Aggressive behavior induces release of nerve growth factor from mouse salivary gland into the bloodstream

(submaxillary salivary gland/adrenal gland)

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ABSTRACT   Intraspecific fighting induced by 6–8 weeks of social isolation results in massive release of nerve growth factor (NGF) into the bloodstream of adult male mice. The amount of circulating NGF is highly correlated with the number of fighting episodes. Biological, radioimmunological, immunohistochemical, and ultrastructural studies show that NGF is discharged from the salivary gland into the blood within minutes after fighting and reaches the highest level 3–4 hr later. Adrenergic innervation of the salivary gland or adrenalectomy does not abolish the NGF release. Corticotropic hormones do not induce NGF increase in the blood. Daily administrations of highly purified NGF (3 µg per g of body weight) result in a considerable increase in the volume of adrenal glands. These findings are unequivocable evidence for a physiological role of the mouse salivary glands as a major source of blood NGF.

Almost 3 decades ago, it was reported that mouse submaxillary salivary glands (SSGs) synthesize and release an extraordinarily large amount of a nerve growth factor (NGF) molecule (1, 2) endowed with a potent nerve growth-promoting activity on sympathetic and sensory nerve cells (3–6). This finding raised the question whether this molecule serves any specific function and, if so, whether it is discharged into the saliva, into the blood, or into both. All the results obtained so far seem to favor the hypothesis that this salivary product, the NGF, is routed from the gland secretory ducts into the saliva, where it plays some still uncertain function (7, 8). This hypothesis springs from the almost generally agreed upon observation that the injection of pharmacological agents shows NGF not to be present in mouse blood, whereas it is present in large amounts in the saliva (9–11). Structural, ultrastructural, and immunohistochemical studies have also favored this hypothesis and shown that the granular products are localized in the apical part of the cell lining in the gland tubular portion where NGF has been identified (11, 12). However, results from this laboratory provided convincing evidence that, upon pharmacological stimulation (13, 14), NGF from SSG can also be found in the bloodstream.

A recent report that aggressive behavior results in the release into the blood of a large amount of another biologically active protein synthesized in the gland tubular portion, renin (15), prompted us to investigate the effects of aggressiveness induced by social isolation on NGF content in the blood. The present study was aimed at the investigation of this problem and, more importantly, at the possible functional significance of such a large amount of NGF synthesis and release by mouse salivary glands. The results of these studies showed that aggressive behavior induces massive NGF discharge and that the adrenal gland might be a primary target for this endogenous NGF release.

MATERIALS AND METHODS

Behavioral Studies. Male mice of a Swiss-derived (CD-1) strain (25–27 g), purchased from Charles River Italy, were used. They were either individually housed for 6–8 weeks or maintained in groups of three (nonisolated mice) in opaque Plexiglas boxes (33 × 13 × 14 cm) with metal tops. Water and standard pellet food were freely available. The animals were kept in an air-conditioned room at 21 ± 1°C and 50% ± 10% relative humidity, using a 9:30 a.m. to 9:30 p.m. lighting schedule. Isolated and nonisolated mice were randomly assigned to the control or fighting condition. Fighting mice were introduced in pairs in a clean box of the same type as the home box, and the number of fighting episodes was recorded. A pilot study showed a high consistency of the scores assigned by four different observers during testing or by the same observer in repeated examinations of videotape records. All scores in the present study were assigned by the same observer. Tests were carried out between 10:00 a.m. and 1:00 p.m. Unless otherwise indicated, the fighting sessions lasted 20 min. (Under these conditions, no significant sign of dominance was observed.) Blood was collected from the retro-orbital plexus with a glass pipette at the end of the fighting sessions (with the exception of the time-course study). All samples were used for biological (5) and radioimmunological (16) assays. Each mouse was used only once.

Surgical and Chemical Treatments. Mice were sialoadenectomized or adrenalectomized under deep sodium pentobarbital (Nembutal) anesthesia 2 days before fighting. For immunosympathectomy, neonates were injected daily during the first postnatal week with antibodies to NGF (17). At the age of 10 weeks they were isolated, and 6 weeks later they were used in fighting sessions. For chemical sympathectomy (18), 6-hydroxydopamine (50 µg per g of body weight) obtained from Fluka was injected intraperitoneally (i.p.) into isolated mice for 2 consecutive days; these animals underwent a fighting session 2 days later. Eye ptosis and the absence of adrenergic nerve terminals in the iris were used as confirmation that complete sympathectomy was achieved (19). Porcine corticotropin (ACTH) purchased from Sigma was dissolved in 0.09% saline [270 international units (IU) in 1 ml of saline]. Adrenal gland (30 in 4 ml) and hypophysis (20 in 2 ml) of adult male mice were homogenized in phosphate-buffered saline in an ice bath using a glass and Teflon homogenizer. The insoluble material was removed by centrifugation at 3000 rpm for 15 min at 4°C. Isolated nonfighting male mice were injected i.p. with (*i*) 0.5 ml of extract of adrenal gland, (*ii*) 0.2 ml of extract of hypophysis, or (*iii*) 27 IU of ACTH. Blood was collected 20–60 min and 120 min after the injection, and serum was assayed for the presence of NGF.

Abbreviations: NGF, nerve growth factor; SSG, submaxillary salivary gland; ACTH, corticotropin.

NGF was prepared by the method of Bocchini and Angeletti (20) and further purified by an additional passage through CM-52 column chromatography resulting in a NGF with undetectable renin activity (21). Adult male mice were injected i.p. for 10 consecutive days with NGF or vehicle solution (saline, 0.09%) (3 μg per g of body weight) and sacrificed on the 11th day for adrenal gland histological examination.

Histological and Histometrical Studies. Adrenal glands and SSGs were fixed in alcoholic Bouins' solution, washed, dehydrated, embedded in Paraplast, sectioned (7 μm thick), and stained in hematoxylin/eosin or toluidine blue. To determine the relative volume of adrenals, serial sections were projected with a camera lucida, and outlines of the cortical and medullary zones were traced on paper. The outlines of all sections were cut out and then weighed on a torsion balance (22). SSGs were also fixed and processed for immunohistochemical localization of NGF (14).

Ultrastructural Studies. Fighting and nonfighting mice were perfused with buffered paraformaldehyde (4%) and glutaraldehyde (1%) fixative. SSGs were removed, washed, and postfixed with 1% OsO$_4$. For light microscopic observation, tissues were dehydrated, embedded in Epon 812, sectioned (1 μm), and stained with toluidine blue. Ultrathin sections were stained with uranyl acetate and lead citrate and observed with a Philyps 200 electron microscope.

RESULTS

There is no detectable NGF in the whole (100%) blood serum of mice according to our biological and radioimmunological assays (Table 1; see also refs. 4–6). Likewise, blood serum collected from mice isolated for several weeks (control) contains no bioassayable NGF. On the contrary, blood samples from isolated animals that experience fighting (fighting mice) contain high levels of NGF ($\approx$100 ng/ml) and stimulate the outgrowth of nerve fibers in cultured chicken sympathetic and sensory ganglia when they are added to the medium in appropriate dilutions (Fig. 1). This finding indicates that biologically active NGF has been released into the bloodstream. A time-course study showed that the level of NGF released into the blood increases gradually up to 300 ng/ml during the first 2–3 hr and then decreases steadily, reaching basal levels 12–24 hr later (Fig. 2). As shown in Fig. 3, the concentration of released NGF is positively correlated with the frequency of fighting episodes, indicating some relationship between aggressive behavior and serum NGF concentration.

To determine how closely the amount of NGF found in the blood after fighting is related to the release of the NGF present in the SSG, we made histological, immunoperoxidase, and electron microscopic comparisons of the

Table 1. Relationship between number of fighting episodes and NGF serum concentrations of male mice subjected to different experimental conditions

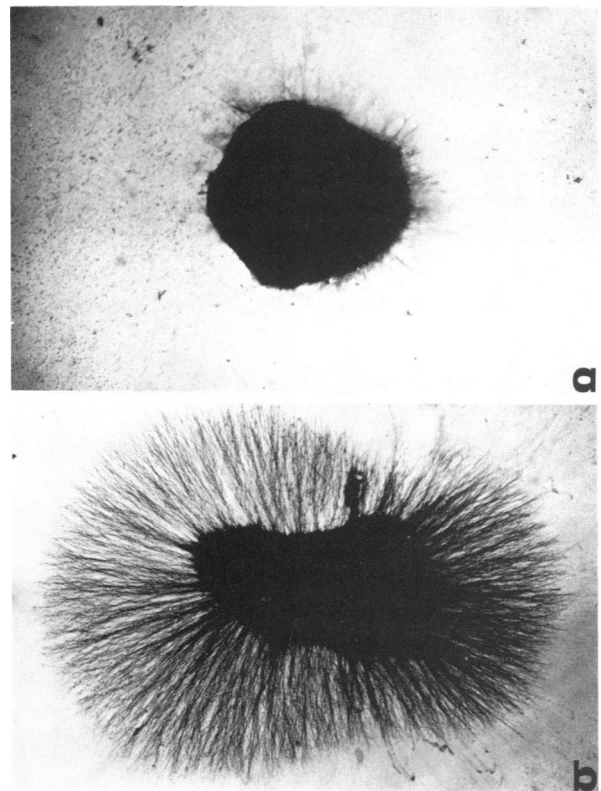| Condition	*n*	Fighting episodes	Serum NGF, ng/ml
Control	6	—	ND
Fighting	10	32.9 ± 3.2	103.5 ± 20.1
Nonisolated	7	31.6 ± 3.3	88.4 ± 12.3
Sialoadenectomized	5	37.2 ± 4.4	ND
Immunosympathectomized	6	35.7 ± 5.1	101.7 ± 23.6
6-Hydroxydopamine	6	38.2 ± 5.9	93.3 ± 35.5
Adrenalectomized	5	32.2 ± 8.9	52.5 ± 13.5
Adrenal extract	8	—	ND
Hypophysis extract	8	—	ND
ACTH	6	—	ND

Values are means ± SEM. ND, not detectable.

FIG. 1. Neurite outgrowth from chicken sensory ganglia cultured for 24 hr in the presence of 20 μl of blood serum collected from control (*a*) and fighting (*b*) mice. Silver stain. ($\times$60.)

submaxillary granular convoluted tubules of fighting and nonfighting mice. The results of the three morphological studies clearly indicate that there is a depletion of NGF-containing granules in the SSG of fighting mice (Fig. 4). Furthermore, mice sialoadenectomized two days before isolation retained high levels of fighting behavior, but no increase in their blood NGF concentration was found. As shown in Table 1, blood samples collected from fighting mice that underwent chemical or surgical sympathectomy still
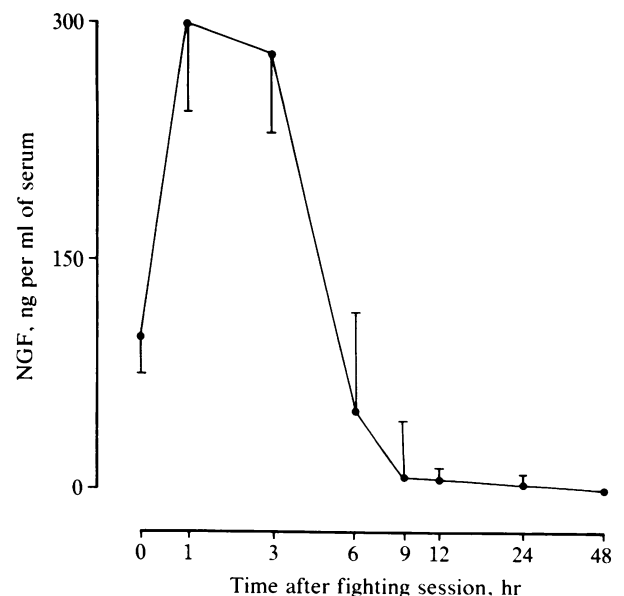

FIG. 2. Time course of NGF release in the bloodstream of fighting mice. Each point represents the mean ± SEM of six mice. Different animals were used for each time point.
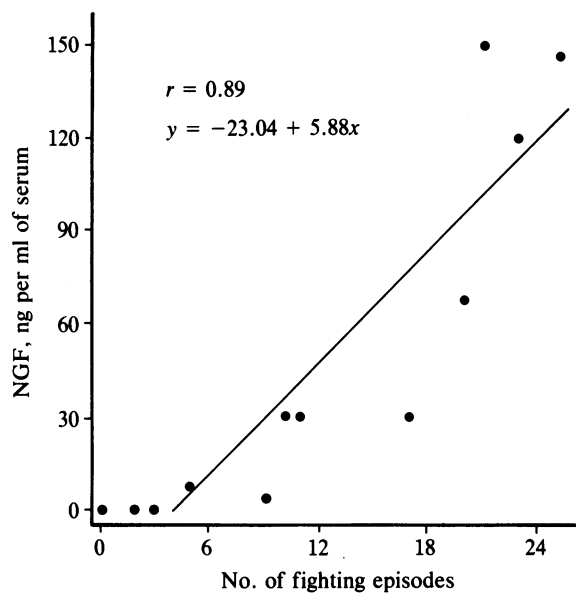
FIG. 3.   NGF levels in blood serum of fighting mice as a function of aggressive behavior scored during a 6-min session. NGF values refer to mean levels of the two fighting animals.

contain large amounts of NGF. To assess the role of ACTH and adrenal gland hormones on NGF release, the blood serum of adrenalectomized fighting mice or that of nonfighting mice previously injected with ACTH, hypophysis extract, and adrenal gland extracts was assayed for the presence of NGF. The results show that adrenalectomized fighting mice maintain high levels of NGF in their blood serum and that
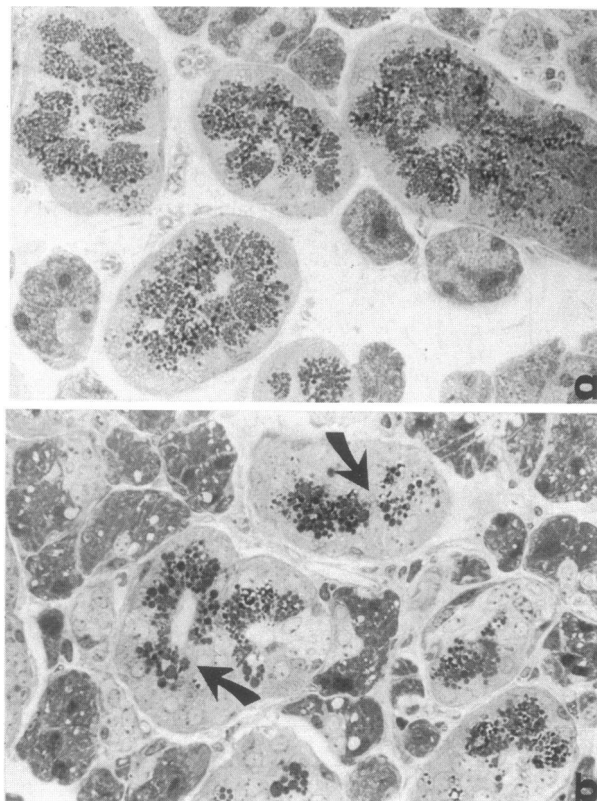

FIG. 4.   Histological sections of SSG from control (*a*) and fighting (*b*) mice stained with toluidine blue. Note the depletion of NGF-containing granules in the convoluted tubules of the gland in the fighting animal (arrows). (×320.)

ACTH injection does not cause any NGF release into the serum. Moreover, injection of adrenal gland or hypophysis extract in adult mice did not result in NGF release into the blood (Table 1). It appears, therefore, that neither corticosteroid excess nor ACTH is involved in the NGF release into the blood serum.

As shown in Fig. 5, daily injections of NGF (3 μg per g of body weight) for 10 consecutive days result in marked weight and volume increases of these glands. Both the cortical and medullary zones are larger in weight and volume than those of vehicle-injected and unhandled controls. This effect is more pronounced in the medullary than in the cortical zones of the glands. In all cases, adrenals of NGF-treated mice differed significantly (paired t tests, $P < 0.01$ or less upon Bonferroni's adjustment) from those of the other groups, while glands of saline-injected and unhandled groups did not differ from each other ($P < 0.05$).

DISCUSSION

The results demonstrate that intraspecific fighting induced by social isolation (23, 24) causes massive NGF release from mouse SSG. Nonisolated male mice paired with isolated ones engage in aggressive behavior and likewise show high levels of NGF release. Thus, fighting *per se*, rather than a combined effect of isolation and fighting, produces the NGF release. This release appears to be specifically induced by intraspecific fighting, a form of psychosocial stress (25). An extensive series of experiments using a different stressful condition—i.e., inescapable footshock treatment (26, 27) (number of shocks, 5–100; intensity, 0.1–5 mA; duration, 1–60 sec; intershock interval, 15–600 sec; 1 or 3 days of treatment)—failed to evidence any NGF release into the blood. Moreover, another series of tests using stresses such as cold water swimming, forced immobilization, and forced biting yield similarly negative results.

The release of NGF does not appear to be mediated by the adrenergic innervation of the salivary glands. In fact, blood samples collected from fighting mice immunosympathectomized with antibody to NGF since birth, or chemically sympathectomized with 6-hydroxydopamine as adults, still contain large amounts of NGF. These findings suggest that under the present conditions NGF release into the bloodstream from SSG is not mediated by the adrenergic nerves innervating their tubular portion or, alternatively, by an enhancing of norepinephrine release from postganglionic nerves and the consequent potentiation, as first hypothesized by Cannon and Rosenblueth (28), of the defensive mechanism and/or aggressiveness. Since adrenalectomy did not block NGF release in fighting mice, and injections of commercially available ACTH or adrenal gland and hypophysis extracts in isolated adult male mice did not result in NGF release into the bloodstream (Table 1), it is unlikely that circulating corticosteroids or ACTH could account for this effect.

While the nature and mechanism triggering the NGF release into the bloodstream remain unknown, increase of circulating NGF results in adrenal gland hypertrophy, following administration of NGF, as demonstrated in these studies and suggested in a previous report (22). It is also known that repeated fighting causes an increase in size and weight of adrenal glands (25, 29–31), an effect that in light of present results could be elicited by circulating NGF. The hypertrophic hyperplastic effect, as well as the selective induction of tyrosine hydroxylase caused by NGF treatment in rodent adrenal medullary cells (32–34), lend support to the hypothesis of the role of NGF in the stress syndrome accompanying the aggressive behavior. It remains to be seen whether the adrenal cortical hypertrophy produced by NGF administration to adult mice is a direct NGF-specific corticotropic effect.
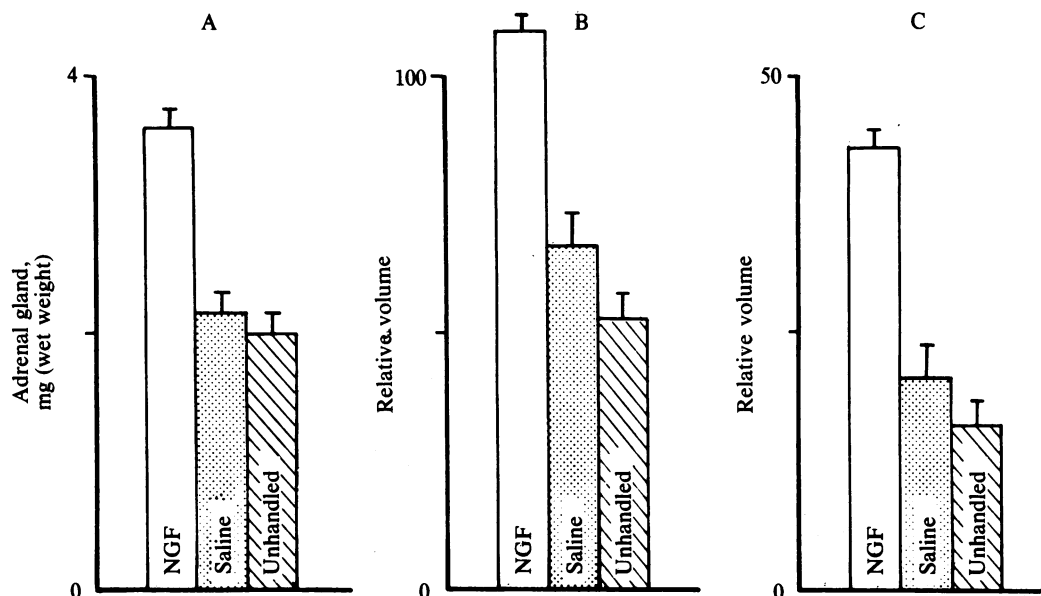
FIG. 5.   Histogram of adrenal gland (wet weight) (A) and relative volumes of cortical (B) and medullary (C) zones of mice injected i.p. for 10 consecutive days with purified mouse NGF or vehicle (Saline) solution (3 μg per g of body weight) or left undisturbed (Unhandled). Relative volumes refer to area of paper covered by microscopic projections (×40) of adrenal sections. Values represent weight of paper (mg). At least 12 glands in each treatment group were used.

The results of these studies demonstrate that in adult male mice aggressive behavior, but not other kinds of stressful events, leads to a massive release of NGF from SSG into the blood. Concomitant evidence that exogenous NGF produces a marked increase in the medullary zone of the adrenal gland suggests an effect similar to that of the endogenous release of NGF. To what extent the latter effect may be related to the well-known aggressive behavior of males of this species (24, 35) remains to be investigated.

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