports suggest that this might indeed be the case (23,40,35), such an hypothesis needs to be tested experimentally.

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