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PHYSIOLOGICAL ROLES OF NERVE GROWTH FACTOR IN ADULT RODENTS: A BIOBEHAVIORAL PERSPECTIVE

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ABSTRACT: The present review is concerned with the biological role(s) exerted by Growth Factor (GF) protein molecules in adult rodents. In fact, despite the increasing amount of papers published in the last two-three decades about the physiological roles played by Nerve GF and Epidermal GF (as well as by related polypeptide molecules) on the ontogenesis of rodent peripheral and central nervous systems, very little attention has been given to adult regulations involving these two factors. We here report about our studies concerning the biological significance of the huge quantity of NGF stored in the submaxillary salivary glands of the adult male mouse. When released into the bloodstream as a result of psychosocial stress, salivary NGF affects peripheral nervous structures (chromaffine cells and ganglia) and peritoneal mast-cells. Following psychosocial stress, NGF production is enhanced in specific hypothalamic zones. Adult regulations regarding the concomitant EGF release from salivaries are also discussed.

RIASSUNTO: La presente rassegna è centrata sul ruolo esercitato da fattori di crescita polipeptidici (NGF ed EGF) sulle regolazioni fisiologiche di roditori nello stadio adulto. Infatti, nonostante la mole crescente di lavori pubblicati negli scorsi tre decenni sul ruolo biologico esercitato da tali fattori sull'ontogenesi dei sistemi nervosi periferico e centrale di roditori, poca o nulla attenzione e stata invece prestata riguardo al ruolo esercitato da NGF ed EGF in regolazioni fisiologiche nell'adulto. Vengono riportati i risultati di una serie di studi condotti dagli Autori sul rilascio del NGF accumulato in elevatissime quantità all'interno delle ghiandole sottomascellari del topo maschio adulto, e che viene rilasciato in circolo esclusivamente in seguito a stress di tipo psicosociale. Tale rilascio causa ipertrofia di vari tessuti surrenalici, attiva i gangli periferici, e causa una selettiva degranulazione in elementi mastocitari. Lo stress psicosociale eleva nel contempo la produzione di NGF in particolari aree ipotalamice. Vengono infine discussi anche dati riguardanti il ruolo biologico dell'EGF nel roditore adulto, proteina anch'essa immagazzinata nelle sotto-mascellari murine e rilasciata in circolo in seguito a stress psicosociale.

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INTRODUCTION

Nerve Growth Factor (NGF) is a neurotrophic factor with a well established physiological role in promoting and/or maintaining survival of specific portions of the peripheral and central nervous systems (Levi-Montalcini & Angeletti, 1968; Calissano, Cattaneo, Aloe, and Levi-Montalcini, 1984; Black, 1986; Levi-Montalcini, 1987; Thoenen et al., 1987). NGF exerts trophic, tropic, and differentiative effects on specific cellular targets, and mitogenic properties on developing chromaffine cells (Zaimis, 1971; Aloe and Levi-Montalcini, 1979; Lillien and Claude, 1985). In addition, evidence accumulated over the past few years shows that NGF is capable of exerting specific effects on basal forebrain cholinergic neurons of the CNS (Gnahn et al., 1983; Seiler and Schwab, 1984; Korshing, 1986; Shelton and Reichardt, 1986), opening new perspectives about its role in biobehavioral studies.

Although the physiological role of NGF in living organisms is still a matter of great debate, what we would like to stress here is that most of the current debate is centered on the developmental properties of NGF, and that very little room has been left for speculations about NGF (or other well-known GFs) roles on adult CNS/behavioral regulations. For example, Epidermal Growth Factor (EGF), which was considered almost exclusively a GF specific for epidermal-derived tissues has been recently proved to have several target tissues at the CNS level (Fallon et al., 1984, Gomez-Pinilla, 1988; Pioro and Cuello, 1988; Werner et al, 1988). Such a picture is now changing, since, e.g., review articles on NGF effects at the brain level tend to include effects on adult brains (Korshing, 1986), but behavioral functions involving NGF have not been taken into consideration.

Presence of GFs in Adult Vertebrates

It is well known that several vertebrate species synthesize and/or store at adulthood large amounts of GFs in particular structures. As far as NGF is concerned, as an example (NGF is the best known GF among the ever increasing GF molecule "family") (Mercola and Stiles, 1988; Sporn and Roberts, 1988), it is present in the salivary/venom gland of snake species belonging to the *Viperidae, Crotalidae,* and *Elapidae* families (Cohen, 1959; Levi-Montalcini and Cohen, 1965; Hogue-Angeletti et al., 1976; Levi-Montalcini and Angeletti, 1968) in the prostate gland of the Guinea pig, and in seminal plasma of Guinea pigs and the bull (Harper et al, 1979; Harper and Thoenen, 1980; Thoenen et al, 1987). In particular, NGF, together with EGF is produced and stored in very high amounts within the submaxillary salivary glands in the mouse species.

Historically, these murine glandular structures have been extremely important, since they provide the richest and the most easily available

and accessible source of both NGF and EGF. A "gift of nature," easy to extract, and extremely useful in the past for their biochemical characterization and for studying their biological roles. Only in very recent years techniques of recombinant DNA may have represented a powerful alternative in producing several GFs, and providing, as an example, highly purified EGF for human therapeutical uses (Nakagawa et al., 1985; Ito et al., 1986; Brown et al., 1986). Despite the fact that most of the scientific investigations carried out during the past thirty years concerned developmental aspects of NGF biology, the actual molecules used in these studies were from adult structures (salivary glands) of unknown functioning. It is really amazing to note how little curiosity originated from this discrepancy between actual biological investigation and actual source of biological material.

Despite the ever increasing number of investigations carried out in the field of GF biology (it has been estimated that more than one EGF paper per day is published) (Carpenter and Zendegui, 1986), no sound hypothesis has been produced until very recent years to explain why rodent salivary glands contain NGF and EGF in very high amounts, ranging from 350 to 500 ng/mg of wet weight. In the meanwhile, the ontogeny of the storing processes was fully characterized: NGF presence is sexually dimorphic, since male mice have up to ten-fold more salivary GF than female individuals (Caramia et al., 1962; Levi-Montalcini and Angeletti, 1964; Levi-Montalcini and Angeletti, 1968). GF production and storage closely follow testosterone hormone ontogeny, reaching the plateau stage when full sexual maturity is achieved (Levi-Montalcini and Angeletti, 1964). Adult castration abolishes (or at least, markedly reduces) salivary levels (Levi-Montalcini and Angeletti, 1968; Aloe & Levi-Montalcini, 1980; Barka, 1980). Breeding experience also affects salivary NGF levels. Salivaries of male and female mice without sexual experience, in fact, contain less NGF than those of experienced breeders (Aloe, personal communication, 1985).

Some of the hypothetical points of view expressed in the last decades stated that such a storage phenomenon in mice could be explained in terms of an epiphenomenal accumulation of biomaterial without any actual role in adult physiological regulation. In fact, glandular tissues evolve through rapid multiplication of unitary substructures; and such a multiplication could explain the high GF levels as accumulation of molecules of no biological value, in terms of "relics" of previous developmental stages, where they exerted an actual physiological role, and then "dragged" in the evolutionary process of multiplication (Barka, 1980; Thoenen & Barde, 1980; Purves & Lichtman, 1984). The bigger the glandular structure, the more GF molecules will be contained in it.

Other explanations have also been proposed, but none of them was consistent with a proposition of a physiological role for adult GF. All

hypotheses turned around the idea of the *lusus naturae*/freak molecule accumulation leitmotif.

A Biological Role for Mouse Salivary NGF

In 1983 Rita Levi-Montalcini and we started a new research program, aimed at assessing whether or not a series of behavioral syndromes could cause NGF release from male mouse salivary glands into the bloodstream. This idea arose out of several observations in the literature, claiming (Caramia et al., 1962; Aloe et al, 1985; Hendry & Iversen, 1973; Wallace & Partlow, 1976;), or negating (Ogata, 1955; Murphy et al., 1977b; Burton et al., 1978; Murphy et al., 1980) NGF presence in the bloodstream of mice. It appeared to us that such a series of conflicting results could be produced by some uncontrolled (behavioral) variable. Moreover, Bing and co-workers (Bing & Poulsen, 1979; Bing et al., 1980) found that renin, another protein molecule exerting *in vivo* strong regulatory properties, (which is also stored in male mouse salivary glands), was released into the bloodstream following intermale aggressive behavior caused by a prolonged period of individual housing.

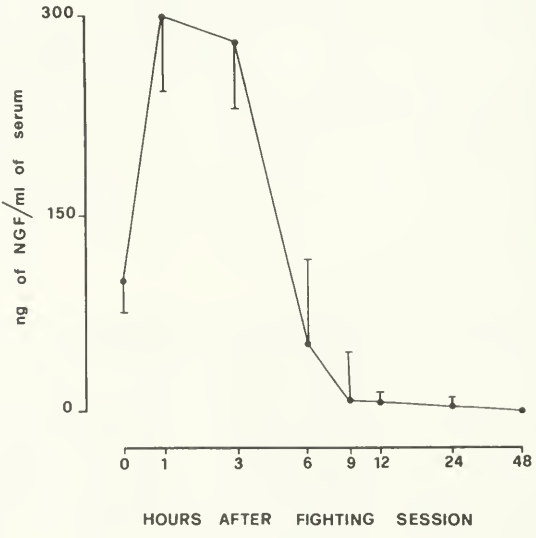

FIGURE 1

Time course of NGF release in the bloodstream of fighting mice. Fighting mice were paired for 20 min with an 8-week isolated adult male mouse, resulting in 32 to 38 separate fighting episodes. All mice used were of the Swiss-derived CD-1 outbred strain. Each point represents the mean ± SEM of six mice. Different animals were used for each time point. For more details see Aloe et al. 1986.

We found that NGF is released into the circulation following a minimum of 12-20 fighting episodes occurring within minutes; that NGF release is rapid (we found circulating NGF after about fifteen minutes from the fighting episodes), reaching a peak blood value about two-three hours later, and returning to basal levels in about 48 hours (Fig. 1 and 2). The peak value was about 300 μl/ml of serum, demonstrating that a massive NGF release actually occurred (Aloe et al., 1986).

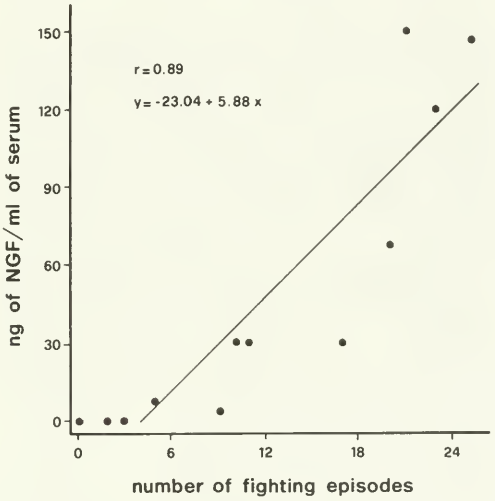

FIGURE 2

NGF levels in blood serum of fighting mice as a function of aggressive behavior scored during a 6 min session. For more details see Aloe et al., 1986.

Sialoadenalectomy (i.e. complete removal of salivary glands) results in undetectable NGF levels after fighting, although it cannot be ruled out that, using more sensitive techniques than those now available, small differences in NGF baseline quantities could be determined. There are many examples showing that endogenous NGF is secreted from source(s) other than the salivary glands (Murphy et al., 1977a; Thoenen & Barde, 1980; Taniuchi et al., 1988). It has been shown in fact that NGF is synthesized and released continuously in minute quantities by sympathetic and sensory neuronal target cells, and following nerve injury or axotomy (Goedert et al., 1986; Thoenen et al., 1987). However, whether or not these different sources of NGF are directly or indirectly activated by fighting behavior has to be established. Following fighting, immunohistochemical and electron microscopy examination (Fig. 3 and 4) of the glandular tissue confirmed a marked depletion of the ductal granules containing NGF, thus indicating that the great increase of blood NGF level was mainly due to secretion from salivary sources (Aloe et al., 1986).

From a behavioral point of view we found that NGF is released only by psychosocial stressors (Weiss, 1968; Axelrod, 1983; Axelrod & Reisine, 1984), i.e., following stress conditions involving behavioral interactions among conspecifics. In fact, we were unable to detect any difference in circulating NGF levels using stress conditions such as "cold water swim," escapable or inescapable footshock, forced biting, or forced restraint (Aloe et al., 1986). For ethical reasons we never tested shock-induced fighting.

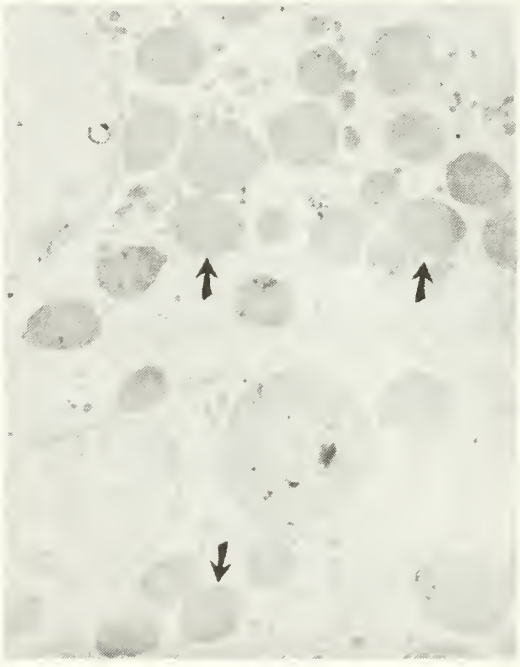

FIGURE 3

Electron micrograph of granule tubule cells from an adult male mouse salivary gland (control). NGF is stored within the electron-dense granules shown by arrows (x 6000). For more details see Aloe et al., 1986.

In contrast, both isolation-induced intermale fighting (and, at lower blood levels, interfemale aggressive behavior) caused a rise in NGF blood levels, as did also precopulation sexual arousal (Alleva & Aloe, 1988). It is rather well known that profound differences exist, in the physiological mechanisms involved in psychosocial stress syndromes and non-psychosocial syndromes (Coe & Levine in press; Henry et al., 1971; Axelrod & Reisine, 1984; Levine & Coe, 1985). Moreover, the specificity of the NGF release can be easily related to the way the animal copes with

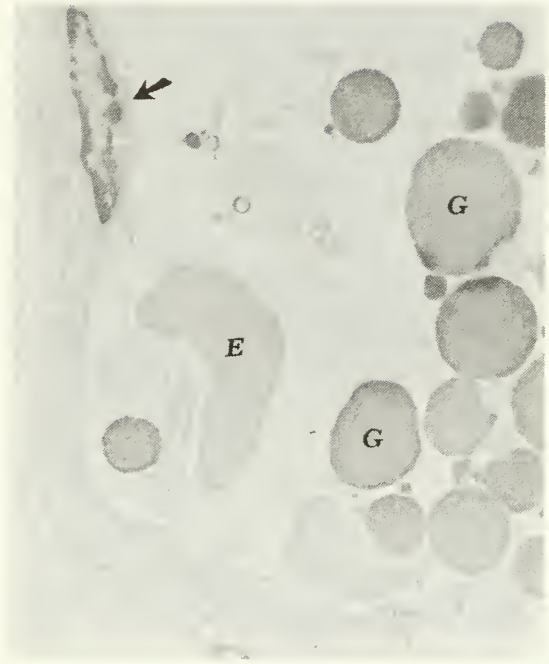

FIGURE 4

Electron micrograph showing a portion of blood vessel within the submaxillary salivary gland of a fighting male mouse. Electron-dense granules similar to those in the granular tubule cells are seen inside the blood vessel, indicating that these granules are released into the circulation. Arrow points to an endothelial cell lining a thin-walled vein, filled with erythrocytes (E) and electron-dense (salivary) granules (G). (x 7000).

the "stressing" situation, in terms of active attempts to put the situation under control, rather than to cope with it through a passive habituation to an unavoidable set of negative stimuli (Levine et. al., 1979; Levine, 1983; Levine & Coe, 1985). In other words, we can expect the animal will be able to display a particular set of social responses toward conspecifics, while interactions with the human experimenter or with inanimate objects will elicit a totally different cascade of physiological events. NGF release, which is accompanied by a concomitant release of salivary EGF (Bing & Poulsen, 1979, Bing et al., 1980; Aloe et al., 1986; Lakshamanan, 1986a), appears then to be causally linked to the first (social) category of "stressing" events. NGF and EGF now seem to be rather specific mediators in rodents (and useful markers) of a series of behavioral/physiological events triggered by a social confrontation inducing behavioral arousal (Fig. 5 and 7).

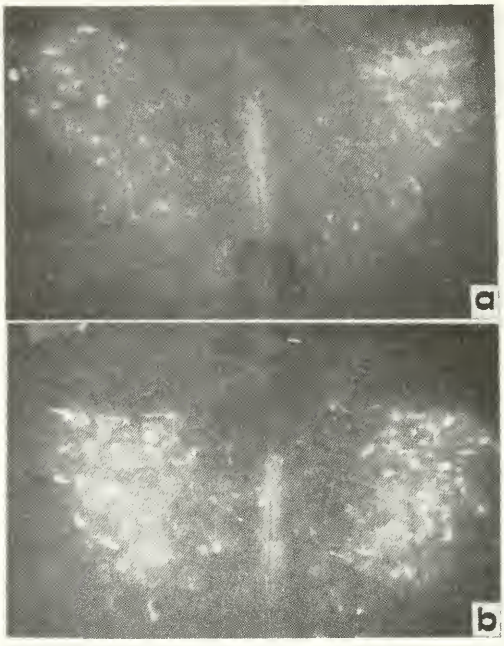

FIGURE 5

Immunofluorescence-stained sections of the anterior hypothalamic area of a control (a) and a fighting (b) adult male mouse showing more numerous NGF positive cells in (b) than in (a). (x 60). For more details see Aloe and Alleva, 1987.

A First Biological Target of Mouse NGF Release: The Adrenals

Several *in vivo* studies and miriads of *in vitro* investigations carried out in the past, at first by the pioneering work of Rita Levi-Montalcini and co-workers, and then by dozens of laboratories in the last three decades, have shown that NGF acts as a trophic and differentiative agent for the peripheral nervous system (Levi-Montalcini & Angeletti, 1968; Levi-Montalcini, 1987; Thoenen et al., 1987), and for the chromaffine tissue contained in the adrenal gland (Zaimis, 1971; Aloe & Levi-Montalcini, 1979). At the beginning it was quite obvious to look at these tissues as putative targets of a massive release of salivary NGF into the blood-stream following fighting.

In fact, it is known from the literature that male mouse adrenals change rather markedly (and rather quickly) following fighting behavior, while female adrenal morphology seems to be much more stable (Welch & Welch, 1969; Brain 1971; 1972; Hucklebridge, et al. 1981). As we reported above, male mice store an enormous amount of NGF in their salivary glands. It appeared therefore worthwhile to hypothesize that salivary NGF controls adrenal morphology (as well as adrenal functional

FIGURE 6

Peritoneal mast cells of isolated (a) and fighting (b) adult male mice showing plasma membrane disruption and cell degranualtion (arrows) following aggressive behavior. (x 160). A concomitant histamine release in the peritoneal fluid was found. For more details see Aloe, 1987.

status) through blood NGF levels, which in turn are regulated by the social/aggressive behavior (see the right part of Fig. 7). Moreover, mouse strains characterized by high levels of aggressive behavior have higher NGF levels in the bloodstream, as reported by Stephani et al., 1987.

We showed that exogenous NGF administrations (given intraperitoneally for ten consecutive days) markedly increase adrenal size (Aloe et al., 1986). It is interesting to note that both the medullary and the cortical layers increase their size following NGF exposure. These findings open the field to subsequent analyses of the putative mechanisms regulating relative balance between medullary and cortical zones of the adrenal gland, and involving interactions with NGF molecules.

It is almost trivial to underline the profound endocrine and behavioral changes produced by such a dramatic enlargement of the adrenals. We could expect both short-term changes in behavioral reactivity at the individual level and long-term changes in mouse population structure (Christian, 1965; Brain, 1971; Bronson, 1987). The latter could be caused by endocrine changes evoked by NGF release, affecting both peripheral nervous system reactivity, and adrenal hormonal levels. These changes are finely tuned by the frequency and

intensity of NGF release, i.e., by the fighting behavior displayed by each single animal within the population (Bronson, 1987; Drickamer, 1987). Moreover (see later) the concomitant EGF release affects male breeding capability directly (Tsutsumi et al., 1986). In other words, GF role in adult mice can be easily explained using an *in vivo* approach, and having in mind sound biological perspectives.

The Hypothalamic NGF Increase

We have recently reported that isolation-induced aggressive behavior causes a marked increase in hypothalamic NGF levels (Aloe & Alleva, 1987). This finding has been confirmed by several independent lines of evidence. NGF was first detected using immunohistochemical localization (Fig. 5), then confirmed by the classical NGF bioassay (halo effect in sympathetic chick ganglia) and radioimmunoassay. We are now carrying out *in situ* hybridization studies in order to measure normal $mRNA^{NGF}$ levels and/or fighting-induced changes of $mRNA^{NGS}$ levels in the hypothalamus.

The tentative explanation for such a difference in brain NGF could be, at first, that NGF interacts with other polypeptides, peptides, and/or hormones which are present in the hypothalamic area, affecting some feedback mechanism, and in turn changing the endocrine status of the organism (Swanson & Sawchenko, 1983; Albert, 1987). A series of interactive effects between NGF and thyroid hormones, ACTH, and peptides, have been reported in the past (Otten et al., 1979; Aloe & Levi-Montalcini, 1980; Otten, 1984; Wion et al., 1985). Recent *in vivo* findings have shown that NGF and thyroid hormones exert a synergistic effect on cholinergic cells of the CNS, suggesting an interaction between NGF and thyroid hormones in the regulation of choline acetyltransferase activity (Hayashi & Patel, 1987).

Such a brain change could in turn cooperate with the concomitant release in the periphery. In fact, we do not expect NGF to cross the blood/brain barrier, and we still do not know if any bioactive NGF fragment could actually enter the brain. However, it has been shown that monoclonal antibodies to the NGF receptor can pass from the cerebrospinal fluid to the region of the brain which contains NGF receptors (Schweitzer, 1987), suggesting that the NGF molecule could enter the brain in a similar way.

Another biological explanation, much more speculative than the former one (but not excluding it), is that the sudden presence of relatively high NGF levels within the brain could serve some still unknown process leading to a renewal of the brain plasticity at the adult stage. We know in fact that under particularly arousing behavioral contexts a "renewed plasticity" of the brain/behavioral condition has been reported

(Black, 1986; Black et al., 1987). It appears that stress can cause imprinting-like phenomena in adults, in which some environmental stimuli suddenly became relevant, and produce long-lasting behavioral alterations (Bateson, 1981; Bateson 1982; Albonetti & D'Udine, 1986). Furthermore, physiological significance of endogenous NGF in regulating the mechanisms of neuronal plasticity in the CNS of adult rodents has been recently suggested. It has been hypothesized that normally ongoing release of endogenous NGF evokes collateral sprouting from the terminal field in the adult nervous system, and that NGF could be involved in adult brain plasticity (Diamond et al., 1987).

Hypothalamic NGF could also represent a functional link between the "emotional" status caused by a psychosocial stressor and the biological needs of the organism (and of its brain) to "remember" the events leading to an appropriate (or inappropriate) coping with the stressor itself. NGF could shift some still unknown brain zones backwards to an "immature-like" stage. The only evidence we have at the present time are that (1) NGF-sensitive brain zones include cholinergic areas (cortex, hippocampus, nucleus basalis magnocellularis, etc.) (Gnahn et al. 1983; Shelton & Reichardt, 1986; Thoenen et al., 1987; Whittemore et al., 1987), and (2) these zones are classically considered the substrates of associative and retention processes. Experimental evidence published very recently have shown that several other structures within the CNS (both cholinergic and non-cholinergic) express NGF receptors (Korsching et al., 1985; Goedert et al., 1986; Shelton & Reichardt, 1986; Raivich & Kreutzberg, 1987; Pioro & Cuello, 1988), suggesting a rather complex role of the NGF molecule in regulating adult rodent CNS.

Endogenous NGF Release Induces Histamine Release from Mast Cells

The first evidence that NGF molecules interact with elements of the immune system was reported by Aloe & Levi-Montalcini (1977), showing that systemic treatment of newborn rats with NGF caused widespread accumulation of mast cells in subepiderma layers and other tissues. Subsequent *in vitro* and *in vivo* studies showed that other cell lines of the immune system of young and adult rodents, such as mast cells (Bruni et al., 1982; Sugiyama et al., 1985), lymphocytes (Abramchik et al., 1988), and platelets (Gudat et al., 1981) also respond to the action of NGF. Further evidence confirmed that NGF stimulates histamine release from isolated adult mast cells and induces plasma extravasation in rat skin. It has also been shown that NGF causes phenotypic conversion of spleen cells into mast cells (Böhm et al., 1986) and potentiates the blastogenic response (an *in vivo* model of immune system response) (Mazurek et al., 1986).

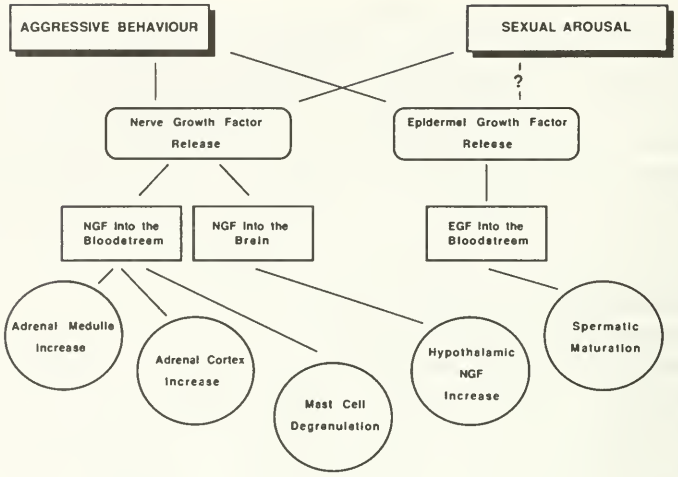

FIGURE 7

Diagram showing the behavioral states and the resulting physiological changes in which NGF and EGF are involved. No information is so far available on EGF release following (pre-copula) sexual arousal. See text for more details.

We have recently studied the structural, ultrastructural, and pharmacological response of peritoneal mast cells after massive release of endogenous NGF from mouse salivary glands. We observed that following isolation-induced aggressive behavior, male mouse mast cells of the peritoneum are highly degranulated (Fig. 7), and as a result of such a degranulation process, a concomitantly higher histamine level in their peritoneal fluid was found (to be published). Exposure to anti-NGF antibodies blocks mast-cell degranulation, indicating that salivary NGF is specifically involved in mast-cell activation. Furthermore, sialoadelanectomy in adult male mice or chronic exposure to NGF antibodies causes size reduction in peritoneal cells, particularly mast cells.

Such a series of results suggest a functional role of NGF, occurring as a result of psychosocial stress, i.e., the potentiation of the immune defenses of the organism, when it faces a potentially damaging life event. However, it has to be stressed that specific stress syndromes cause rather specific changes in specific portions of the immune systems (Stein et al., 1985; Coe & Levine, in press). In particular, as far as intermale fighting in mice is concerned, evidence that fighting results in immunological activation has been reported (Hadry et al., 1987). The NGF release, and the resulting mast-cell degranulation, appears to be a rather selective and limited effect of immunological defence activation.

NGF and EGF Release as Factors Controlling Mouse Populations

Other authors have reported that EGF is similarly co-released with NGF into the bloodstream following isolation-induced intermale fighting (Lakshamanan, 1986b). EGF is also dimorphically contained in much higher amounts in the salivary glands of male mice, while females show low storage levels (Cohen, 1962). It is not yet clear whether or not salivary EGF is stored in the same granules which contain NGF, or in different granules, but their release after aggressive encounters follows approximately the same pattern. Such a co-release poses questions as to whether or not the same cell targets are involved, since a number of different nerve cell types exhibit both NGF and EGF receptors (Levi-Montalcini, 1987; Gomez-Pinilla, 1988; Pioro & Cuello, 1988; Werner et al., 1988). Another point, supposing that both GFs are stored in the same granule, is whether their releases are functionally linked, or viceversa elicited by independent mechanisms. Very recent observations indicate that both EGF and NGF, and $mRNA^{NGF}$ are present in rat testes and seminal fluid (Tsutsumi et al. 1986; Olson et al., 1987), raising some interesting questions about NGF involvement in sperm maturation and motility.

Tsutsumi et al. (1986) recently provided a sound biological explanation for EGF presence in mouse salivary glands. These authors showed that EGF is continuously released into circulation, where it can be detected at the 5 ng/ml level. Sialoadenalectomy abolishes circulating EGF levels, since no detectable EGF was found in serum following salivary gland removal. More interestingly, the number of mature sperm in the epididymis decreased by as much as 55%. At the same time, the number of spermatid in the testes decreased by 40 to 50%, while the number of spermatocytes increased by about 20%. EGF administration to sialoadenalectomized mice restored to normal values both the sperm content of the epididymis and the number of spermatids in the testes. This study demonstrated that, in adult mice, EGF may play a key role in male reproductive function by stimulating the meiotic phase of spermatogenesis.

A subsequent study showed that EGF is also released in male mice following aggressive interactions caused by social isolation (Lakshamanan, 1986b). It is possible to speculate that EGF, together with NGF, exert a "tonic" influence on male reproductive/endocrine status, which in turn depends on the frequency of (and/or amount of) aggressive encounters. Such a functional role could be an essential feature in regulating mouse population levels under natural conditions. Aggressive behavior caused by overcrowding, limited resources, etc. could in fact shape the composition of murine population through hormonal/repro-

ductive/behavioral alterations (Christian et al. 1965; Brain, 1971; Bronson, 1987; Drickamer, 1987) modulated by NGF/EGF release.

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