Effects of adenylyl cyclase-linked neuropeptides on the expression of ciliary neurotrophic factor-mRNA in cultured astrocytes

Hiroshi Nagao, Ichiro Matsuoka*, Kenzo Kurihara

Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo, 060 Japan

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Abstract Ciliary neurotrophic factor (CNTF) is a molecule which has profound effects on various neural cell types. In the central nervous system, expression of CNTF-mRNA is highly concentrated in olfactory bulb. In the present study, we examined the regulatory mechanism of CNTF-mRNA expression in cultured astrocytes from newborn rat brain. Cultured astrocytes from new born rat brain expressed CNTF-mRNA at levels comparable to the level in olfactory bulb in vivo. Treatment of the astrocytes with forskolin, an activator of adenylyl cyclase, led to a decrease of CNTF-mRNA level. The effect of forskolin was mimicked by cAMP-linked agonists, such as VIP, PACAP, isoproterenol and dopamine. Cycloheximide, an inhibitor of protein synthesis, did not abolish the forskolin-induced decrease of CNTF-mRNA. Measurement of the half-life of CNTF-mRNA in the presence of actinomycin D, an inhibitor of transcription, indicated that the degradation of CNTF-mRNA is not destabilized by the forskolin-treatment. These data taken together suggest that the cAMP-induced suppression of CNTF-mRNA is mainly caused by the inhibition of CNTF gene transcription.

Key words: CNTF; cAMP; VIP; PACAP; Astrocyte; Olfactory bulb

1. Introduction

CNTF was originally identified as a target derived neurotrophic molecule supporting the survival of parasympathetic ciliary neurons [1,2]. Now it became aware that CNTF exerts a broad spectrum of biological actions on various types of neuronal cells as well as on glial cells in culture [3–5]. The expression of CNTF in the CNS is highly concentrated in olfactory bulb suggesting that CNTF has unique functions in this region of brain [2,6]. To elucidate the function of CNTF in the olfactory system, it is important to know how the expression of CNTF is regulated in situations linked to the physiological activities of the olfactory system. Using immunohistochemical technique, it is shown that, within olfactory bulb, the expression of CNTF is localized to subpopulation of astrocytes [6]. In the present study, as a first step to investigate the function of CNTF in the olfactory system, we analyzed the regulatory mechanism of the CNTF expression in the cultured astrocytes prepared from new born rat olfactory bulb. The results indicated that the astrocytes from olfactory bulb expressed CNTF-mRNA at high level comparable to that in olfactory bulb in vivo. Forskolin, an activator of adenylyl cyclase, as well as cAMP-linked agonists suppressed the expression of CNTF-mRNA in the olfactory bulb astrocytes. The mechanism and the physiological implications of the forskolin-induced suppression of CNTF-mRNA level are discussed.

*Corresponding author. Fax: (81) (11) 706 4991.

Abbreviations: CNTF, ciliary neurotrophic factor; NGF, nerve growth factor; VIP, vasoactive intestinal polypeptide; PACAP, pituitary adenylyl cyclase-activating polypeptide; IBMX, 3-isobutyl-1-methylxanthine.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), poly-L-ornithine, ionomycin, dideoxyforskolin, 3,4-dihydroxyphenethylamine (dopamine) were purchased from Sigma (St. Louis), forskolin, actinomycin D, cycloheximide, 3-isobutyl-1-methylxanthine (IBMX), *dl*-isoproterenol were from Wako Pure Chemical (Tokyo), vasoactive intestinal polypeptide (VIP), carnosine, and substance P were from Peptide Institute (Osaka), fetal calf serum was from Gibco (New York), penicillin and streptomycin were from Meiji Seika (Tokyo). Pituitary adenylyl cyclase-activating polypeptides (PACAP-27 and PACAP-38) were generous gift from Dr. Watanabe, T. of Takeda Pharmaceutical Industries (Osaka). Plasmid (pBluescript SK(−)) containing rat CNTF-cDNA [2] used for making sense and antisense cRNA was kindly provided by Dr. P. Caroll (Martinsried, FRG).

2.2. Cell culture

Astrocytes were prepared essentially as described previously [7]. Olfactory bulbs and cerebral cortices were dissected from 2-day-old Wistar rats and treated with 0.25% trypsin in DMEM for 20 min. After trituration with pipette, dissociated cells were collected by centrifugation, resuspended with DMEM supplemented with 10% fetal calf serum and antibiotics (50 U/ml penicillin and 100 μg/ml streptomycin) and plated on 35 mm culture dishes (approximately 1×10^6 cells/dish) which were precoated with 100 μg/ml poly-L-ornithine. The medium was changed on the day following plating and thereafter every third day. Cells were grown for 7–10 days before stimulation with various compounds.

2.3. RNA analysis

Total RNA was prepared from the cells according to the method of Chomczynski and Sacchi [8]. The extraction buffer contained 20 pg of 0.6-kb long CNTF standard RNA to evaluate the recovery of RNA. RNA samples were glyoxylated, electrophoresed through a 1.5% agarose gel, and then transferred to a nylon membrane (Hybond-N, Amersham). Following transfer, membranes were fixed by UV irradiation. Membranes were prehybridized, and hybridized with ^{32}P-labeled antisense CNTF-cRNA probe (0.6 kb) at 65°C in 50% formamide. Membranes were washed and exposed to Kodak X-OMAT AR. The radioactivity bound to CNTF-mRNA was quantified with Bio-image analyzer (Fuji Photo Film) and corrected for recovery of RNA.

3. Results

3.1. Expression of CNTF-mRNA in rat brain and cultured astrocytes

Quantitative Northern blot analysis was performed to evaluate the expressions of CNTF-mRNA in different regions of adult rat brain and cultured astrocytes (Fig. 1). Olfactory bulb expressed the highest level of CNTF-mRNA in brain, while the levels of CNTF-mRNA in other brain regions such as cerebrum

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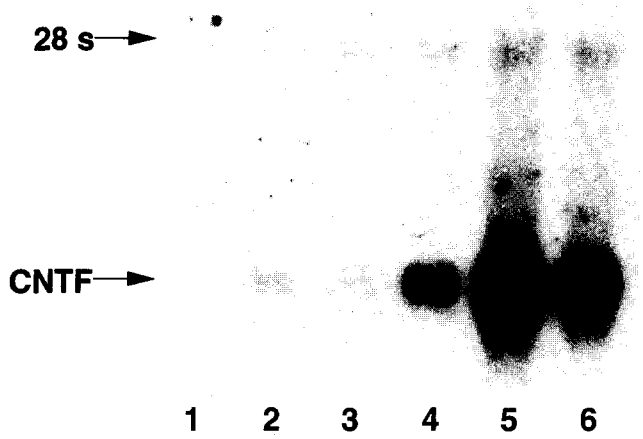

Fig. 1. Northern blot analysis of CNTF-mRNA in adult rat brain and cultured astrocytes. The positions of CNTF-mRNA (1.2 kb) and 28s ribosomal RNA were indicated by arrows. Lane 1, cerebral cortex; lane 2, midbrain; lane 3, cerebellum; lane 4, olfactory bulb; lane 5, cultured olfactory bulb astrocytes; lane 6, cultured cerebral cortex astrocytes. Each lane contained 10 μg of total RNA. Even recovery of loaded RNA on the membrane was confirmed by staining of ribosomal RNA with methylene blue.

cortex, mid brain and cerebellum were very low. This result confirms the observation by Stöckli et al. [6] that olfactory bulb expresses the highest level of CNTF-mRNA in central nervous system. Cells were dissociated from newborn rat olfactory bulb and cerebral cortex and were maintained in DMEM containing 10% fetal calf serum. During the culture period for one week, the confluent monolayer of flat cells was obtained. Most of neuron-like cells were eliminated during this period by massive proliferation of the flat cells. Immunostaining with anti-GFAP antibody revealed that the monolayer of flat cells obtained from both olfactory bulb and cerebral cortex consisted of Type 1-astrocytes with purity of >95%. The cultured olfactory bulb astrocytes expressed a relatively high level of CNTF-mRNA (approximately 1 pg/μg total RNA), which was 7.2-fold higher than the level in adult rat olfactory bulb in vivo (Fig. 1). Interestingly, astrocytes obtained from cerebral cortex also expressed the CNTF-mRNA at the level comparable to that expressed by olfactory bulb astrocytes.

3.2. *Suppression of CNTF-mRNA in cultured astrocytes by cAMP producing agents*

cAMP has been shown to mediate the increase in NGF-mRNA levels in cultured astrocytes and Schwann cells [9–11]. Contrary, CNTF-mRNA in the cultured astrocytes from both olfactory bulb and cerebral cortex was abolished by treatment with forskolin, an activator of adenylyl cyclase. In cultured olfactory bulb astrocytes CNTF-mRNA was decreased to less than 20% of the original level with 10 μM forskolin at 9 h of the treatment and half-maximal concentration of forskolin was approximately 0.3 μM (data not shown). Analysis of the time course of the forskolin-suppression of CNTF-mRNA indicated that the level of CNTF-mRNA was decreased by 50% at 4.7 h after addition of 20 μM forskolin and to less than 20% of the original level at 9 h of the treatment (Fig. 4). 3-Isobutyl-1-methylxanthine (IBMX), an inhibitor of phosphodiesterase, mimicked the effect of forskolin on the suppression of CNTF-mRNA at concentrations above 10 μM. Thus, CNTF-mRNA was decreased to 46.5% of the original level at 9 h after addition of 100 μM IBMX (Table 1). Alternatively, dideoxyforskolin, an analog of forskolin which has no ability to activate adenylyl cyclase, but shares other cellular effects with forskolin, did not affect the levels of CNTF-mRNA in olfactory bulb astrocytes (Table 1). These results suggest that the accumulation of intracellular cAMP led to the abolishment of CNTF-mRNA in the cultured astrocytes from olfactory bulb and cerebral cortex.

3.3. *Effects of cAMP-linked agonists on the CNTF-mRNA levels in cultured astrocytes*

Variety of receptors for transmitters and neuropeptides which are linked to adenylyl cyclase are known to be expressed in brain astrocytes [12–16]. Therefore, we attempted to identify major cAMP-linked agonists capable to down-regulate CNTF-mRNA in olfactory bulb astrocytes. As a result, agonists for dopamine, β-adrenergic, vasoactive intestinal polypeptide (VIP) and pituitary adenylyl cyclase-activating polypeptide (PACAP) receptors were effective in decreasing the CNTF-mRNA levels (Table 1, Fig. 2). Dopamine and isoproterenol, a β-adrenergic agonist, showed modest effects. Thus, the CNTF-mRNA level was decreased to 72% and 75% of the original level by 9-h treatments with 100 μM isoproterenol and 100 μM dopamine, respectively. Calcitonin gene-related peptide (CGRP), known as an adenylyl cyclase-linked neuropeptide [17], did not affect the suppression of CNTF-mRNA in the olfactory bulb astrocytes.

VIP and PACAP exerted the prominent effects on the CNTF-mRNA levels. That is, in the cultured astrocytes from olfactory bulb, CNTF-mRNA level was decreased to 66.6% of the original level by a 9 h-treatment with 100 nM VIP while it was decreased to 49 and 66% at 9-h treatments with 100 nM PACAP27 and PACAP38, respectively (Fig. 2A). Olfactory bulb astrocytes responded to PACAP27, PACAP38 and VIP with approximate EC_{50} value of 0.1, 0.1 and 10 nM, respectively. Similarly, in the cultured astrocytes from cerebral cortex, the CNTF-mRNA level was decreased to 25.4% of original level at 9 h after the addition of 100 nM VIP while it was decreased to 14.3 and 22.5% at 9-h treatments with 100 nM PACAP27 and PACAP38, respectively (Fig. 2B). Cerebral

Table 1
Effects of various compounds on the CNTF-mRNA levels

Compounds	Concentration	CNTF-mRNA	Range
None	–	100.0	–
FK*	20 μM	19.3	± 4.2
DFK*	20 μM	96.6	± 10.0
IBMX*	100 μM	46.5	± 1.7
Dopamine	100 μM	74.7	± 8.5
Isoproterenol	100 μM	72.4	± 0.2
CGRP	50 nM	99.1	± 2.9
VIP	100 nM	66.6	± 2.3
PACAP38	100 nM	65.9	± 4.9
PACAP27	100 nM	49.0	± 9.2
Substance P	100 μM	95.6	± 5.5
Carnosine	100 μM	106.3	± 0.3
Ionomycin	0.1 mg/ml	100.1	± 0.4

Cultured astrocytes from olfactory bulb were incubated with the neurotransmitters and neuropeptides at the indicated concentrations for 9 h. Each data represents the mean and range of duplicate samples except for compounds with asterisk where data represents the mean ± S.D. (n = 4). FK, forskolin; DFK, dideoxyforskolin.

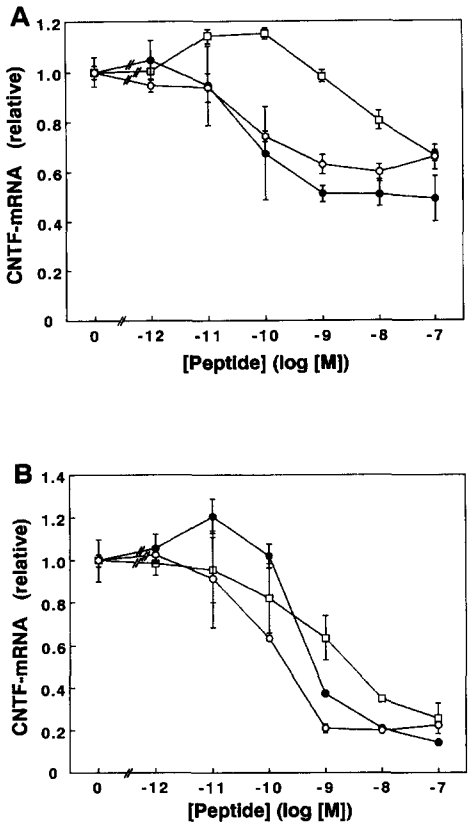

Fig. 2. Dose-dependence of neuropeptides on CNTF-mRNA levels. Cultured astrocytes from olfactory bulb (A) or cerebral cortex (B) were incubated with indicated concentrations of neuropeptides for 9 h: VIP (□), PACAP 27 (●), PACAP 38 (○). Each data point represents the mean and range of duplicate samples.

cortex astrocytes responded to all the three peptides with an approximate EC_{50} value of 0.1 nM. Analysis of the time course of the changes in the CNTF-mRNA levels revealed that the effects of neuropeptides were transient (Fig. 3). That is, the CNTF-mRNA level was decreased to the minimum at 9 h after addition of 10 nM VIP, PACAP27 or PACAP38, but, after that time point, the CNTF-mRNA levels began to recover and the original level was almost regained at 24 h.

On the other hand, a treatment of the astrocytes with 0.1 mg/ml ionomycin, a Ca-ionophore, did not change the CNTF-mRNA level (Table 1). Similarly, Substance P, a neuropeptide known to be coupled to phosphatidyl inositol turnover and mobilization of intracellular Ca^{2+} [18], did not affect the CNTF-mRNA level in the cultured astrocytes. Incidentally, carnosine, the putative transmitter of olfactory neuron [19,20], did not change the CNTF-mRNA level in olfactory bulb astrocytes.

3.4. Regulatory mechanism of CNTF-mRNA expression by cAMP in olfactory bulb astrocytes

We examined whether de novo protein synthesis is required for the forskolin-induced process. Treatment of the olfactory bulb astrocytes with cycloheximide, an inhibitor of protein synthesis, slightly increased the CNTF-mRNA level (120% of the original level) up to 12 h of the treatment (Fig. 4A). Thereafter, the CNTF-mRNA level began to decrease slowly. When cycloheximide was added in combination with forskolin, the CNTF-mRNA level decreased with a time course very similar to the case where forskolin alone was added. Thus, the forskolin-induced suppression of CNTF-mRNA was not canceled by the cycloheximide-treatment. Therefore, it is strongly suggested that the forskolin-induced suppression of CNTF-mRNA was not resulted from de novo protein synthesis.

There are two possibilities for forskolin to suppress the expression of CNTF gene at mRNA level. The first possibility is the destabilization of the CNTF-mRNA and the other possibility is the inhibition of CNTF gene transcription. First, we examined whether the destabilization of CNTF-mRNA participates in the forskolin-induced suppression of CNTF-mRNA in the olfactory bulb astrocytes. The half-life of the CNTF-mRNA was measured by following the time course of decay of CNTF-mRNA after addition of actinomycin D, an inhibitor of transcription, to the cells treated with or without forskolin. As shown in Fig. 4B, addition of actinomycin D to the untreated astrocytes induced the decay of CNTF-mRNA with a half-life ($t_{1/2}$) of 7 h. This half-life corresponds to the native stability of CNTF-mRNA. But, in the presence of actinomycin D, forskolin did not accelerate the decay of CNTF-mRNA. Thus, the half-life became rather slightly longer ($t_{1/2}$ = 10 h) than that of the cells treated with actinomycin D alone. Since forskolin abolishes CNTF-mRNA even in the presence of cycloheximide and forskolin does not promote degradation of CNTF-mRNA in the presence of actinomycin D, it is suggested that forskolin decreases the level of CNTF-mRNA via inhibition of transcription of CNTF gene in a de novo protein synthesis independent manner as a major pathway. Further confirmation of the inhibition of CNTF gene transcription by forskolin and its contribution to the forskolin-induced suppression of CNTF-mRNA should await the nuclear run-off assay. Alternatively, the slight acceleration of the degradation of CNTF-mRNA by forskolin alone ($t_{1/2}$ = 4.7 h) and its cancellation by actinomycin D suggest that forskolin destabilized the CNTF-mRNA in a protein synthesis dependent manner to contribute partly to the forskolin-induced suppression of CNTF-mRNA.

4. Discussion

The goal of the present study was to obtain information on the mechanism involved in the regulation of CNTF expression in the cultured brain astrocytes as a basis for understanding of

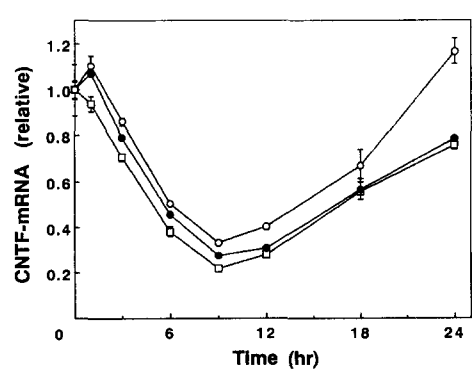

Fig. 3. Time courses of changes in CNTF-mRNA levels induced by neuropeptides. olfactory bulb astrocytes were treated with 10 nM of neuropeptides for the indicated time: VIP (□), PACAP 27 (●), PACAP 38 (○). Each data point represents the mean and range of duplicate samples.

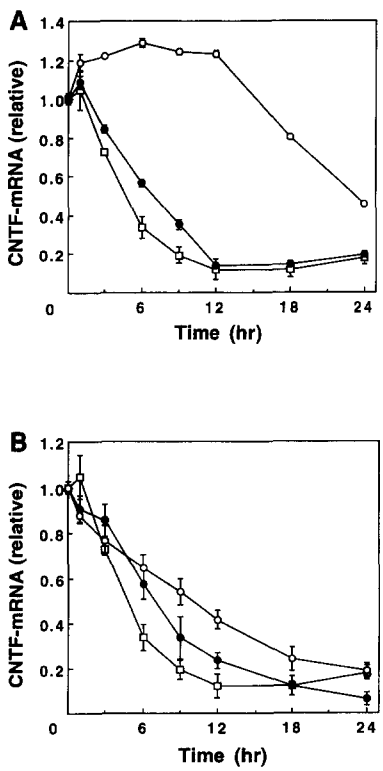

Fig. 4. Effects of cycloheximide and actinomycin D on the forskolin-induced suppression of CNTF-mRNA. (A) cultured astrocytes from olfactory bulb were pretreated with 10 mg/ml cycloheximide for 0.5 h. Then, the astrocytes were further incubated with cycloheximide alone (○) or with 20 μM forskolin (●) for the indicated time. Time course of change in the CNTF-mRNA levels of the cells treated with forskolin alone is also indicated (□). (B) olfactory bulb astrocytes were pretreated with 10 μg/ml actinomycin D for 0.5 h. Then, the astrocytes were further incubated with actinomycin D alone (●) or with 20 μM forskolin (○) for the indicated time. Time course of changes in CNTF-mRNA levels of the astrocytes treated with forskolin alone is also indicated (□). Each data point represents the mean and range of duplicate samples.

the in vivo role of CNTF in the olfactory system. CNTF has been shown to be expressed in glial cells of both central and peripheral nervous system such as astrocytes and Schwann cells [6,21]. We also confirmed the high level of CNTF-mRNA expression in the cultured astrocytes prepared from olfactory bulb and cerebral cortex. Using cultured astrocytes which mimic reactive astrocytes in vivo [22], we first looked for extra- and intra-cellular signals regulating CNTF-mRNA.

The high level of CNTF-mRNA expression in cultured astrocytes was suppressed by accumulation of cAMP. Among various cAMP-linked agonists, PACAPs and VIP were the most prominent suppresser of the CNTF-mRNA in the cultured astrocytes. Recently it was reported that Type I PACAP receptor which preferentially utilizes PACAPs over VIP is expressed in limited regions in the brain including glomerular layer of olfactory bulb [23]. Two types of VIP receptors (VIP1 and VIP2) which bind PACAPs and VIP with the same affinity are also expressed in olfactory bulb [24,25]. On the other hand, in cerebral cortex, the expression of only the VIP1 receptor is reported [26]. In the present study, PACAPs were 100 times more effective than VIP on suppressing the CNTF-mRNA in the cultured astrocytes from olfactory bulb, while PACAPs and VIP were equally effective in the cultured astrocytes from cerebral cortex. Hence, our results suggest that PACAPs and VIP activate Type I PACAP receptor and VIP1 receptor to suppress CNTF-mRNA in the cultured astrocytes from olfactory bulb and cerebral cortex, respectively. It is important to clarify whether these neuropeptides and their receptors are involved in the regulation of CNTF expression in vivo. Especially, it is interesting to examine whether changes in the levels of these neuropeptides in brain during development and injury correlate with those of CNTF expression.

Intracellular mechanism in which activation of adenylyl cyclase leads to the suppression of CNTF-mRNA levels seems to be complex. It is generally accepted that cAMP exerts its cellular effects mostly via activation of cAMP-dependent protein kinase (PKA) [27] . However, elucidation of the roles of PKA in the suppression of CNTF-mRNA is ambiguous. Treatment of the cultured astrocytes with H-89, a selective inhibitor of PKA [28], as well as H-85, an analog of H-89 which is devoid of the action on PKA [28], did not reverse the forskolin-induced suppression of CNTF-mRNA (data not shown). Furthermore, H-89 and H-85 themselves decreased the CNTF-mRNA levels to 30.3 and 61.5% of original level, respectively with the 9 h-incubation. We also found that other protein kinase inhibitors such as H-7 and staurosporine also decreased the CNTF-mRNA levels (data not shown). Complex cascades of protein phosphorylation seem to be involved in the cAMP-induced up-regulation of CNTF-mRNA expression as well as in its basal expression.

The transient nature of the effect of VIP and PACAPs might have resulted from desensitization processes between activations of the receptor and adenylyl cyclase while forskolin activated adenylyl cyclase persistently to induce long lasting suppression of CNTF-mRNA level. Alternatively, neuropeptides added to the culture medium might have been degraded by proteases on the surface of astrocytes. Interestingly, removal of forskolin from the culture medium of astrocytes also quickly recovered the expression of CNTF-mRNA indicating the reversible nature of the action of forskolin (data not shown). Hence, it is suggested that persistent activation of adenylyl cyclase is required for the suppression of CNTF-mRNA expression.

Our experiments suggest that elevated cAMP induces the inhibition of transcription of CNTF gene as a major pathway to quench the CNTF-mRNA level. However, it is also suggested that the promotion of degradation of CNTF-mRNA by forskolin contributes subsidiary. Since, stably and strongly expressed CNTF-mRNA has longer half life ($t_{1/2}$ = 7 h) compared with that of NGF-mRNA ($t_{1/2}$ = 0.67 h [11]), it is reasonable to utilize the mRNA destabilizing machinery in addition to transcriptional inhibition to quench the CNTF-mRNA expression rapidly in physiological events. The effects of cycloheximide on the CNTF-mRNA level suggested that the inhibition of CNTF transcription did not require de novo protein synthesis and was evoked by post translational modification of constitutively expressed transcription factor. Future analysis of the sequence elements on the promoter region of CNTF gene responsible for the action of cAMP would reveal the identity of transcription factors regulating CNTF gene expression.

Regulatory mechanisms of CNTF-mRNA expression in the CNS and PNS in vivo are largely unknown. In the peripheral nervous system, after transection or crush of the sciatic nerve, CNTF immunoreactivity and CNTF-mRNA levels expressed

in Schwann cells fall dramatically while expressions of NGF immunoreactivity and NGF-mRNA in Schwann cells are induced dramatically [29–32]. Expression of CNTF is regained when the nerve is regenerated [31,33]. Therefore, CNTF is thought to be a trophic factor for the intact sciatic nerve and may function also as a lesion factor to exert acute trophic effects when released from Schwann cells in response to injury, while NGF released from Schwann cells is thought to promote the subsequent nerve regeneration.

But, the regulatory mechanisms of the CNTF expression in CNS and PNS may be different. Recently it is reported that after injury to cerebral cortex and hipocampus where basal level of CNTF-mRNA is very low, astrocytes are activated to proliferate to fill in the lesioned sites and increase the expression of CNTF-mRNA concomitantly with expression of NGF-mRNA [34]. Hence, it is suggested that astrocytes in general express very low level of CNTF-mRNA in differentiated or resting state while they express high level of CNTF-mRNA upon transfer to culture or after injury. In this context, the high level of CNTF-mRNA in olfactory bulb in vivo could be a reflection of the presence of astrocytes around glomerulli activated by the continuous degeneration of olfactory nerve. The remarkable difference between the CNTF-mRNA levels in the cultured cerebral cortex astrocytes and cerebral cortex in vivo could be explained accordingly. PACAP, VIP and other cAMP-linked agonists which are released from olfactory nerve or certain type of cells in olfactory bulb may convey the signal of olfactory nerve degeneration or regeneration upon astrocytes to alter the CNTF and NGF expressions. In conclusion, we demonstrated the high level of CNTF expression in the cultured brain astrocytes and analyzed its cAMP-dependent regulatory mechanism which we hope applicable to the in vivo regulation of this unique neurotrophic factor in the olfactory system.

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