

ACTH₁₋₂₄ Down-Regulates Expression of Ciliary Neurotrophic Factor mRNA in Cultured Rat Astrocyte

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ABSTRACT

We examined the effects on astrocytes of ACTH, which is used to treat West syndrome. We stimulated cultured rat astrocytes with ACTH₁₋₂₄, corticotropin-releasing factor, and dexamethasone, and examined changes in neurotrophic factor mRNAs by reverse transcription-PCR. Down-regulation of ciliary neurotrophic factor mRNA expression was observed by stimulation with ACTH₁₋₂₄, but the expression of nerve growth factor, brain-derived neurotrophic factor, and neurotrophin-3 mRNAs was unaffected. Northern blot analysis revealed that the decrease in ciliary neurotrophic factor mRNA occurred 4 h after stimulation with more than 10 nM of ACTH₁₋₂₄. Up-regulation of neurotrophin-3 mRNA expression was found after stimulation with 1 mM dexamethasone. These results suggest that ACTH₁₋₂₄ administered in West syndrome may influence the expression

of neurotrophic factors in astrocytes *in vivo*. (*Pediatr Res* 52: 950-957, 2002)

Abbreviations

CRF, corticotropin-releasing factor
Dex, dexamethasone
RT-PCR, reverse transcription-PCR
NGF, nerve growth factor
BDNF, brain-derived neurotrophic factor
NT-3, neurotrophin-3
CNTF, ciliary neurotrophic factor
PBS(-), Ca²⁺/Mg²⁺-free PBS
GFAP, glial fibrillary acidic protein
PKA, protein kinase A
PKC, protein kinase C

ACTH is a 39-amino acid peptide that is derived from the precursor peptide pro-opiomelanocortin. Hypothalamic neurons contain most of the brain pro-opiomelanocortin-related peptides, but ACTH is also found in the amygdala, cerebral cortex, brainstem, and cerebellum (1). In addition to its classic function, ACTH is thought to act as a neurotransmitter, neuromodulator, and growth factor (1). ACTH has been demonstrated to have a neurotrophic effect, enhancing recovery from damage in both the peripheral nervous system and the CNS (2).

West syndrome is an intractable epileptic encephalopathy in infants. The seizures are lessened by ACTH and glucocorticoids, both major components of the brain-adrenal axis (3), but

ACTH is reported to be more effective than glucocorticoids (4, 5). However, the mechanism of action of ACTH on immature damaged brain, which may lead to West syndrome, has not been thoroughly investigated. ACTH and adrenal steroids may be important regulators of trophic processes operative in synaptic plasticity (6). It has been reported that not only neurons but also astrocytes express ACTH-receptors and that ACTH induces morphologic changes in astrocytes (7). Thus, ACTH₁₋₂₄ administered in West syndrome may have some influence on astrocytes *in vivo*. Astrocytes express several kinds of neurotrophic factors and extracellular matrices that regulate axonal growth or regeneration after injury. In view of the therapy for West syndrome, it might be important to examine the influence of ACTH on astrocytes, especially with respect to the expression of neurotrophic factors.

METHODS

Astrocyte culture. Astrocytes were prepared from d 20 embryonic Wistar rat brains according to the method of Nakanishi *et al.* (8). The tissues were dissected and dissociated

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with trypsin. The dissociated cells were suspended in low-glucose (1000 mg/L) Dulbecco's modified Eagles' medium (D-MEM, GIBCO BRL, Rockville, MD, U.S.A.) supplemented with 10% fetal bovine serum (Microbiological Associates, Sigma, St. Louis, MO, U.S.A.), penicillin G (Meiji Seika, Tokyo, Japan, 80 U/mL), and streptomycin (Meiji Seika, 0.2 mg/mL). After culturing in 25-cm² flasks (Costar, Cambridge, MA, U.S.A.) for 6 d, the glial components were dislodged with 0.1% trypsin and replated. The medium was changed twice a week. Cultures were grown at 37°C in a humidified atmosphere of 7% CO₂. After 14 d, the astrocytes formed a confluent monolayer and were prepared for the drug stimulation experiment. To check the cell population, cells were rinsed with PBS(−) and then fixed with 4% paraformaldehyde in PBS(−) for 15 min at room temperature. GFAP was visualized immunohistochemically by the streptavidin-biotin method using rabbit anti-GFAP antibody (Chemicon International Inc., Temecula, CA, U.S.A.) and Histofine SAB-PO Kit (Nichirei, Tokyo, Japan) according to manufacturer's protocol. Diaminobenzidine tetrahydrochloride was used as the chromogen and methyl green was used as a counterstain. More than 95% of the cultured cells were positive for GFAP (data not shown).

Drug stimulation experiment. Twenty-four hours before treatment of the confluent astrocyte monolayer, the medium was replaced to serum-free high-glucose (4500 mg/L) D-MEM containing penicillin G, streptomycin, and 1% N-2 Supplement (100× N-2 Supplement, GIBCO BRL). CRF (Research Biochemicals International, Natick, MA, U.S.A.) in distilled water, ACTH₁₋₂₄ (Calbiochem-Nova Biochem International, San Diego, CA, U.S.A.) in distilled water, or Dex (Wako Pure Chemical, Osaka, Japan) in ethanol was added to the cultures. Controls for CRF and ACTH₁₋₂₄ were treated with distilled water, and those for Dex with ethanol (0.01%). To investigate whether the ACTH₁₋₂₄-induced effect is mediated by activation of PKA or PKC, astrocytes were pretreated with protein kinase inhibitors, H-89 or H-7 (Seikagaku Corporation, Tokyo, Japan), for 1 h before ACTH₁₋₂₄ stimulation.

Reverse transcription-PCR. Total RNA was isolated from control and drug-stimulated astrocytes using Isogen (Wako) according to the manufacturer's instructions. Reagents used for RT-PCR were deoxyribonuclease I (DNase I, amplification grade, GIBCO BRL); 10× DNase I reaction buffer; 25 mM EDTA (GIBCO BRL); 25 mM MgCl₂ (TaKaRa, Kusetsu, Japan); 10× PCR buffer (TaKaRa); dNTP mixture (TaKaRa); oligo (dT)₁₂₋₁₈ primer (GIBCO BRL); 0.5 μg/μL Superscript II RNase H[−] reverse transcriptase (GIBCO BRL); and 0.1 M DTT (GIBCO BRL).

One microgram of RNA, 1 μL of 10× DNase I reaction buffer, 1 μL of DNase I, and diethylpyrocarbonate-treated water to 10 μL were mixed in a 0.5-mL tube, and the tube was incubated for 15 min at room temperature. DNase I activity was inactivated by addition of 1 μL of 25 mM EDTA and heating for 10 min at 70°C. Reverse transcription of RNA samples was performed in 20 μL of the reaction buffer containing 1 μL of oligo (dT)₁₂₋₁₈ primer, 2 μL of 10× PCR buffer, 1 μL of 25 μM MgCl₂, 2 μL of dNTP mixture, 2 μL of DTT, and 1 μL of Superscript II RNase H[−] reverse transcriptase. First-strand cDNAs were synthesized at 42°C for 50 min. mRNA-cDNA chains were denatured, and the reverse transcriptase activity was arrested by heating at 70°C for 10 min.

Primer pairs for NGF, BDNF, NT-3, and CNTF (all Promega, Madison, WI, U.S.A.) were used for amplification and yielded a single band corresponding to 189-bp, 296-bp, 176-bp, and 168-bp DNA fragments, respectively (Table 1). The reaction was carried out for 35 cycles (NGF, BDNF, NT-3) or 27 cycles (CNTF) using a denaturing step at 94°C for 30 s, an annealing step at 65°C for 1 min, and an extension step at 68°C for 2 min. β-Actin mRNA detection was used to show equal amounts in the control and stimulated groups. The rat β-actin-specific upstream and downstream primers are listed in Table 1. They yielded a single band corresponding to a 542-bp DNA fragment. The reaction was carried out for 25 cycles, using a denaturing step at 94°C for 30 s, an annealing step at 63°C for 1 min, and an extension step at 68°C for 2 min.

Northern blot analysis. Aliquots of total RNA (10 μg) were denatured with formaldehyde, fractionated on 0.9% agarose/2.1 M formaldehyde gels, and blotted onto nylon membranes (Micron Separations Inc., Westborough, MA, U.S.A.), which were fixed by UV irradiation. Equal loading of RNA samples was confirmed by ethidium bromide staining. Membranes were prehybridized for 18 h at 42°C in a solution containing 50% formamide, 5× standard saline citrate (SSC), 5× Denhardt's solution, 500 μg/mL salmon sperm DNA, 1% glycine, and 0.1% SDS, and hybridized at 42°C for 24 h with ³²P-labeled cDNA probe for CNTF (0.6 kb). Plasmids containing rat CNTF-cDNA were kindly provided by Professor Shouei Furukawa (Laboratory of Molecular Biology, Gifu Pharmaceutical University). The hybridization solution consisted of 50% formamide, 5× SSC, 1.5× Denhardt, 100 μg/mL salmon sperm DNA, 0.1% SDS, and 10% dextran sulfate. The membranes were washed twice at room temperature for 5 min in 2× SSC containing 0.1% SDS, once at 65°C for 15 min in 2× SSC containing 0.1% SDS, and once at 65°C for 5 min in 1× SSC containing 0.1% SDS. The membranes were then exposed to x-ray Omat AR film (Eastman Chemical Company, Rochester, NY, U.S.A.) for 2 d.

Western blot analysis. To determine the content of CNTF protein in astrocytes stimulated with ACTH₁₋₂₄, cells were washed with PBS(−) and scraped into 50 μL of lysis buffer (5 mM Tris-HCl pH 7.4, 2 mM phenylmethanesulfonyl fluoride (PMSF), 10 μM pepstatin A, 10 μM leupeptin, 2 mM EDTA, and 0.5% NP-40). After sonication, total protein in the sonicates was determined using a BCA protein assay kit (Pierce, Rockford, IL, U.S.A.). For Western blot analysis, 50 μg of protein per lane was electrophoresed in 12% polyacrylamide gels. Recombinant CNTF was electrophoresed in a separate lane. After blotting on polyvinylidene difluoride membranes (Millipore, Bedford, MA, U.S.A.)

Table 1. Primers for detection of NGF, BDNF, NT-3, CNTF and β-actin

NGF/5' primer	CCA AGG GAG GAG CTT TCT ATC CTG G
NGF/3' primer	GGC AGT GTC AAG GGA ATG CTG AAG T
BDNF/5' primer	AGC CTC CTC TGC TCT TTC TGC TGG A
BDNF/3' primer	CTT TTG TCT ATG CCC CTG CAG CCT T
NT-3/5' primer	TTT CTC GCT TAT CTC CGT GGC ATC C
NT-3/3' primer	GGC AGG GTG CTC TGG TAA TTT TCC T
CNTF/5' primer	GGC TAG CAA GGA AGA TTC GTT CAG A
CNTF/3' primer	TGA AGG TTC TCT TGG AGT CGC TCT G
β-actin/5' primer	GAC CTG ACT GAC TAC CTC AT
β-actin/3' primer	TCG TCA TAC TCC TGC TTG CT

for 45 min at 180 mA, the membranes were blocked with Tris-buffered saline containing 5% dry milk powder (Snow Brand, Sapporo, Japan) and incubated with anti-rat CNTF antibody (Pepro Tech, Inc., London, England) diluted 1:1000. Bound antibody was detected using anti-rabbit IgG horseradish peroxidase-linked F(ab')₂ fragment and ECL Western blotting detection reagents (Amersham Life Science, Buckinghamshire, England).

Statistical analysis. X-ray films were scanned and analyzed using NIH image 1.59. For Northern blot analysis, film background was subtracted and the density of 28S

ribosomal RNA was used for normalization. All data were entered into computer and analyzed using the StatView Program (Abacus Concepts, Berkeley, CA, U.S.A.). The effect of ACTH₁₋₂₄ or drug stimulation was compared with control values using *t* test for Figures 1, 4, and 6, or multivariate analysis of variance (MANOVA) for Figures 2, 3, and 5. When statistical significance was observed, one-way ANOVA with Scheffé *post hoc* test was performed for each set of variables. Probability values less than or equal to 0.05 were considered significant.

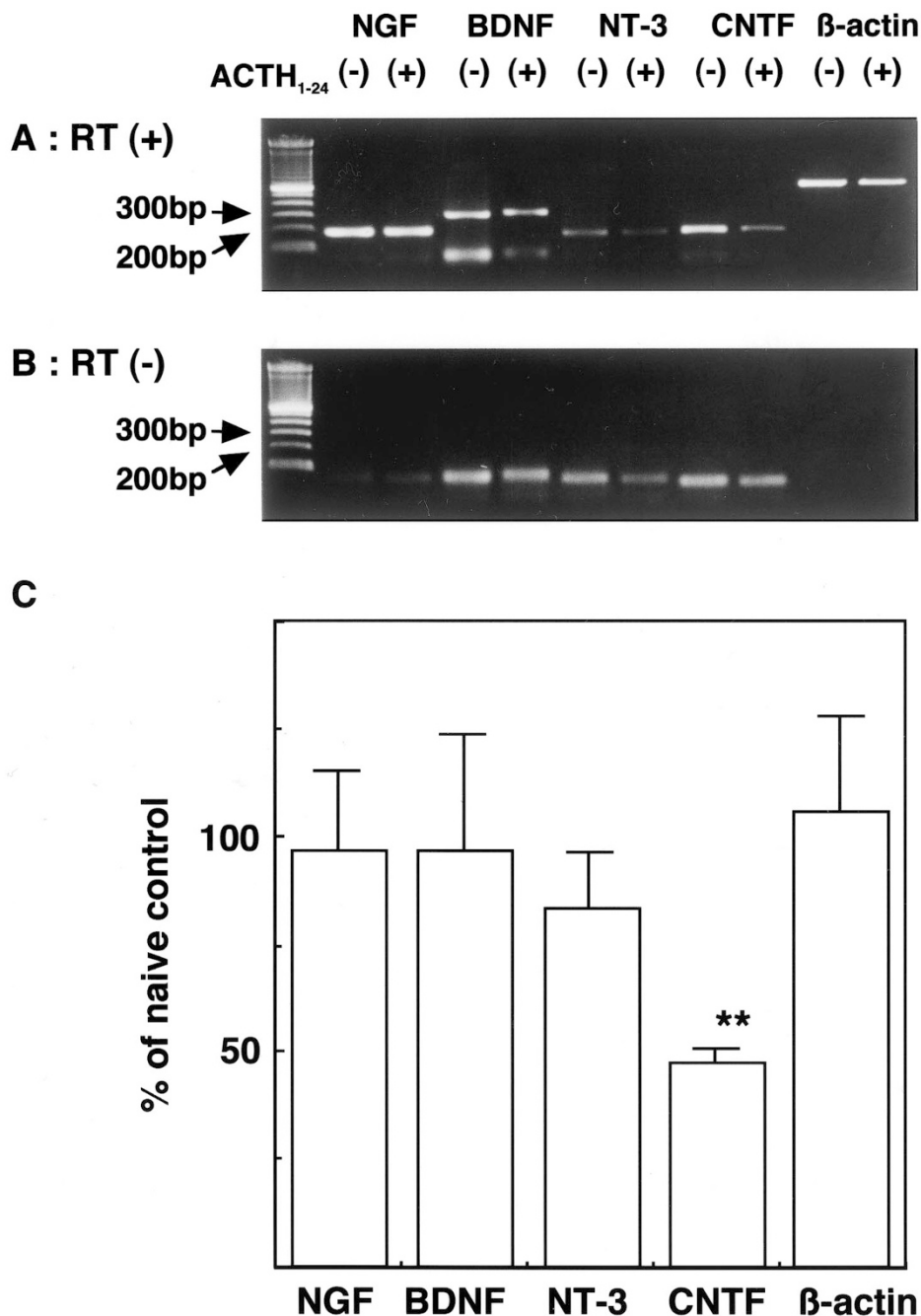


Figure 1. Expression of neurotrophic factor mRNAs in astrocytes treated with ACTH₁₋₂₄. Cultured astrocytes from whole brain of embryonic d 20 Wistar rats were stimulated by 100 nM ACTH₁₋₂₄ for 8 h. PCR products were electrophoresed on 1.5% agarose gels and visualized by ethidium bromide staining. *A*, PCR products from the samples with reverse transcription. *B*, PCR products from the samples without reverse transcription. *C*, values, presented as ratios relative to controls which received no ACTH₁₋₂₄ treatment, are mean \pm SEM (*bars*) of three experiments. ***p* < 0.01 compared with the control (*t* test).

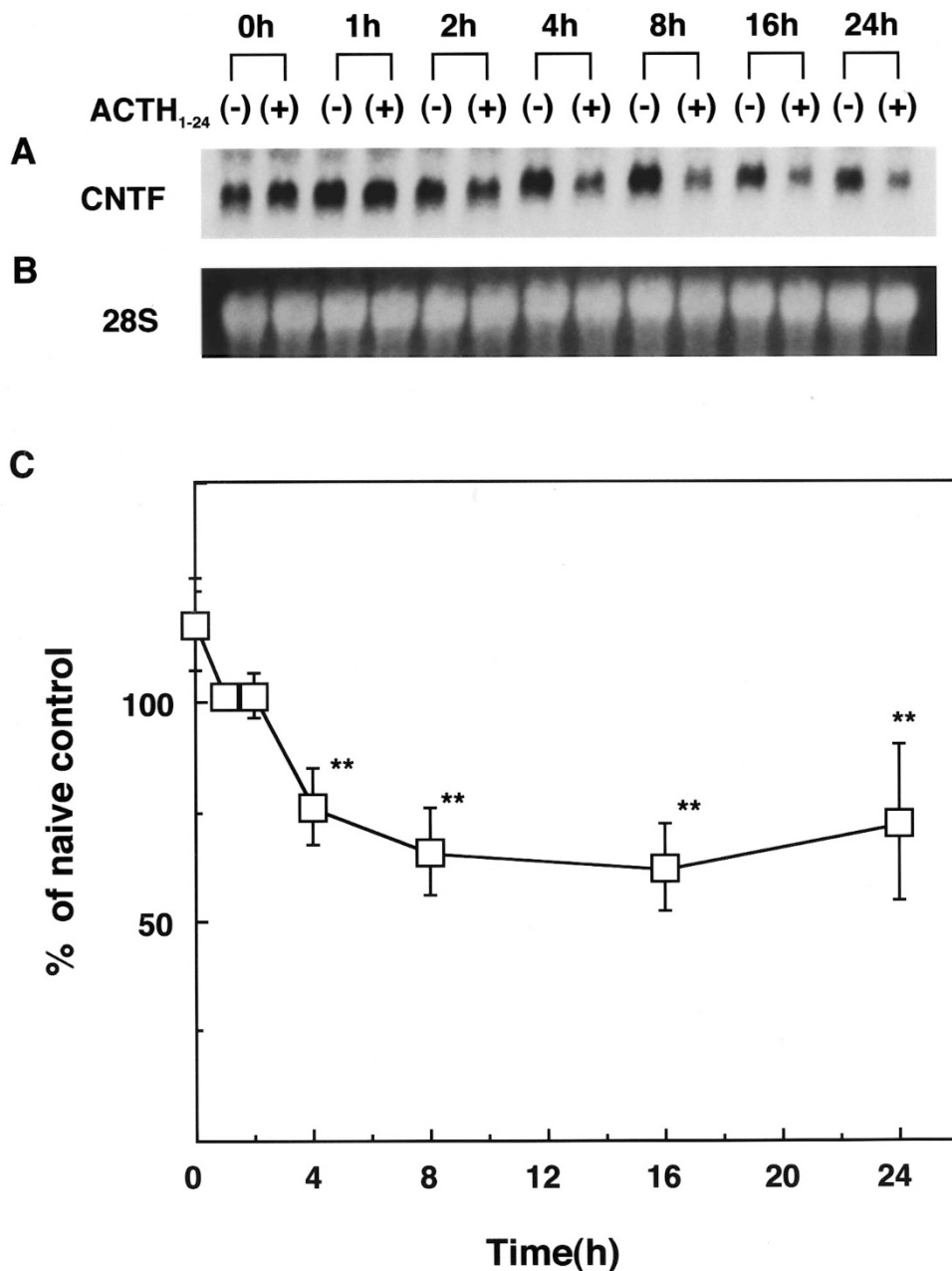


Figure 2. Time-dependent changes in CNTF mRNA in astrocytes treated with ACTH₁₋₂₄. Cultured astrocytes were exposed to 100 nM ACTH₁₋₂₄ for the indicated periods of time. Total RNA was extracted and subjected to Northern blot analysis as described in the text. *A*, autoradiogram of a blot hybridized with CNTF probe. *B*, 28S ribosomal RNA in an ethidium bromide-stained gel, allowing for comparison of the total amount of RNA (10 μ g) per sample. *C*, time-dependent changes in CNTF mRNA expression were relative to controls. Values are mean \pm SEM (*bars*) of three experiments. ***p* < 0.05 compared with the control (Scheffé *post hoc* test).

RESULTS

ACTH₁₋₂₄-induced expression of neurotrophic factor mRNAs in astrocytes. NGF, BDNF, NT-3, and CNTF are well-characterized neurotrophic factors and known to be produced by astrocytes. At first, we investigated the effects of ACTH₁₋₂₄ on the expression of NGF, BDNF, NT-3, and CNTF in astrocyte. We used RT-PCR to detect NGF, BDNF, NT-3, and CNTF mRNAs. Total RNA from astrocytes stimulated by 100 nM ACTH₁₋₂₄ for 8 h was amplified by RT-PCR, and PCR products for NGF, BDNF, NT-3, CNTF, and β -actin were determined by electrophoresis. It had been confirmed, in ad-

vance, by RT-PCR without the transcription step, that the PCR products were not derived from concomitant DNA fragments. Figure 1 (*A* and *B*) shows the representative results of triplicate experiments. The amounts of PCR products relative to the control (*n* = 3) are shown in Figure 1*C*. There was an apparent down-regulation of CNTF mRNA caused by stimulation with 100 nM ACTH₁₋₂₄.

Time-dependent reduction of CNTF mRNA caused by ACTH₁₋₂₄. To characterize the effects of ACTH₁₋₂₄ on the expression of CNTF mRNA, astrocytes were incubated for 0 to 24 h with 100 nM ACTH₁₋₂₄. Figure 2 shows the time-

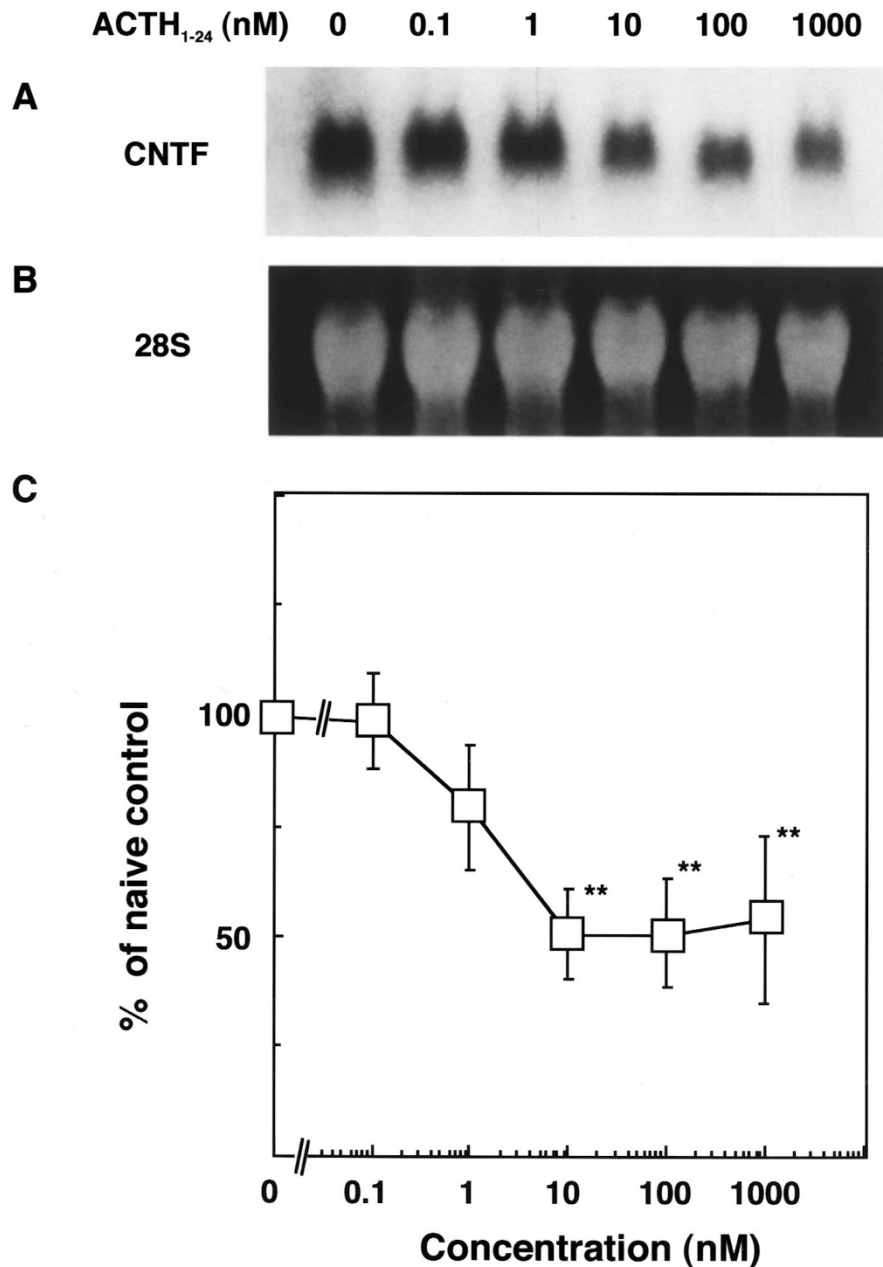


Figure 3. Dose-dependent changes in CNTF mRNA in astrocytes treated with ACTH₁₋₂₄. Cultured astrocytes were exposed to ACTH₁₋₂₄ at indicated concentrations for 8 h. Total RNA was extracted and subjected to Northern blot analysis as described in the text. *A*, autoradiogram of a blot hybridized with CNTF probe. *B*, 28S ribosomal RNA in an ethidium bromide-stained gel. *C*, dose-dependent changes in CNTF mRNA expression relative to controls. Values are mean \pm SEM (bars) of three experiments. ** $p < 0.05$ compared with the control (Scheffé *post hoc* test).

dependent changes in CNTF mRNA in response to 100 nM ACTH₁₋₂₄. An apparent down-regulation of CNTF mRNA was observed after 4 h of treatment of 100 nM ACTH₁₋₂₄ (Fig. 2C).

Dose-dependent reduction of CNTF mRNA caused by ACTH₁₋₂₄. Astrocytes were exposed for 8 h to various concentrations of ACTH₁₋₂₄ ranging from 100 pM to 1 μ M. Figure 3 shows the dose-dependent reduction of CNTF mRNA in response to treatment with ACTH₁₋₂₄ for 8 h. At concentrations greater than 10 nM ACTH₁₋₂₄, down-regulation of CNTF mRNA was observed (Fig. 3C).

Effect of ACTH₁₋₂₄ on intracellular CNTF protein. To investigate whether ACTH₁₋₂₄ reduces intracellular CNTF pro-

tein, Western blot analyses were performed. Astrocytes were treated with 100 nM ACTH₁₋₂₄ every 24 h and harvested at 8 h, 24 h, and 72 h after the first ACTH₁₋₂₄ treatment, and cell extracts were subjected to Western blot analyses. Figure 4A shows a representative result of four experiments. At 72 h, after three treatments with 100 nM ACTH₁₋₂₄, the band of CNTF decreased slightly compared with control, but this is not significant (Fig. 4B).

Effects of a PKA and a PKC inhibitor on down-regulation of CNTF mRNA. ACTH₁₋₂₄ has been reported to induce cAMP, which exerts its effects mostly via activation of PKA. Therefore, we determined whether the down-regulation of CNTF mRNA by ACTH₁₋₂₄ treatment was mediated via PKA.

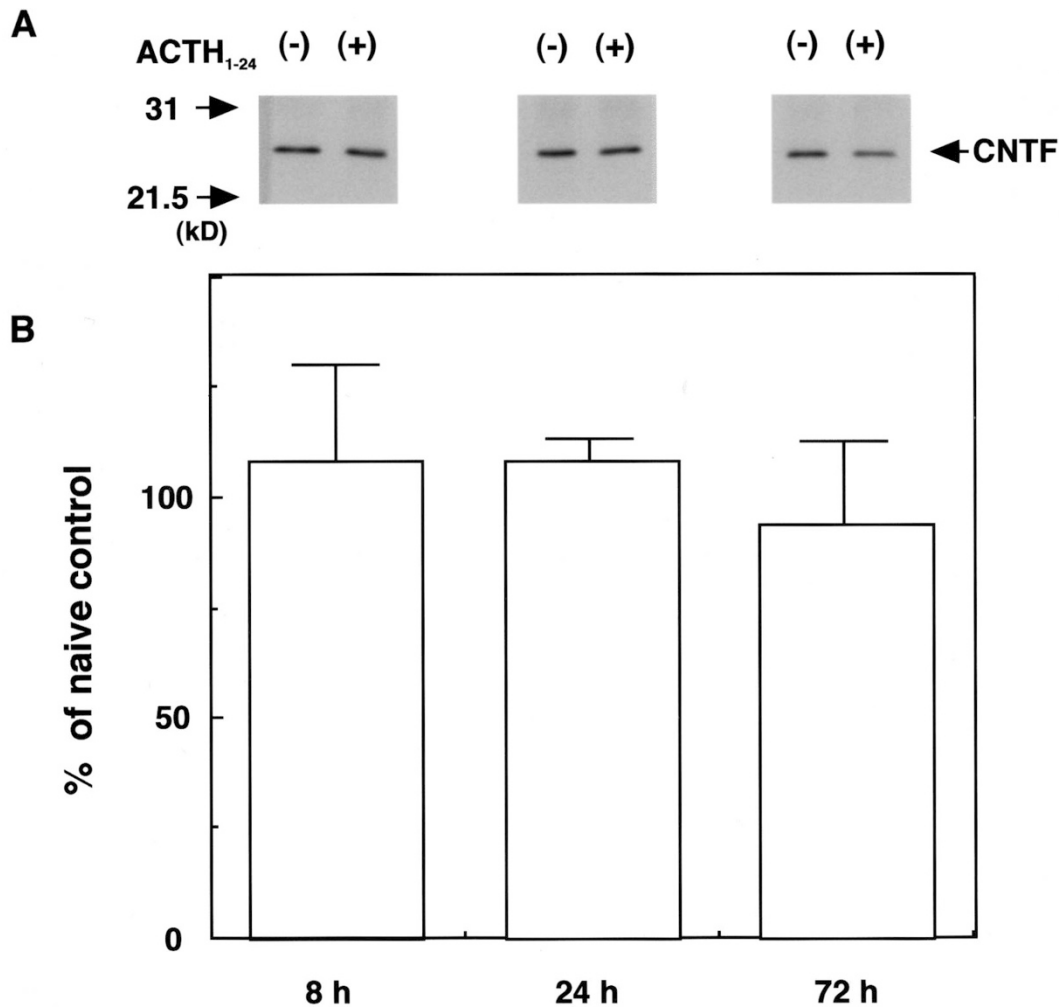


Figure 4. Western blot analysis of intracellular CNTF protein in astrocytes treated with ACTH₁₋₂₄. Astrocytes were harvested at 8 h, 24 h, and 72 h after ACTH₁₋₂₄ treatment and cell extracts were subjected to Western blot analysis as described in the text. *A*, representative result of four experiments. *B*, changes in CNTF band relative to controls. Values are mean \pm SEM (bars) of four experiments.

Treatment of cultured astrocytes with H-89, a selective inhibitor of PKA, did not reverse the ACTH₁₋₂₄-induced suppression of CNTF mRNA. Treatment of the astrocytes with H-7, an inhibitor of PKC, slightly reduced CNTF mRNA, but this is not significant (Fig. 5).

CRF- and Dex-induced expression of neurotrophic factor mRNAs in astrocytes. To investigate the indirect effect of ACTH, we examined the effects of CRF and Dex on the expression of neurotrophic factors in astrocytes. In this study we used Dex to check the typical glucocorticoid effect on astrocyte, because Dex has approximately 8 \times stronger effects than prednisolone. As with ACTH₁₋₂₄ treatment, RT-PCR for NGF, BDNF, NT-3, and CNTF was performed after treating with 1 μ M CRF or 1 μ M Dex for 8 h. An apparent up-regulation of NT-3 mRNA by treatment with 1 μ M Dex was observed, but CRF had no effect on the expression of neurotrophic factor mRNAs (Fig. 6).

DISCUSSION

With our RT-PCR results we observed down-regulation of CNTF mRNA by ACTH₁₋₂₄ treatment, but expression of

NGF, BDNF, and NT-3 mRNAs was unaffected. Therefore, we focused on the changes in expression of CNTF mRNA induced by ACTH₁₋₂₄ treatment. Northern blot analyses revealed that down-regulation of CNTF mRNA occurred in a time- and dose-dependent manner. By Western blot analysis, a slight decrease in CNTF protein was observed at 72 h. These results suggest that repeated administration of ACTH₁₋₂₄, as a therapy for West syndrome, is needed for down-regulating CNTF protein because astrocytes express high amounts of CNTF, especially under reactive conditions.

The capacity to synthesize CNTF mRNA in cultures of neural tissue appears to be confined mainly to astrocytes, as cultured fibroblasts, neurons, and macrophages do not show appreciable levels of CNTF mRNA (9, 10). CNTF is a pleiotropic cytokine that supports survival or differentiation of a variety of neuronal cell types including sensory, sympathetic, and motor neurons, and it induces type-2 astrocyte differentiation in culture (11). On the other hand, CNTF has been implicated as an injury factor involved in regulating astrogliosis in the CNS (12). In a recent report, CNTF reduced survival of isolated Purkinje cells (13). Taken together with our results, administration of ACTH under

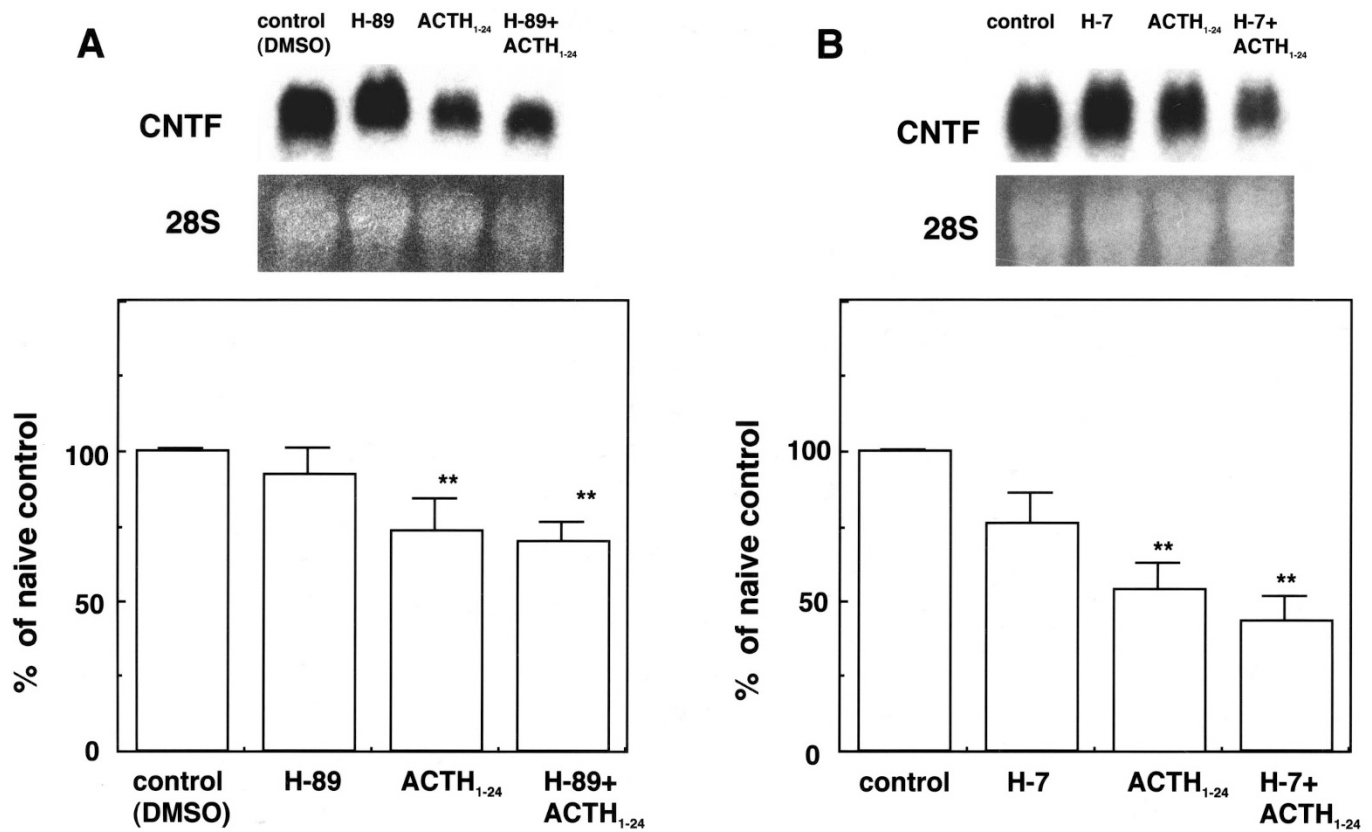


Figure 5. Effect of protein kinase inhibitors on CNTF mRNA expression in astrocytes treated with ACTH₁₋₂₄. Cultured astrocytes were preincubated with protein kinase inhibitors and then exposed to ACTH₁₋₂₄ for 8 h. Total RNA was extracted and subjected to Northern blot analysis as described in the text. *A*, cells were exposed to 1 μ M H-89 for 30 min before being treated with 10 nM ACTH₁₋₂₄ for 8 h. *B*, cells were exposed to 6 μ M H-7 for 30 min before being treated with 100 nM ACTH₁₋₂₄ for 8 h. Pictures are representative results of three experiments. Values, presented as ratios relative to controls, are mean \pm SEM (bars) of three experiments. ***p* < 0.05 compared with the control (Scheffé post hoc test).

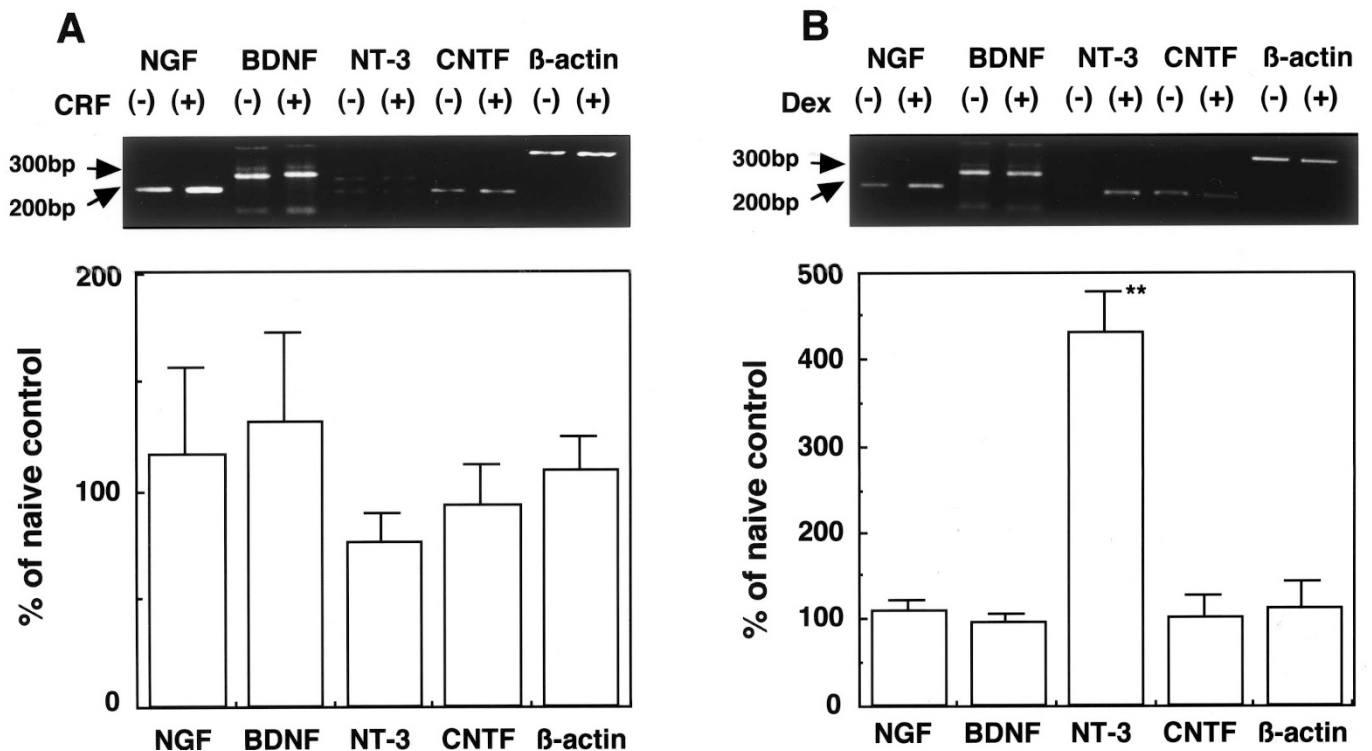


Figure 6. Expression of neurotrophic factors mRNAs in astrocytes treated with CRF or Dex. Cultured astrocytes were treated with 1 μ M CRF (*A*) or 1 μ M Dex (*B*) for 8 h. The expression of neurotrophic factor mRNAs was analyzed by RT-PCR. Pictures are representative results of three experiments. Values, presented as ratios relative to controls, are mean \pm SEM (bars) of three experiments. ***p* < 0.01 compared with the control (*t* test).

the condition of gliosis after brain damage may reduce expression of CNTF to appropriate levels that may be neuroprotective.

In adult rats less than 1% of an injected dose of ACTH is found in the brain (14, 15). Recently, Brunson *et al.* (16) reported that administered ACTH acts directly on limbic neurons to decrease the expression of CRF. At injured lesions or after a convulsion, especially in immature brain, administered ACTH₁₋₂₄ may reach astrocytes easily because of dysfunction of the blood-brain barrier. However, further research is needed to determine the permeability of immature or damaged brain to ACTH.

Melanocortin receptors have seven membrane-spanning domains coupled to adenylate cyclase (17). Five members of this receptor family have been cloned, and MC3, MC4, and MC5 receptors are expressed in brain. ACTH at concentrations from 10 nM to 1 mM can bind to these receptors and activate adenylate cyclase (18). These concentrations correspond to those that reduce CNTF mRNA in astrocytes. In this study, treatment of cultured astrocytes with H-89, a selective inhibitor of PKA, did not reverse ACTH₁₋₂₄-induced suppression of the expression of CNTF mRNA. This indicates that cascades other than PKA are involved in the ACTH-induced down-regulation of CNTF mRNA and is consistent with previous reports (19, 20). Recently, Kawasaki *et al.* (21) reported that cAMP activates a Rap1 in a PKA-independent manner, a new cAMP-mediated signaling pathway. According to this evidence, a cAMP-Rap1 cascade might be one of the important pathways for regulation of CNTF mRNA. It has been reported that the PKC pathway is also involved in the regulation of CNTF mRNA expression (22). Therefore, we examined the effect of a PKC inhibitor on the down-regulation of CNTF mRNA caused by ACTH₁₋₂₄ treatment. An inhibitor of PKC, H-7, slightly reduced CNTF mRNA, and H-7 together with ACTH₁₋₂₄ showed a tendency of an additive effect on the down-regulation of CNTF mRNA. However, there is no statistical significance between control and H-7 alone, or between H-7 alone and H-7 with ACTH₁₋₂₄.

It has been proposed that enhanced expression of CRF is one of the causes of West syndrome. Therefore, we investigated the effect of CRF on the expression of neurotrophic factors in astrocytes. Expression of the CRF receptor is high in extrahypothalamic regions, and astrocytes from fetal extrahypothalamus brain cell cultures contain CRF receptors that are coupled to adenylate cyclase (23). However, in this study no significant changes in NGF, BDNF, NT-3, and CNTF mRNAs were observed in astrocytes stimulated by CRF, because we used astrocytes from whole brain that might express only low levels of CRF receptors.

In addition to ACTH, glucocorticoid hormones have been used to treat West syndrome. We found that Dex induced the expression of NT-3 mRNA in cultured astrocytes. We reported previously that Dex induces lipocortin-1 mRNA in astrocytes and that lipocortin-1 has a neurotrophic effect on cultured cortical neurons (24). These data suggest that glucocorticoid induced by ACTH₁₋₂₄ may also have neurotrophic effects by inducing NT-3 and lipocortin-1 in astrocytes.

CONCLUSIONS

Both ACTH and glucocorticoids have been shown to be effective in treating West syndrome, and the major effect of

ACTH and glucocorticoids is down-regulation of CRF concentration in the CNS (25), but ACTH is reported to be more effective than glucocorticoids (4, 5). This study may provide evidence for one of the mechanisms by which ACTH is advantageous for treating West syndrome, as ACTH may influence the gene expression in astrocyte. *In vivo* studies are needed to confirm our data.

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