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Stimulation of Nerve Growth Factor Biosynthesis in Developing Rat Brain by Reserpine: Steroids as Potential Mediators

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SUMMARY

The stimulation of β-adrenergic receptors by isoproterenol increases nerve growth factor (NGF) biosynthesis in C6 rat glioma cells, suggesting that norepinephrine may regulate NGF biosynthesis *in vivo*. We have tested this hypothesis in 21-day-old rats by depleting catecholamine stores with reserpine. Northern blot analysis of NGF mRNA, in combination with a two-site enzyme immunoassay for NGF, showed that depletion of catecholamines was associated with a 3-fold increase in NGF mRNA, which was followed by a significant increase in the NGF content of cerebral cortex. The increase in NGF mRNA was most marked 9 hr after reserpine administration (2 mg/kg, subcutaneously) and was no longer apparent 24 hr after drug administration, when brain monoamine stores were still depleted. Moreover, the lowest dose of reserpine that significantly increased NGF mRNA levels induced only a small change in the content of cortical catecholamines. These results suggest that reserpine mediates the increase in NGF production by a mechanism other than monoamine depletion. Because reserpine increases plasma glucocorticoid concentrations through the pituitary-adrenal axis, we investigated whether adrenal steroids could be responsible for the induction of NGF biosynthesis. The effect of reserpine on NGF biosynthesis was abolished in adrenalectomized rats. Moreover, dexamethasone, a synthetic glucocorticoid, given at a dose of 0.5 mg/kg, subcutaneously, increased the amount of NGF mRNA and NGF in cerebral cortex. NGF biosynthesis in the central nervous system may, thus, be regulated by adrenocortical hormonal secretion.

Recent findings have suggested that NGF may prove to be useful as a therapeutic tool for neurodegenerative diseases characterized by defective neuronal plasticity (1, 2). However, NGF cannot be administered parentally because of difficulties imposed by the blood-brain barrier, and so pharmacological agents that enhance brain NGF biosynthesis might represent an appropriate alternative.

In C6 rat glioma cells, the synthesis and release of NGF are increased by BAR stimulation and by the activation of molecular mechanisms that lead to an increase in the concentration of intracellular cAMP (3-5). The increase in NGF mRNA mediated by isoproterenol, a BAR agonist, is blocked by the BAR antagonist propranolol (6). These results suggest that catecholamines may also regulate NGF biosynthesis in brain glial cells *in vivo*. However, because C6 glioma cells, unlike brain astrocytes, operate in the absence of catecholamines, it is not clear whether a physiological significance can be inferred from the C6 glioma cells data.

To gain insight into the mechanisms that are operative in the regulation of brain NGF biosynthesis, we have investigated whether the induction of NGF biosynthesis can be regulated *in vivo* by catecholamines. We have attempted to deprive BAR of endogenous catecholamines by depleting catecholamine stores with reserpine. This drug reduces brain catecholamine and serotonin content by blocking storage of these amines in synaptic vesicles. We now report that reserpine injection causes a dose-dependent activation of brain NGF biosynthesis.

Materials and Methods

Treatment of animals. We used 21-day-old male Sprague-Dawley rats (Zivic Miller, Allison, PA) for this study. Adrenalectomized and sham-operated rats were received directly from Zivic Miller (adrenalectomy was performed at the age of 13 days), and treatment began 1 week later. Adrenalectomized and sham-operated rats received saline in place of drinking water. During the treatments, all rats were maintained five/cage in a temperature-controlled room with a 12-hr light-dark cycle. Food and water were provided *ad libitum*. Reserpine (Sigma, St. Louis, MO) was dissolved in 0.1 M acetic acid, and dexamethasone in 1:9 (v/v) ethanol/saline; both drugs were injected subcutaneously, with control rats receiving the appropriate drug vehicle. Rats were killed by decapitation at the indicated times after a single dose of the drugs or vehicle. The brains were quickly removed, and specific brain

ABBREVIATIONS: NGF, nerve growth factor; BAR, β-adrenergic receptor; 50× Denhardt's, 1% bovine serum albumin, 1% Ficoll, 1% polyvinylpyrrolidone; BSA, bovine serum albumin; HPLC, high performance liquid chromatography; ACTH, adrenocorticotropic hormone; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ANOVA, analysis of variance; NE, norepinephrine; DA, dopamine; 5-HT, 5-hydroxytryptamine.

structures were dissected as previously described (7) and stored at −70° for analysis at a later time.

Isolation of RNA and blotting procedure. Tissues were homogenized in 5 M guanidine isothiocyanate, and total RNA was isolated by the cesium chloride method (8). Poly(A)$^+$ RNA was separated by two cycles of chromatography on oligo(dT) cellulose (9), denatured in 50% formamide, 6% formaldehyde, for 15 min at 65°, and subjected to electrophoresis in a 1.1% agarose gel containing 6% formaldehyde. After electrophoresis, RNA was transferred to a nylon membrane (Nytran; Schleicher and Schuell, Keene, NH), by the capillary-blot procedure, and fixed. Blots were prehybridized at 63–65° for at least 4 hr in 50% formamide, 5× Denhardt's solution, 0.5× standard saline citrate, 0.1% sodium dodecyl sulfate, 25 mM EDTA, 12.5 mM Tris·HCl (pH 7.4), 100 μg/ml denatured salmon sperm DNA, and then hybridized with a ^{32}P-labeled NGF antisense RNA (6), in fresh prehybridizing solution but containing 1× Denhardt's, at 63–65° for at least 18 hr. Blots were then washed in 0.1× standard saline citrate, 0.1% sodium dodecyl sulfate, at 68° and, finally, exposed to Kodak X-OMAT film with intensifying screens at −70°. After exposure, the radioactivity was removed from the blots by washing, as previously described (10). The blots were then rehybridized with a ^{32}P-labeled nick-translated p1B15 cDNA (see below) (11, 12), washed, and exposed to a new X-ray film.

Probe preparation. The clone pGEM-NGF is a derivative of pGEM-3Z (Promega, Madison, WI) containing a 543-base pair cDNA (a gift from Dr. A. Ullrich, Genentech, San Francisco, CA) encoding mouse β-NGF (13). The transcript generated by the SP6 polymerase is the β-NGF antisense RNA (6). The *in vitro* transcription reaction was performed as previously described (14), to yield ^{32}P-labeled cRNA at a specific activity of 8×10^8 cpm/μg of RNA (5, 6). Clone p1B15 (a gift from Dr. R. Milner, Research Institute of Scripps Clinic, La Jolla, CA) contains the cDNA encoding the stable structural protein cyclophilin (11, 12). This plasmid was labeled by nick translation (15).

Quantitation of NGF mRNA. The intensity of the autoradiographic hybridization band was quantified by laser densitometry. The amount of the NGF mRNA detected in the blot was estimated in arbitrary units that were defined as the ratio between the densitometric peak of the NGF mRNA band and that of the cyclophilin mRNA band. Cyclophilin mRNA has been used as a reference standard to correct for experimental artifacts and losses of poly(A)$^+$ RNA during mRNA extraction, purification, and blotting (5, 6, 10, 16). Therefore, our relative units allow estimation of a change in the amount of specific mRNA after correction for experimental errors due to variations in purification, hybridization, or extraction procedures.

NGF two-site enzyme immunoassay. Tissues were sonicated in 50 mM Tris·HCl (pH 7.0), 150 mM NaCl, 1% BSA, 1% Triton X-100, 4 μg/ml aprotinin, 0.5 mM phenylmethylsulfonyl fluoride, 2 mM EDTA, 0.1 mM benzethonium chloride, 0.05% sodium azide (extraction buffer), and centrifuged at 15,000 × *g* for 10 min. $CaCl_2$ (10 mM final concentration) was added to the supernatant before the latter was transferred to the wells of an immunoplate. NGF standards (10 to 320 pg/ml) (a gift from Dr. B. Wise, Fidia-Georgetown Institute for the Neurosciences, Washington, DC), prepared in extraction buffer, were used to generate a standard curve. The quantitative two-site enzyme immunoassay for NGF described by Korsching and Thoenen (17) was performed with the following modifications. Immunoplates were coated for 2 hr at 37° with a purified monoclonal antibody to NGF (0.67 μg/ml, 0.15 ml/well) obtained from Boehringer Mannheim (Indianapolis, IN) and derived from the hybridoma clone 27/21; the plates were then incubated with 1% BSA in 50 mM sodium carbonate (pH 9.6) for 1 hr at room temperature and subsequently washed three times with 50 mM Tris·HCl (pH 7.0), 200 mM NaCl, 10 mM $CaCl_2$, 0.1% Triton X-100, 0.05% sodium azide (buffer A). Tissue extracts or NGF standards were added (0.1 ml/well), and the plates were incubated overnight at 4°. After extensive washing with buffer A, a β-galactosidase-conjugated monoclonal antibody to NGF (Boehringer Mannheim), diluted in buffer A containing 1% BSA (buffer B), was added to the wells (0.133 units/ml, 0.1 ml/well), and the plates were incubated for 4 hr at 37°. After washing with buffer A, the wells were incubated at 37° with the enzyme substrate, chlorophenol red β-D-galactopyranoside (2 mg/ml, 0.2 ml/well) (Boehringer Mannheim), which was prepared in 100 mM HEPES (pH 7.0), 150 mM NaCl, 2 mM $MgCl_2$, 0.1% sodium azide, 1% BSA (buffer C). The reaction product was measured against buffer C at a wavelength of 570 nm, with a Dynatech MR600 microplate reader.

Measurement of catecholamine and indolylalkylamine in rat cerebral cortex. Frozen brain samples were homogenized in 300 μl of 0.4 M perchloric acid containing 0.1 mM EDTA and were then centrifuged at 30,000 × *g* for 30 min at 4°. The supernatant was transferred to filtering tubes with 0.2-μm-pore filters and centrifuged again for 5 min at 6,500 × *g*. NE, DA, and 5-HT in the final filtrate were analyzed by HPLC. The HPLC system used was a Bioanalytical System LC 50, with an ODS phase II column and an LC 4A amperometric detector coupled to a TL 5 glassy carbon electrode. The HPLC mobile phase consisted of 3.5% acetonitrile/96.5% 0.15 M monochloroacetic acid (pH 3) containing 1.08 mM sodium octyl sulfonate and 0.6 mM EDTA (disodium salt). UV-grade tetrahydrofuran (1.8%, v/v) was added after filtering and degassing (18). The electrochemical detector was set on a sensitivity of 1 nA/V for reserpine-treated animals and 5 nA/V for the control animals, at a +0.8-V potential with respect to the reference Ag/NaCl electrode. The amount of monoamines in each sample was calculated per mg of protein. Proteins were measured by the method of Lowry *et al.* (19).

Plasma corticosterone determination. Trunk blood obtained after decapitation of the animals was collected in heparinized glass tubes and centrifuged. Plasma was stored at −20° for corticosterone determination, at a later time, by radioimmunoassay with the RSL 125-I corticosterone kit (ICN, Costa Mesa, CA), which does not require a protein denaturation step.

Statistical analysis. Differences among means were evaluated by ANOVA procedures. When drug treatment elicited significant changes, significance was determined by Dunnett's test (for comparing treatment groups with a single control) or Scheffe's test (for multiple comparisons).

Results

Measurement of brain NGF mRNA and NGF: an index of NGF biosynthesis in developing rats. We used quantitative Northern blot hybridization analysis of NGF mRNA, in combination with a two-site enzyme immunoassay for NGF, to compare the dynamics of NGF biosynthesis in control and treated animals. We elected to use 21-day-old rats because NGF gene expression in the brain is enhanced at this age (20, 21). In poly(A)$^+$ RNA from 21-day-old rats, as well as from adult brain (20, 21) and C6-2B glioma cells (6), the NGF antisense RNA probe detects two hybridization bands (Fig. 1a). The major band (90% of the total hybridization density) corresponds to a transcript of 1.3 kilobases and the minor band (10% of total hybridization) to a transcript of 1.7 kilobases. NGF mRNA expression is high in cerebral cortex and hippocampus and correlates with the amount of NGF in these brain areas (Fig. 1b).

Reserpine stimulation of NGF biosynthesis. We investigated the effect of catecholamine depletion on the amount of NGF mRNA and NGF *in vivo*. Paradoxically, the depletion of catecholamines and 5-HT elicited by a single injection of reserpine (2 mg/kg, subcutaneously) is associated with an unexpected increase (3- to 4-fold) in the amount of NGF mRNA present 9 hr after administration of the drug (Fig. 2a). This increase is not due to a nonspecific change in total RNA synthesis, because the amount of β-actin mRNA (data not shown) or cyclophilin mRNA remained unchanged (Fig. 2a). The reserpine-induced increase in the amount of NGF mRNA

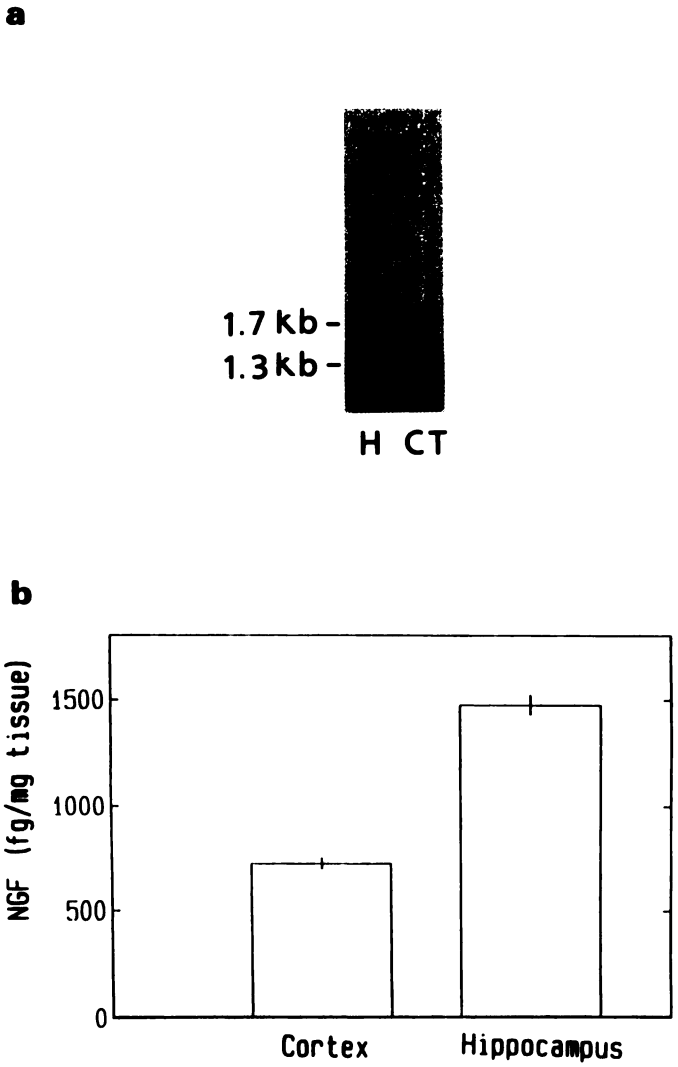

Fig. 1. NGF biosynthesis in cerebral cortex and hippocampus. a, Northern blot analysis of RNA from hippocampus (*H*) and cerebral cortex (*CT*) of 21-day-old rats. Poly(A)$^+$ RNA (15 μg from hippocampus and 20 μg from cerebral cortex) was loaded on a 1.1% agarose-formaldheyde gel and hybridized with an NGF cRNA probe (see Materials and Methods). The sizes of the NGF mRNA bands were calculated on the basis of an RNA ladder (0.24 to 9.5 kilobases; BRL, Gaithersburg, MD). The blot was exposed for 24 hr to Kodak X-OMAT film, with intensifying screens. b, Quantitation of NGF in cerebral cortex and hippocampus of 21-day-old rats by a two-site enzyme immunoassay (see Materials and Methods). Data are means ± standard errors of three separate experiments (seven determinations).

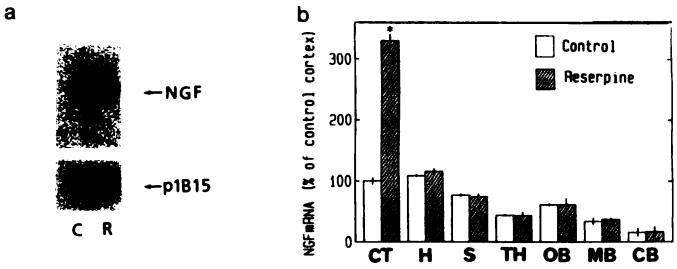

Fig. 2. Reserpine-induced increase in the amount of NGF mRNA in cerebral cortex. a, Northern blot analysis of poly(A)$^+$ RNA from cerebral cortex of 21-day-old rats treated with vehicle (*C*) or reserpine (*R*) (2 mg/kg, subcutaneously) and killed 9 hr after the injection. The blot was hybridized with NGF antisense RNA and exposed to X-ray film. Radioactivity was then removed from the blot by washing (see Materials and Methods), and the blot was rehybridized with nick-translated p1B15 cDNA (which encodes cyclophilin) and exposed to a second X-ray film. b, Quantitation of the relative amounts of NGF mRNA (calculated by using cyclophilin mRNA as a standard reference, as described in Materials and Methods) in cortex (*CT*), hippocampus (*H*), striatum (*S*), thalamus-hypothalamus (*TH*), olfactory bulb (*OB*), midbrain (*MB*), and cerebellum (*CB*). Data, expressed as a percentage of the value for control cortex, represent the means ± standard errors of three independent experiments (nine determinations). *, $p < 0.01$, by ANOVA.

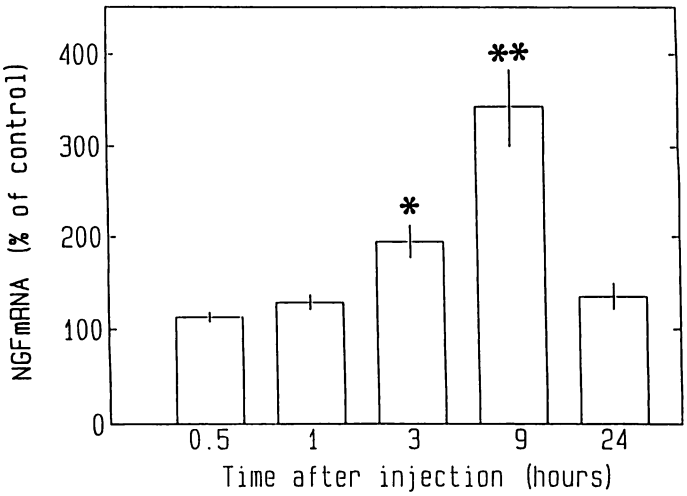

Fig. 3. Time course of reserpine effect on the amount of NGF mRNA in cerebral cortex. Quantitation of the relative amounts of NGF mRNA in the cerebral cortex of 21-day-old rats injected with reserpine or vehicle and killed at the indicated times after injection (see Fig. 2 legend). Data, expressed as a percentage of control, are the means ± standard errors of three independent experiments (three determinations). *, $p < 0.05$; **, $p < 0.01$, versus control (ANOVA and Dunnett's test).

appears to occur specifically in the cerebral cortex; reserpine fails to affect NGF mRNA levels in other brain areas 9 hr after treatment (Fig. 2b). A time course shows that the maximum increase in NGF mRNA induced by reserpine occurs approximately 9 hr after the drug administration (Fig. 3). Although the amount of cortical NGF is not significantly increased at this time, it increases (150% when compared with the appropriate control) 12 hr after administration of the drug and thereafter gradually returns to the control level by 36 hr (Fig. 4).

Reserpine-induced decrease in the amount of cortical monoamines and increase in plasma corticosterone levels. The short lasting changes in cortical NGF mRNA elicited by reserpine prompted us to evaluate whether this effect is independent of the long lasting depletion of monoamine (NE, DA, 5-HT) stores induced by this drug. Reserpine (2 mg/kg, subcutaneously) greatly diminishes the amount of brain monoamines within 1 hr (control, NE = 5.4 ± 0.34, DA = 2.5 ± 0.29, 5HT = 15.2 ± 1.70 ng/mg of protein; with reserpine, NE = 1.2 ± 0.15, DA = 0.6 ± 0.19, 5HT = 4.1 ± 0.56 ng/mg of protein), and this depletion persists for longer than 24 hr (Fig. 5a). Therefore, whereas the brain catecholamine content remains depleted by reserpine, cortical NGF mRNA increases and returns to normal levels. Because of the lack of correlation between these events, we investigated whether other reserpine-induced changes might show a better temporal coincidence with the reserpine-induced changes in NGF mRNA.

Among the biochemical effects induced by reserpine is the release of ACTH (22), which, in turn, increases blood corticosterone levels. Time course studies show that plasma corticos-

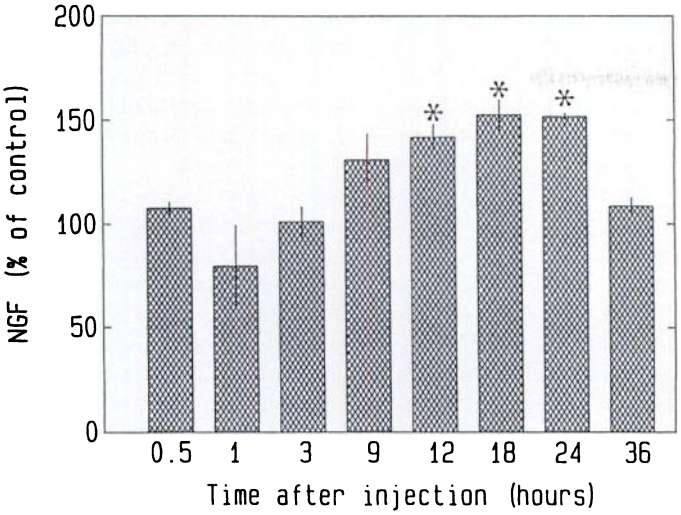

Fig. 4. Time course of reserpine-induced increase in NGF in cerebral cortex. Quantitation of the relative amounts of NGF in the cerebral cortex of 21-day-old rats injected with reserpine or vehicle at the indicated times after injection. NGF was measured by the two-site enzyme immonoassay (see Materials and Methods). Data are expressed as a percentage of control values and are the means ± standard errors of three independent experiments (three determinations). *, $p < 0.05$ versus control (ANOVA and Dunnett's test).

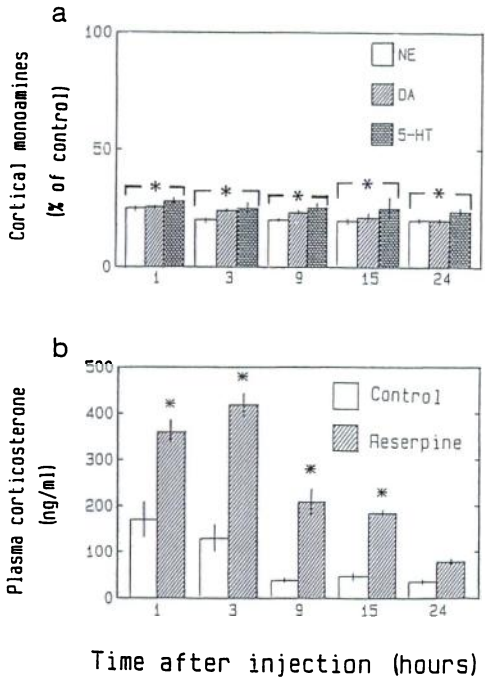

Fig. 5. Effect of reserpine on cerebral cortical monoamine and plasma corticosterone levels. Rats were killed at the indicated times after a single injection of reserpine (2 mg/kg, subcutaneously) or vehicle. a, The amount of cortical monoamines was determined by HPLC analysis. Data are expressed as a percentage of control values and are means ± standard errors (10 determinations). *, $p < 0.01$ versus control (ANOVA and Dunnett's test). b Plasma obtained from trunk blood of the same rats utilized in a was used for the determination of corticosterone levels by radioimmunoassay. Data are means ± standard errors (10 determinations). *, $p < 0.01$ versus control (ANOVA and Scheffe's test).

terone levels are significantly increased 1, 3, 9, and 15 hr after reserpine administration (Fig. 5b). This increase is not due to the stress induced by the injection, because the injection produces only a short lasting (up to 3 hr) effect in control rats (Fig. 5b). At 24 hr, when NGF mRNA has returned to control values, plasma corticosterone also returns to basal levels (Fig. 5b).

Reserpine dose-response studies were performed to help determine whether the induction of NGF synthesis could be ascribed to the depletion of catecholamines or to the increase of plasma corticosteroids. The minimal effective dose of reserpine in changing these three parameters is 0.25 mg/kg, subcutaneously (Fig. 6). However, this dose elicits only a 20% decrease in cortical monoamines (Fig. 6a), whereas it appears to have a greater relative effect in elevating corticosteroid and NGF mRNA levels (Fig. 6b).

Effect of steroids on NGF biosynthesis. Plasma steroid levels are very low 1 week after adrenalectomy. Therefore, we used adrenalectomized rats to test more directly whether the increase of cortical NGF mRNA levels mediated by reserpine could be ascribed to the effect of the drug on the release of adrenal steroids. In these rats, reserpine fails to affect the amount of NGF mRNA in cerebral cortex (Fig. 7), suggesting indeed that its action is mediated through the release of adrenal steroids.

We used dexamethasone, a synthetic glucocorticoid, to further test the hypothesis that reserpine could activate NGF biosynthesis by increasing plasma corticosteroid levels. Dexamethasone (0.5 mg/kg, subcutaneously) elicits a 2- to 3-fold increase in the amount of cortical NGF mRNA within 3 hr, when administered to 21-day-old rats. A smaller, but significant, increase in NGF protein is apparent at 6 and 9 hr after dexamethasone treatment (Fig. 8).

The latency time for the NGF mRNA response to dexamethasone is shorter, compared with that elicited by reserpine. This may be related to a direct effect of dexamethasone on the brain, whereas reserpine must first induce ACTH release, which, in

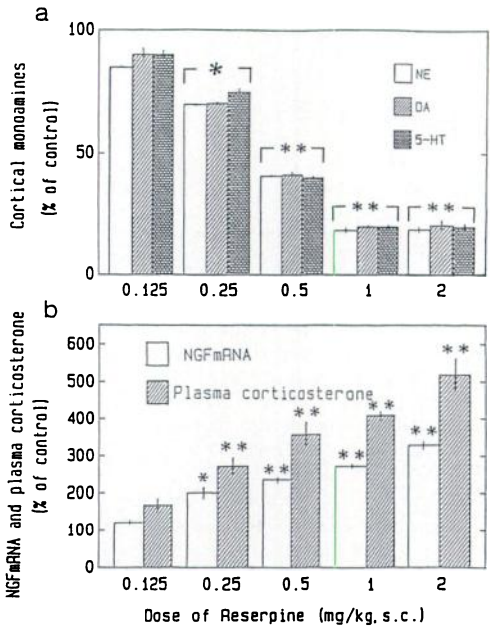

Fig. 6. Dose-response effect of reserpine on cortical monoamines, plasma corticosterone, and cortical NGF mRNA. Rats were treated with the indicated dose of reserpine or vehicle and killed 9 hr after injection. a, Cortical content of NE, DA, and 5-HT. Data are expressed as a percentage of control values and are means ± standard errors (10 determinations). b, Plasma corticosterone and cortical NGF mRNA levels. Data are expressed as a percentage of control values and are the means ± standard errors of three separate experiments (three determinations). *, $p < 0.05$; **, $p < 0.01$, versus control (ANOVA and Dunnett's test).

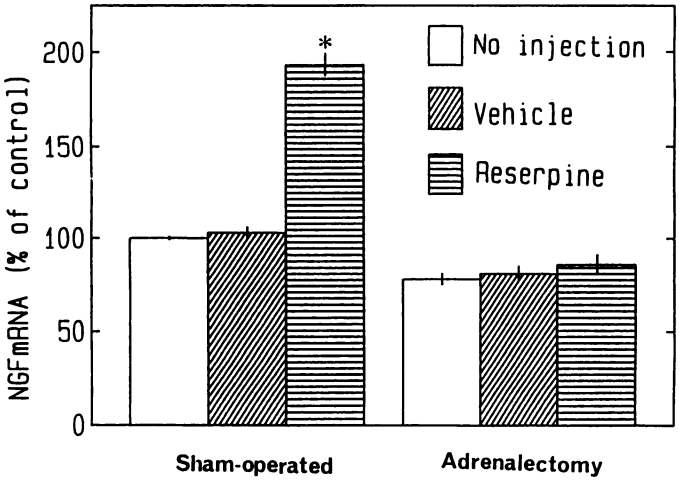

Fig. 7. Failure of reserpine to change cortical NGF mRNA levels in adrenalectomized rats. Adrenalectomized or sham-operated rats were injected at 21 days of age (8 days after operation) with reserpine (0.25 mg/kg, subcutaneously) or vehicle. Rats were killed 9 hr after injection, and the amounts of cortical NGF mRNA were determined. Results are expressed as a percentage of the control values (sham-operated animals receiving no injection) and represent means ± standard errors of three separate experiments (three determinations). *, $p < 0.05$ (ANOVA and Dunnett's test).

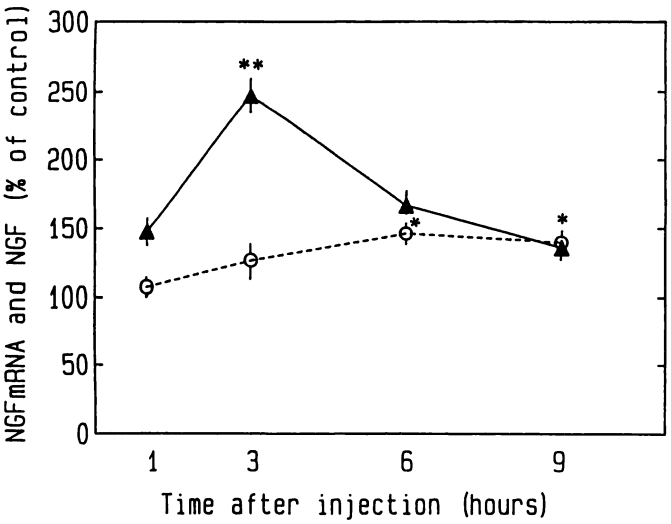

Fig. 8. Time course of induction of NGF synthesis elicited by dexamethasone. Rats were injected with dexamethasone (0.5 mg/kg, subcutaneously) or vehicle and sacrificed at the indicated times. NGF mRNA (▲) and NGF (○) were measured in cerebral cortex. Data are expressed as a percentage of control values and represent the means ± standard errors of three separate experiments (three determinations). *, $p < 0.05$; **, $p < 0.01$ (ANOVA and Dunnett's test).

turn, increases the release of endogenous glucocorticoid from the adrenal gland. Therefore, it is not surprising that the increase of NGF mRNA occurs as early as 1 hr after the injection of dexamethasone (Fig. 8). NGF mRNA levels return to basal values 9 hr after dexamethasone treatment, in accordance with the short half-life of the drug, estimated to be approximately 4 hr (23).

Discussion

In view of the physiological importance of NGF in neuronal trophism during ontogenesis and of the possibility that a similar action may be exerted by NGF in stimulating neuronal plasticity in adult brain, we became interested in investigating whether drugs can facilitate the biosynthesis of NGF in brain. Such pharmacological modification might eventually prove to be useful in the treatment of diseases characterized by a decrease of neuronal plasticity.

Because of the potential role of BAR stimulation in the regulation of NGF biosynthesis (3, 4, 6), we decided to investigate whether blockade of catecholaminergic transmission changes NGF biosynthesis. Surprisingly, when reserpine was administered to 21-day-old rats, it increased the amount of NGF mRNA and NGF in the cerebral cortex but not in other brain areas. Because it is well known that reserpine depletes monoamine stores throughout the brain, the working hypothesis that reserpine could decrease NGF biosynthesis by depleting catecholamine levels was not supported by this finding. However, reserpine, through the initial release of catecholamine, could increase NGF mRNA by increasing noradrenaline receptor occupancy. This possibility was ruled out because a pretreatment with *l*-propranolol, a BAR blocker, or with yohimbine, an α_2-adrenergic receptor blocker, failed to antagonize the effect of reserpine on NGF biosynthesis. The possibility that another mechanism could account for the accumulation of NGF mRNA caused by reserpine was seriously considered when we observed a lack of temporal correlation between the reserpine-induced decrease in cortical monoamine levels and the increase in NGF mRNA. Indeed, whereas a single injection of reserpine elicited a prolonged depletion (longer than 24 hr) of brain monoamines, cortical NGF mRNA had returned to control values by 24 hr after reserpine administration.

Among the central pharmacological effects elicited by reserpine, monoamine depletion and pituitary-adrenocortical system activation (22) have been considered. In fact, a single dose of reserpine, which induces a persistent hypersecretion of ACTH, similar to that evoked by a classical "stressful stimulus," results in an increase of blood corticosterone levels (24). Reserpine dose-response studies allowed us to infer that the increase in plasma corticosterone levels correlated with the increase in NGF mRNA better than did the reduction of cortical monoamine concentrations. Because steroids enhance the expression of a number of genes (25), we tested the hypothesis that adrenal corticosteroids could be responsible for the effect of reserpine on NGF biosynthesis, by administering the drug to adrenalectomized rats. We reasoned that, if the increase in NGF mRNA was linked to the ability of reserpine to release adrenal steroids, the drug should fail to induce NGF mRNA in adrenalectomized animals. Indeed, this was found to be the case. These findings rule out the possibility that the increase of NGF biosynthesis caused by reserpine could be ascribed to the depletion of monoamine stores.

We next investigated the effect of dexamethasone on cortical NGF biosynthesis, by administering a dose roughly comparable to the increase in plasma adrenocortical steroids occurring during stress (26, 27). Dexamethasone increased the amount of cortical NGF mRNA and NGF, supporting our findings that in adrenalectomized rats, where the steroid stores are virtually depleted, reserpine failed to change cortical NGF biosynthesis. Taken together, these data support the hypothesis that adrenal steroids function as a regulatory stimulus to modulate NGF biosynthesis in the brain.

Previous studies have shown that in adrenalectomized rats

the content of hippocampal NGF is significantly reduced (28). It remains to be clarified, however, why dexamethasone, like reserpine, increased NGF biosynthesis in the cerebral cortex but not in the hippocampus, which is an important target site for glucocorticoid action. Because our data were obtained in 21-day-old rats, a specific mechanism linked to brain development could be activated differentially in these brain areas. Indeed, the development of glucocorticoid receptors in the hippocampus is different from that in the cerebral cortex (29). In fact, the cytosol binding capacity for glucocorticoids reaches adult levels in the hippocampus around 4 weeks of age (29), suggesting that, in our experimental model, hippocampal glucocorticoid receptors, although present, are not fully functional. If this is true, then in adult rats dexamethasone and reserpine should also induce NGF biosynthesis in the hippocampus.

The increase in NGF protein always followed the increase in the NGF mRNA. This suggests that an activation of NGF gene expression or stabilization of the mRNA is the primary event induced by reserpine and that the increased production of NGF occurs as a secondary event. It would, therefore, appear that reserpine increases NGF content mainly by inducing NGF biosynthesis, rather than by affecting other parameters of NGF turnover. However, in view of the fact that NGF remains elevated for a longer period of time than does NGF mRNA, an effect of reserpine on the protein half-life cannot be ruled out.

In conclusion, we have presented evidence that, in the developing rat brain, NGF biosynthesis can be pharmacologically regulated. Although the molecular mechanism of this regulation remains to be determined, it appears that steroids may act as a primary stimulus in the induction of NGF gene expression. Whether the induction of NGF mRNA is due to a direct interaction of steroids and/or glucocorticoids with glucocorticoid-responsive elements is still under investigation. However, it is interesting to note that in cultured fibroblasts 17β-estradiol reduces NGF levels (30), whereas in C6 glioma cells, a cell line of neural origin, this hormone increases NGF levels (31). These and our results, while suggesting that NGF biosynthesis might be regulated by a cell-specific mechanism, open a new field in the function of steroids in the brain.

Acknowledgments

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References

1. Levi-Montalcini, R. The nerve growth factor 35 years later. *Science (Washington D. C.)* **237**:1154–1162 (1987).
2. Hefti, F., and W. J. Weiner. Nerve growth factor and Alzheimer's disease. *Ann. Neurol.* **20**:275–281 (1986).
3. Schwartz, J. P., and E. Costa. Regulation of nerve growth factor content in C6 glioma cells by *beta*-adrenergic receptor stimulation. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **300**:123–129 (1977).
4. Dal Toso, R., M. A. De Bernardi, E. Costa, and I. Mocchetti. *Beta*-adrenergic receptor regulation of NGF mRNA in rat C6-2B glioma cells. *Neuropharmacology* **26**:1783–1785 (1987).
5. Mocchetti, I., M. A. De Bernardi, A. M. Szekely, H. Alho, G. Brooker, and E. Costa. Regulation of nerve growth factor biosynthesis by *beta*-adrenergic receptor activation in astrocytoma cells: a potential role of c-Fos protein. *Proc. Natl. Acad. Sci. USA* **86**:3891–3895 (1989).
6. Dal Toso, R., M. A. De Bernardi, G. Brooker, E. Costa, and I. Mocchetti. *Beta*-adrenergic and prostaglandin receptor activation increases nerve growth factor mRNA content in C6-2B rat astrocytoma cells. *J. Pharmacol. Exp. Ther.* **246**:1190–1193 (1988).
7. Glowinski, J., and L. L. Iversen. Regional studies of catecholamines in rat brain: the disposition of ^{3}H-noradrenaline, ^{3}H-dopamine, and ^{3}H-DOPA in various regions of the brain. *J. Neurochem.* **13**:655–669 (1966).
8. Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18**:5294–5299 (1979).
9. Aviv, H., and P. Leder. Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid cellulose. *Proc. Natl. Acad. Sci. USA* **69**:1408–1411 (1972).
10. Mocchetti, I., J. R. Naranjo, and E. Costa. Regulation of striatal enkephalin turnover in rats receiving antagonists of specific dopamine receptor subtypes. *J. Pharmacol. Exp. Ther.* **241**:1120–1124 (1987).
11. Milner, R. J., and J. G. Sutcliffe. Brain gene expression. *Nucleic Acids Res.* **12**:5497–5520 (1983).
12. Danielson, P. E., S. Forss-Petter, M. A. Brown, L. Calavetta, J. Douglass, R. J. Milner, and G. J. Sutcliffe. p1B15: a cDNA clone of the rat mRNA encoding cyclophilin. *DNA* **4**:261–267 (1988).
13. Ullrich, A., A. Gray, C. Bermann, and J. T. Dull. Human *beta*-nerve growth factor gene sequence highly homologous to that of mouse. *Nature (Lond.)* **303**:821–825 (1983).
14. Melton, D. A., P. A. Krieg, M. R. Rebagliati, T. Maniatis, K. Zinn, and M. R. Green. Efficient *in vitro* synthesis of biologically active RNA and RNA hybridization probes from plasmid containing a bacteriophage SP6 promoter. *Nucleic Acids Res.* **12**:7035–7056 (1984).
15. Sambrook, J., E. F. Fritsch, and T. Maniatis. *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989).
16. Gonzales, D., W. Les Dees, J. K. Hiney, S. R. Ojeda, and R. P. Saneto. Expression of β-nerve growth factor in cultured cells derived from the hypothalamus and cerebral cortex. *Brain Res.* **511**:249–258 (1990).
17. Korsching, S., and H. Thoenen. Nerve growth factor in sympathetic ganglia and corresponding target organs of the rat: correlation with density of sympathetic innervation. *Proc. Natl. Acad. Sci. USA* **80**:3513–3516 (1983).
18. Eva, C., M. Fabrazzo, and E. Costa. Changes of cholinergic, noradrenergic, and serotonergic synaptic transmission indices elicited by ethylcholine azidinium ion (AF64A) infused intraventricularly. *J. Pharmacol. Exp. Ther.* **24**:181–186 (1987).
19. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
20. Large, T. H., S. C. Bodary, D. O. Clegg, G. Weskamp, U. Otten, and L. F. Reichardt. Nerve growth factor gene expression in the developing rat brain. *Science (Washington D. C.)* **234**:352–355 (1986).
21. Whittemore, S. R., T. Ebendal, L. Larkfors, L. Olson, A. Seiger, I. Stromberg, and H. Persson. Developmental and regional expression of β-nerve growth factor mRNA and protein in the rat central nervous system. *Proc. Natl. Acad. Sci. USA* **83**:817–821 (1986).
22. Westermann, E. O., R. P. Maickel, and B. B. Brodie. On the mechanism of pituitary-adrenal stimulation by reserpine. *J. Pharmacol. Exp. Ther.* **138**:208–217 (1962).
23. Anderson, W. R., J. W. Simpkins, M. E. Brewster, and S. Boder. Evidence for prolonged suppression of stress-induced release of adrenocorticotrophic hormone and corticosterone with a brain-enhanced dexamethasone-redox delivery system. *Neuroendocrinology* **50**:9–16 (1989).
24. Maickel, R. P., E. O. Westermann, and B. B. Brodie. Effects of reserpine and cold-exposure on pituitary-adrenocortical function in rats. *J. Pharmacol. Exp. Ther.* **134**:167–172 (1961).
25. Harlan, R. E. Regulation of neuropeptide gene expression by steroid hormones. *Mol. Neurobiol.* **2**:183–200 (1988).
26. Sapolsky, R. M., L. C. Krey, and B. S. McEwen. The neuroendocrinology of stress and aging: the glucocorticoid cascade hypothesis. *Endocrinol. Rev.* **7**:284–301 (1986).
27. McEwen B., E. R. De Kloet, and W. Rostene. Adrenal steroid receptors and action in the nervous system. *Physiol. Rev.* **66**:1121–1188 (1986).
28. Aloe, L. Adrenalectomy decreases nerve growth factor in young adult rat hippocampus. *Proc. Natl. Acad. Sci. USA* **86**:5636–5640 (1989).
29. Clayton, C. J., B. I. Grosser, and W. Stevens. The ontogeny of corticosterone and dexamethasone receptors in rat brain. *Brain Res.* **134**:445–453 (1977).
30. Siminoski, K., A. R. Murphy, P. Rennert, and G. Heinrich. Cortisone, testosterone and aldosterone reduce levels of nerve growth factor messenger ribonucleic acid in L-929 fibroblasts. *Endocrinology* **121**:1432–1437 (1987).
31. Perez-Polo, J. R., K. Hall, K. Livingston, and K. Westlund. Steroid induction of nerve growth factor synthesis in cell culture. *Life Sci.* **21**:1535–1544 (1977).

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