

Adrenal Steroids Mediate the Increase of Hippocampal Nerve Growth Factor Biosynthesis Following Bicuculline Convulsions

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Northern blot analysis of nerve growth factor (NGF) messenger ribonucleic acid (mRNA), together with a two-site enzyme immunoassay for NGF protein, showed that a convulsive dose of bicuculline (0.4 mg/kg, IV) induced a rapid (within 1 hour) three-fold increase in hippocampal NGF mRNA. This increase was followed by a significant increase in NGF protein 5 hours later. No changes were detected in the cerebral cortex. The increase of rat hippocampal NGF mRNA and protein content was associated with an increase in plasma corticosterone content. Both responses were completely prevented by a pretreatment with diazepam (5 mg/kg, IP), which suggested that adrenal steroids might be a stimulus underlying the rapid increase in NGF biosynthesis

following bicuculline convulsions. Therefore, we tested to determine whether a convulsive dose of bicuculline could increase NGF mRNA in adrenalectomized and sham-operated rats. Bicuculline increases hippocampal NGF mRNA in sham-operated rats, but not in adrenalectomized rats despite the presence of convulsions in both experimental groups. Moreover, in adrenalectomized rats, dexamethasone (0.5 mg/kg, SC) could partly restore the increase in hippocampal NGF mRNA content induced by bicuculline convulsions; thus, adrenal steroids might have an essential role in the induction of hippocampal NGF biosynthesis elicited by bicuculline convulsions. [Neuropsychopharmacology 8:219–225, 1993]

KEY WORDS: Nerve growth factor; Hippocampus; Convulsions; Steroids; Bicuculline; Benzodiazepine

The physiologic function of nerve growth factor (NGF) for specific populations of neurons of the peripheral nervous system (Levi-Montalcini and Angeletti 1968) and the central nervous system (CNS) (Thoenen et al. 1987) is well documented. Nerve growth factor is an essential neurotrophic factor for the differentiation and sur-

vival of sympathetic neurons, neuronal crest-derived peripheral sensory neurons, and forebrain cholinergic neurons (Levi-Montalcini and Angeletti 1968; Cohen 1960; Aloe et al. 1981; Angeletti et al. 1971; Thoenen and Barde 1980; Thoenen et al. 1987; Whittemore and Seiger 1987). Nerve growth factor can be expressed in specific populations of CNS neurons (Whittemore and Seiger 1987; Whittemore et al. 1988); however, little is known about mechanisms regulating NGF availability in the adult CNS. The modifications induced by NGF in target neurons might participate in enhancing neuronal plasticity; therefore, by learning the molecular mechanisms regulating NGF biosynthesis, we may be able to activate neuronal plasticity in the CNS via a pharmacologically induced increase of NGF availability.

In the adult rat brain, NGF expression can be increased by different types of seizures (Gall and Isack-

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son 1989; Gall et al. 1991; Isackson et al. 1991; Ernfors et al. 1991; Zafra et al. 1990). Moreover, steroids released from adrenal glands during stress can increase the content of NGF messenger ribonucleic acid (mRNA) and NGF immunoreactivity in specific brain regions (Aloe et al. 1989; Fabrazzo et al. 1991; Mocchetti et al. 1991). Several lines of evidence suggest a direct relationship between seizure activity and blood-steroid levels (Craig 1966; Holmes et al. 1984; Backstrom et al. 1985; Bowers et al. 1991; Lee et al. 1987). However, it is not known whether steroids can activate brain NGF biosynthesis following seizures. In the present study, we tested to determine whether adrenal steroids released following bicuculline-elicited convulsions, modulate the increase of NGF synthesis in the CNS.

MATERIALS AND METHODS

Treatment of Animals

Adult male Sprague-Dawley rats weighing between 200 and 260 g (Zivic Miller, Allison, PA) were used. Adrenalectomized and sham-operated rats were received from Zivic Miller 5 days postsurgery and experiments began 2 days later. After adrenalectomy, rats drank ad libitum saline solution instead of water. All rats were housed five per cage in a temperature-controlled room with a 12-hour light/dark cycle. Rats receiving 0.4 mg/kg (10 μ mol/kg) of intravenous (+)bicuculline (Sigma, St. Louis, MO) exhibited tonic-clonic convulsions within 10 seconds of injection, which lasted from 2 to 5 minutes. Dexamethasone (0.5 mg/kg, 1.3 μ mol/kg, SC) or diazepam (5 mg/kg, 18 μ mol/kg, IP, Sigma, St. Louis, MO) was given 3 hours or 10 minutes before bicuculline administration, respectively. Control animals received vehicle injection. Rats were decapitated at the indicated times after treatment and the brains were removed immediately. Cerebral cortex and hippocampus were quickly dissected and stored at -70°C for analysis at a later time.

Probe Preparation

Clone pBS₂ NGF (a gift from Dr. Whittemore, University of Miami, FL) contains an equivalent 770 bp complementary deoxyribonucleic acid (cDNA) encoding rat β -NGF (Whittemore et al. 1988). The transcript generated by T₃ polymerase is the antisense RNA (cRNA) complementary to β -NGF mRNA. The *in vitro* transcription assay was carried out as previously described (Melton et al. 1984) to yield ³²P-cRNA at a specific activity of 5 to 6 $\times 10^8$ cpm/ μ g of RNA. Clone p1B15 (a gift from Drs. Milner and Sutcliffe, Scripps Clinic, La Jolla, CA), which contains the cDNA encoding the structural protein cyclophilin (Danielson et al. 1988),

was labeled by Nick translation procedure (Sambrook et al. 1989).

Preparation of RNA and Northern Blot Analysis

The total RNA was prepared from the cerebral cortex or hippocampus as previously described (Chirgwin et al. 1979; Fabrazzo et al. 1991). The total RNA was size-fractionated by agarose formaldehyde gel electrophoresis and transferred to nylon membrane by the capillary blot procedure. Blots were prehybridized with 50% formamide, 4 \times SSPE, 0.1 mg/ml denatured salmon sperm DNA and 5 \times Denhardt's solution at 65 $^{\circ}\text{C}$ for at least 2 hours. Hybridization with ³²P-labeled NGF probe was carried out overnight at 65 $^{\circ}\text{C}$ in a prehybridization solution containing 1 \times Denhardt's solution. The blots were washed in 0.1 \times standard saline citrate, 0.1% sodium dodecyl sulfate at 68 $^{\circ}\text{C}$ for 2 hours and exposed to Kodak X-OMAT film with an intensifying screen at -70°C . After exposure, the radioactivity was removed by washing the blot at stringency conditions as previously described (Mocchetti et al. 1987). The blots were then rehybridized with a ³²P-p1B15 cDNA (Milner and Sutcliffe 1983; Danielson et al. 1988), washed, and exposed to a new X-ray film. The intensity of the autoradiographic hybridization bands was quantified by laser densitometry.

Calculation of RNA

The amount of NGF mRNA in the blots was estimated in arbitrary units defined as the ratio between the densitometric peak of the major NGF mRNA band (1.3Kb) and that of the cyclophilin (p1B15) mRNA. Cyclophilin was used to correct for recovery variations during extraction, purification and hybridization procedures. The value of each control was set equal to 1 and the other calculated relative to it.

Two-site Enzyme Immunoassay of NGF

Nerve growth factor content was determined by quantitative two-site enzyme immunoassay as previously described (Fabrazzo et al. 1991). Brain extracts for NGF determination were prepared from the hippocampus or cerebral cortex at different times after treatment with either bicuculline (0.4 mg/kg IV) or vehicle. All reagents for the immunoassay were obtained from Boehringer Mannheim (Indianapolis, IN).

Plasma Corticosterone Determination

Trunk blood was collected from decapitated rats in heparinized glass tubes and centrifuged. The plasma was stored at -20°C for corticosterone determination

at a later time. The content of corticosterone in the plasma was measured by radioimmunoassay with the RSL ¹²⁵I-corticosterone Kit (ICN, Costa Mesa, CA). The antibody from the kit is specific against corticosterone with less than 0.01% cross-reactivity to cholesterol, estrone, pregnenolone, and their metabolites. All measurements were within the linear range of the assay as determined by a standard curve.

Data Analysis

Differences among means were evaluated by analysis of variance. When the drug treatment elicited significant changes, significance was determined by Dunnett's test for comparing the treatment and control groups or Scheffé's test for multiple comparisons.

RESULTS

Effect of Bicuculline on Hippocampal NGF mRNA Content

The dose-response relationship between bicuculline-induced convulsions and the increase in hippocampal NGF mRNA content is reported in Table 1. Bicuculline at doses from 0.05 to 0.2 mg/kg (IV) failed to induce convulsions or to increase the hippocampal NGF mRNA content. Only at a dose of 0.4 mg/kg does bicuculline induce tonic-clonic convulsions, which occur 10 seconds after injection and last from 2 to 5 minutes. The hippocampal content of NGF mRNA is increased about three-fold 1 hour after the administration of a convulsive dose (0.4 mg/kg, IV) of bicuculline, whereas the cortical NGF mRNA content remains unchanged (Fig. 1). This dose of bicuculline was used to study the time course of NGF mRNA increase following convulsions. As shown in Fig. 2, the hippocampal NGF mRNA con-

Table 1. Dose Response of Bicuculline-Induced Convulsions and Hippocampal NGF mRNA Expression

Treatment	Convulsions	NGF mRNA (Arbitrary Units)
Control	No	1
Bicuculline		
0.05 mg/kg	No	1.1 ± 0.12
0.1 mg/kg	No	1.2 ± 0.11
0.2 mg/kg	No	1.3 ± 0.0070
0.4 mg/kg	Yes	3.2 ± 0.25**

Animals were injected intravenously with the indicated doses of bicuculline or vehicle and decapitated 1 hour after the injection. Nerve growth factor mRNA was estimated by Northern blot analysis. Control value was set equal to 1 and the other value calculated relative to it. Data are the mean ± SEM of three independent experiments. ***p* < 0.01 versus control group.

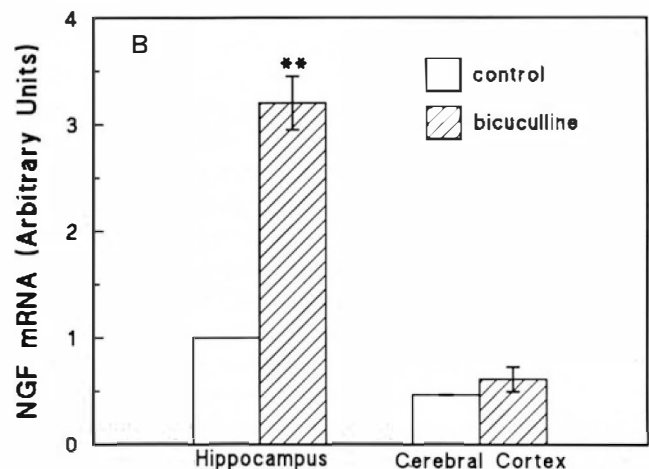
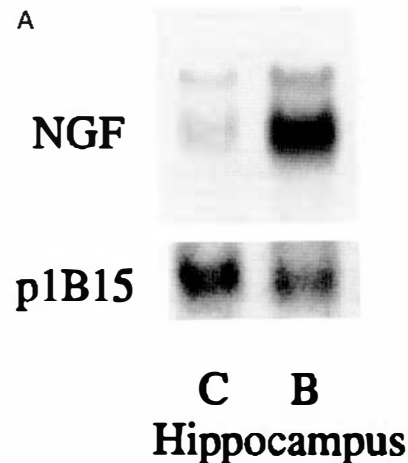


Figure 1. Bicuculline increases NGF mRNA in rat hippocampus. (A) Northern blot analysis of RNA from hippocampus of adult rats 1 hour after bicuculline (B = 0.4 mg/kg, IV) or vehicle (C) treatment. Blot was hybridized with NGF cRNA probe (NGF), washed, exposed to x-ray film for 24 hours, and washed again. Blot was rehybridized with cyclophilin cDNA (p1B15), washed, and exposed for 6 hours. (B) Densitometric analysis of NGF mRNA content in hippocampus and cerebral cortex. Nerve growth factor mRNA content is expressed in arbitrary units. Data are the mean ± SEM of three independent experiments. ***p* < 0.01 versus control group.

tent is already significantly increased above control levels 30 minutes after bicuculline injection, reaches peak values at 1 hour, and returns to control levels within 9 hours (Fig. 2A). The hippocampal content of NGF protein increases at 6 hours and returns to the basal level within 24 hours postadministration of bicuculline (Fig. 2B). However, in the cerebral cortex, neither NGF mRNA nor NGF protein content changes during this time period (Fig. 2A and B).

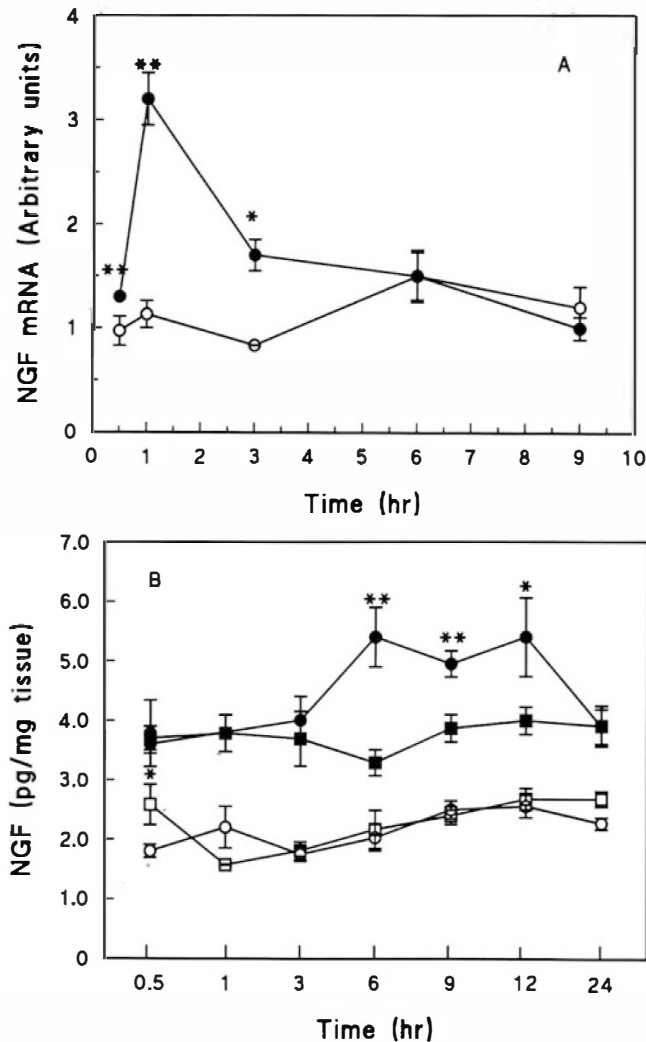


Figure 2. Time course of bicuculline-elicited increase of NGF mRNA **A** and NGF Protein **B** contents in the cerebral cortex and hippocampus. Rats were decapitated at the indicated times after a single injection of bicuculline (0.4 mg/kg, IV) or vehicle. Filled circles indicate hippocampus of bicuculline-treated rats; filled squares indicate hippocampus of vehicle-treated rats; open circles indicate cerebral cortex of bicuculline-treated rats; and open squares indicate cerebral cortex of vehicle-treated rats. (A) The amount of NGF mRNA was estimated by Northern blot hybridization analysis. Arbitrary units were calculated as described in the Materials and Methods section. The value for control rats (each time point) was set equal to 1 and other values calculated relative to it. Data are the mean \pm SEM of three independent experiments. (B) The content of NGF protein was measured by a two-site enzyme immunoassay. The mean \pm SEM ($n = 6$) are shown. * $p < 0.05$; ** $p < 0.01$ versus control group.

Diazepam Antagonism of Bicuculline-Mediated Increase of Hippocampal NGF mRNA Content

Several lines of investigation have shown that benzodiazepine recognition sites are located in the gamma aminobutyric acid-A (GABA_A) receptor structure and,

when bound by various drugs, they can modulate channel gating by GABA in a positive or negative modulation. Diazepam, a positive allosteric modulator of GABA action in several GABA_A receptor subtypes, has been tested to evaluate the role of convulsions in eliciting the increase of hippocampal NGF mRNA content. Diazepam at a dose of 5 mg/kg (IP) prevents bicuculline-induced convulsions, the increase in plasma corticosterone levels, and also inhibits the induction of hippocampal NGF mRNA (Table 2). Diazepam completely prevents both of these biochemical changes mediated by bicuculline convulsions without any effect per se on the basal levels of the abovementioned parameters (Table 2).

Effects of Adrenal Steroids on Bicuculline-Mediated Induction of Hippocampal NGF mRNA

Plasma corticosterone levels increase following bicuculline-induced convulsions (Table 2), which suggests that convulsions induce the release of adrenal steroids. To ascertain whether the increase of the hippocampal content of NGF mRNA elicited by bicuculline is related to the increase of blood-borne adrenal steroids, we experimented with bilaterally adrenalectomized and sham-operated rats. Bicuculline increases hippocampal NGF mRNA content in sham-operated rats, but not in adrenalectomized rats (Table 3), despite the presence of convulsions in both groups. These results support the view that the increase of blood-borne corticosteroid plays a role in bicuculline's induction of hippocampal NGF mRNA. Dexamethasone, a synthetic glucocorticoid, was given to adrenalectomized and sham-operated rats 3 hours before bicuculline administration. Figure 3 shows that dexamethasone (0.5 mg/kg, SC) alone elicits an increase of approximately 1.5-fold of NGF mRNA content in the cerebral cortex, but not in

Table 2. Antagonism by Diazepam of Bicuculline-Induced Convulsions and Increase of Hippocampal NGF mRNA and Plasma Corticosterone Contents

Treatment	Convulsions	NGF mRNA (Arbitrary Units)	Corticosterone (ng/ml Plasma)
Control	No	1	33 \pm 8.7
Bicuculline	Yes	3.2 \pm 0.25	63 \pm 7.7
Diazepam	No	0.73 \pm 0.12	30 \pm 6.3
Diazepam + Bicuculline	No	0.67 \pm 0.009**	35 \pm 3.8

Diazepam (5 mg/kg, IP) was injected 10 minutes before the administration of bicuculline (0.4 mg/kg, IV). Rats were decapitated 1 hour after bicuculline injection. Nerve growth factor mRNA was estimated by Northern blot analysis ($n = 3$). Plasma corticosterone was determined by radioimmunoassay ($n = 7$). Data are the mean \pm SEM. * $p < 0.05$ or ** $p < 0.01$ versus bicuculline.

Table 3. Bicuculline Fails to Increase NGF mRNA in Adrenalectomized Rats

Groups	NGF mRNA (Arbitrary Units)					
	Hippocampus		Cerebral Cortex		Convulsions	
	Sham-Operated	Adrenalectomized	Sham-Operated	Adrenalectomized	Sham-Operated	Adrenalectomized
Control	1	1.2 ± 0.14	1	1.0 ± 1.4	No	No
Bicuculline	2.5 ± 0.13**	1.5 ± 0.21	0.98 ± 2.26	0.80 ± 0.008	Yes	Yes

Experiments on adrenalectomized and sham-operated rats began 1 week after surgery. Rats were decapitated 1 hour after bicuculline administration. Nerve growth factor mRNA was estimated by Northern blot analysis. Data represent the mean ± SEM of three experiments. ***p* < 0.01 versus control group.

the hippocampus of sham-operated rats. Co-administration of bicuculline and dexamethasone can further elicit an increase in cortical NGF mRNA content of about 3- and 1.8-fold in sham-operated and adrenalectomized rats, respectively. Bicuculline or dexamethasone given alone, fails to change the basal levels of hippocampal NGF mRNA in adrenalectomized rats. However, in these animals, bicuculline co-administered with dexamethasone significantly increases the hippocampal NGF mRNA content by about 1.7-fold. These data suggest that in adrenalectomized rats dexamethasone can partially restore the ability of bicuculline to increase the hippocampal NGF mRNA expression.

DISCUSSION

Drugs that decrease brain GABAergic tone usually elicit seizures (Loscher 1982; Kendall et al. 1981; Loscher and Frey 1979). In the present study, bicuculline, an iso-steric GABA antagonist at GABA_A receptors, was used

in normal, adrenalectomized, and sham-operated rats to induce tonic-clonic convulsions. We investigated whether convulsions induce the expression of NGF in the adult CNS and we characterized the mechanisms whereby convulsions increase NGF. Bicuculline, at the convulsive dose, increased plasma corticosterone, hippocampal NGF mRNA, and protein contents. Diazepam, a positive allosteric modulator of the GABA_A receptor (Costa and Guidotti 1979), not only prevented the bicuculline-induced convulsions and the increase in plasma corticosterone levels, but also inhibited the induction of hippocampal NGF mRNA. These results demonstrate that behavioral and biochemical changes induced by bicuculline are antagonized by enhancing GABAergic transmission and suggest that the cascade of events leading to the accumulation of NGF is initiated by GABA_A receptor blockade.

Nerve growth factor protein accumulates following the increase in NGF mRNA. These data suggest that, whether bicuculline increases gene expression or mRNA stabilization, it enhances NGF production.

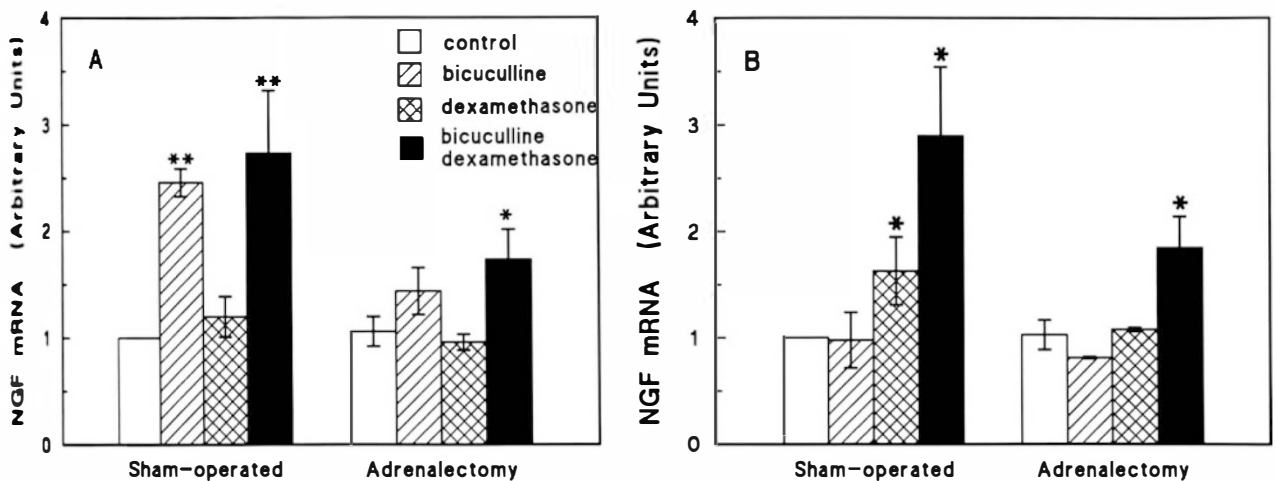


Figure 3. Dexamethasone partially restores bicuculline-induced increase of brain NGF mRNA in adrenalectomized rats. Experiments on adrenalectomized and sham-operated rats began 1 week after operation. Dexamethasone (0.5 mg/kg, SC) or vehicle was administered to the animals 3 hours before administration of bicuculline or vehicle. Rats were decapitated 1 hour after bicuculline or vehicle treatment. Nerve growth factor mRNA contents in hippocampus A and cerebral cortex B were estimated by Northern blot analysis. Data represent the mean ± SEM of four independent experiments. **p* < 0.05 and ***p* < 0.01 versus each control group.

Thus, it might be concluded that bicuculline increases hippocampal NGF biosynthesis rather than decreasing NGF utilization or degradation. However, the selective action of bicuculline on hippocampal NGF mRNA content contradicts a direct action of bicuculline and favors an indirect action. A direct relationship between seizure activities and adrenal steroids has been demonstrated (Craig 1966; Holmes et al. 1984; Backstrom et al. 1985; Bowers et al. 1991; Lee et al. 1987). Bicuculline given at the convulsive dose induces the release of adrenal steroids. Thus, we considered the possibility that the GABAergic receptor antagonist may act on brain NGF biosynthesis via an increase in plasma corticosterone levels. Previous data have shown that glucocorticoids increase NGF in the CNS (Fabrazzo et al. 1991). To evaluate whether corticosteroid released from the adrenal gland following bicuculline-induced convulsions participate in the enhancement of NGF mRNA, we resorted to the strategy of testing to determine whether bicuculline increases brain NGF mRNA in adrenalectomized rats. Interestingly, we observed that adrenalectomy could abolish the bicuculline-induced increase in hippocampal NGF mRNA content, despite the presence of convulsions. Actually, convulsions appeared more severe and longer lasting in adrenalectomized rats, thus confirming the report of Bowers et al. (1991). In adrenalectomized rats, dexamethasone can partially restore the bicuculline enhancement of hippocampal NGF mRNA content. These results demonstrate that blood-borne steroids play a major role in modulating the increase in NGF mRNA content elicited by bicuculline convulsions. However, dexamethasone alone failed to change the content of NGF mRNA, suggesting that in the hippocampus, glucocorticoids are not the main adrenal steroids involved in the bicuculline-mediated induction of NGF mRNA. Indeed, our experiments failed to elucidate how adrenal steroids regulate hippocampal NGF gene expression and which adrenal steroids (mineralocorticoids?), specifically participate in this regulation.

In the cerebral cortex, bicuculline failed to change NGF mRNA and protein levels. However, in this brain area, but not in the hippocampus, dexamethasone increases NGF availability in developing (Fabrazzo et al. 1991) and adult rats. This anatomic specificity of NGF induction suggests that NGF expression is regulated in different brain areas by different neural circuitry. Our studies failed to demonstrate whether bicuculline-mediated NGF expression occurs in neurons or in astrocytes. Nevertheless, we cannot rule out the possibility that in the cerebral cortex, cell synthesizing NGF might express both GABA_A and steroid receptors. Indeed, when bicuculline and dexamethasone were concomitantly administered, dexamethasone elicited a stronger effect on cortical NGF expression, suggesting that induction of glucocorticoid-mediated NGF expres-

sion is potentiated when the GABAergic transmission is blocked.

Bicuculline-induced convulsions elicit a rapid increase in NGF mRNA and protein in the hippocampus, similar to the accumulation of NGF mRNA previously observed (Gall et al. 1989, 1991; Isackson et al. 1991; Ernfors et al. 1991). However, unlike the other seizure models, the intravenous injection of bicuculline elicits convulsions only for a relatively short time, and NGF mRNA content returns to the basal levels within 3 hours. These data suggest that continuous seizures most likely are responsible for prolonged induction of NGF mRNA. Moreover, the induction of NGF following convulsions might represent an adaptive event that is activated in selective brain circuitry. Hence, the rapid and transient increase in NGF availability may be important for protecting and maintaining the integrity of the brain circuitry via which seizures propagate.

In summary, in adult rat CNS, bicuculline-induced convulsions elicit hippocampal NGF biosynthesis in a manner inhibited by diazepam or adrenalectomy. In adrenalectomized rats, dexamethasone can partly restore the bicuculline-induced increase in hippocampal NGF mRNA. These data indicate that blood-borne adrenal steroids may play a role in the increase of hippocampal NGF content following bicuculline convulsions. We cannot conclude whether adrenocorticotrophic hormone participates in this regulation because such a notion requires further experiments with hypophysectomized rats.

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