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Short communication

Ciliary neurotrophic factor receptor α mRNA in NB41A3 neuroblastoma cells: regulation by cAMP

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Abstract

Ciliary neurotrophic factor (CNTF) affects the in vitro and in vivo survival and differentiation of several classes of neurons by binding to the CNTF receptor α. We examined the possibility that intracellular cAMP can regulate CNTF receptor α mRNA levels in two neuronal cell lines that display cAMP-dependent process outgrowth. Dibutyryl cAMP did not affect CNTF receptor α mRNA levels in PC12 cells but elicited a dose- and time-dependent increase in NB41A3 cell CNTF receptor α mRNA levels. Forskolin similarly increased CNTF receptor α mRNA levels in NB41A3 cells. The data suggest that signal transduction mechanisms involving cAMP may 'crosstalk' with CNTF-initiated signal transduction in a cell type-specific manner and that CNTF receptor α expression is not generally linked to neuronal process outgrowth.

Keywords: CNTF (ciliary neurotrophic factor receptor α); cAMP; NB41A3 cell; PC12 cell; mRNA

1. Introduction

Ciliary neurotrophic factor (CNTF) influences the in vitro survival, differentiation and proliferation of a wide variety of cell types (Louis et al., 1993; Magal et al., 1993; and for review, Richardson, 1994). In vivo, CNTF reduces the naturally occurring developmentally associated death of spinal motorneurons, and decreases the axotomy-induced death of neurons in the facial nucleus, thalamus, medial septum, and substantia nigra (Richardson, 1994, for review). In addition, CNTF administration has been shown to slow the development of behavioral and anatomical defects associated with three genetic mouse models of human motor disease (Lindsay, 1994, for review) and arrest the development of these symptoms in one model when co-administered with brain-derived neurotrophic factor (Mitsumoto et al., 1994). Together, the above data suggest that endogenous CNTF may participate in the development and maintenance of neurons while exogenously administered CNTF may serve a therapeutic role in the treatment of conditions involving neuronal loss.

Molecular cloning and heterologous expression studies indicate that CNTF produces most, and possibly all, of its effects by binding to a protein referred to as CNTF receptor α (Stahl and Yancopoulos, 1994, for review). The CNTF-CNTF receptor α complex, when combined with two transmembrane proteins, leukemia inhibitory growth factor receptor β (LIFRβ) and gp130, initiates a series of tyrosine phosphorylations which ultimately regulate cellular gene expression (Stahl and Yancopoulos, 1994). When CNTF receptor α is expressed by the same cells as LIFRβ and gp130, the three proteins form a 'tripartite' receptor complex with CNTF receptor α anchored to the extracellular surface of the cellular membrane by a glycosyl-phosphatidylinositol linkage. In this case extracellular CNTF binds to the complex to activate it. However, CNTF receptor α can also be released from the membrane by phosphatidylinositol-specific phospholipase C digestion, bind to CNTF with the same affinity as it does when combined with LIFRβ and gp130, and initiate CNTF signal transduction events in cells that express LIFRβ and gp130 but otherwise lack CNTF receptor α (Gimble et al., 1994; Panayotatos et al., 1994; Stahl and Yancopoulos, 1994). Furthermore, studies suggest that this soluble form of CNTF receptor α is produced and active in vivo (Stahl and Yancopoulos, 1994).

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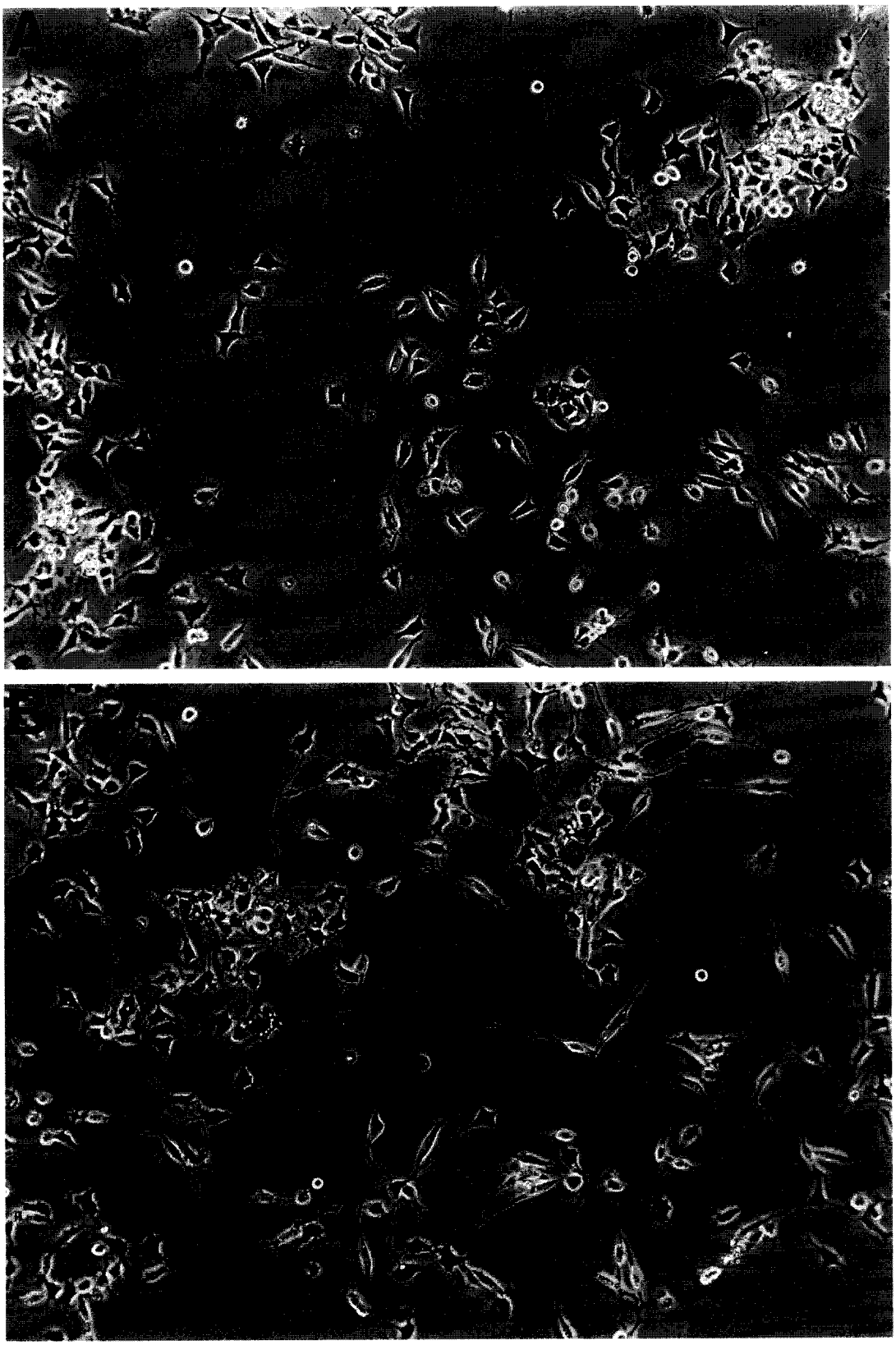

In summary, most if not all the in vivo effects of endogenous and exogenous CNTF are dependent on CNTF receptor α expression. As part of an effort to explore how other signal transduction mechanisms may modulate CNTF-initiated signal transduction, the experiments presented here were designed to determine whether cellular CNTF receptor α mRNA concentrations can be regulated by changes in intracellular cAMP concentrations. The potential role of cAMP was studied because this second messenger participates in many forms of growth factor and neurotransmitter-initiated signal transduction. We report that CNTF receptor α mRNA levels are increased in NB41A3 mouse neuroblastoma cells following their treatment with either the stable, cell-permeable cAMP analogue, dibutyryl cAMP, or the adenylate cyclase activator, forskolin. The data raise the possibility that a wide variety of growth factors and neurotransmitters may regulate CNTF responses through cAMP-mediated changes in CNTF receptor α expression.

2. Materials and methods

2.1. Materials

All materials used in cell culture were purchased from Gibco/BRL with the following exceptions: dibutyryl cAMP, forskolin, penicillin, streptomycin and collagen were from Sigma and all plates and disposable plasticware were from Fisher. The random hexamer priming oligolabelling kit from Pharmacia was used with [α-^{32}P]deoxycytidine 5′-triphosphate purchased from Amersham. Biotrans membranes were purchased from ICN. The cyclophilin cDNA was a gift from Dr. J. Gregor Sutcliffe (Scripps Clinic, La Jolla, CA, USA). The c-fos clone was purchased from ATCC (Cat. No. 41041) and the CNTF was a gift from Regeneron Pharmaceuticals.

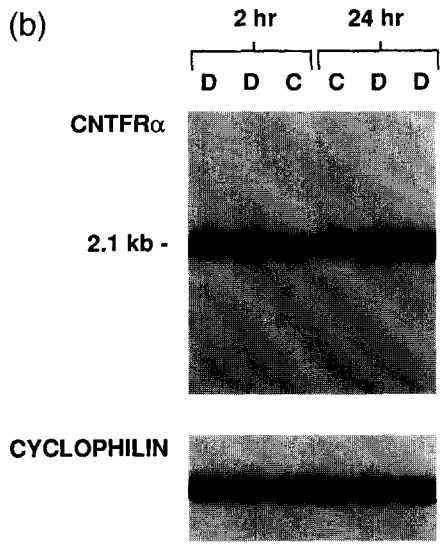

Fig. 1. (a) Increase in NB41A3 cell process outgrowth elicited by treatment with dibutyryl cAMP. (A) NB41A3 cells treated with vehicle for 24 h. (B) NB41A3 cells treated with 1.0 mM dibutyryl cAMP for 24 h. Scale bar equals 100 μm. (b) Northern blot analysis of CNTF receptor α mRNA in NB41A3 cells following dibutyryl cAMP treatment. Cells were treated with vehicle (C) or 1.0 mM dibutyryl cAMP (D) for 2 h or 24 h and then extracted for analysis. The blots were probed with a CNTF receptor α cDNA (upper panel), stripped and then probed with a cyclophilin cDNA (lower panel) to correct for any intersample variability in extraction, loading, or transfer.

2.2. Cell culture and treatment

NB41A3 mouse neuroblastoma cells were grown on standard tissue culture plates in Dulbecco's modified Eagle medium containing 10% fetal bovine serum. PC12 rat pheochromocytoma cells were grown on collagen-coated plates in RPMI medium containing 10% horse serum and 5% fetal bovine serum. All media were also supplemented with 1 unit/ml penicillin and 1 μg/ml streptomycin.

Cells were treated with dibutyryl cAMP or forskolin by adding freshly dissolved drug to plates of approximately 50% confluent cells. Controls were treated in parallel with an equal volume of vehicle and medium. All cells in each experiment were plated from a single pool of cells.

2.3. Northern blot analyses

After treatment, cells were washed with 1 × phosphate-buffered saline (PBS), pH 7.4 while anchored to plates. They were then mechanically dislodged and collected by centrifugation, frozen in liquid nitrogen and stored at −80°C. Poly A RNA was extracted with the FastTrack kit (Invitrogen Corp.) and quantities were estimated spectrophotometrically from absorbance at 260 nm.

To construct Northern blots, poly A RNA (5–15 μg per lane) was denatured in 50% deionized formamide, 6.0% formaldehyde at 65°C for 5 min and then size-fractionated by electrophoresis on a horizontal agarose gel (1.25%) containing 6.0% formaldehyde. The RNA was subsequently transferred to nylon Biotrans membranes, which were then dried and baked at 80°C for 2 h under vacuum. Membranes were incubated for 2 h at 42°C in 5 × standard saline citrate buffer (SSC), 50% formamide, 0.5% sodium dodecyl sulfate (SDS), 50 mM sodium phosphate (pH 6.5), 250 μg/ml denatured salmon sperm DNA, 5 × Denhardt's solution, and 100 μg/ml polyadenylic acid. The CNTF receptor α cDNA probe (MacLennan et al., 1994) (purified on two consecutive agarose gels and labeled with ^{32}P by random hexamer priming) was then added to the solution. After incubation at 42°C overnight, the membranes

were washed twice for 30 min at room temperature in 2 × SSC and twice for 45 min at 60°C in 0.1 × SSC, 0.1% SDS.

Membranes were exposed to X-ray film with two intensifying screens at −80°C for various lengths of time. The probe was subsequently removed from the membranes by washing at 65°C in 50% formamide, 10 mM sodium phosphate, pH 6.5, for 1 h. The membranes were then probed with rat cyclophilin cDNA, 1B15 (Danielson et al., 1988), to correct for any inter-sample variability in extraction, loading or transfer of RNA. Densitometric analyses of the resulting autoradiographs were performed on a Microscan 1000 Gel Analyzer (Technology Resources) by individuals unaware of the experimental design. The resulting data were statistically evaluated with Fisher's protected least significant differences post-hoc analysis.

The c-fos Northern blots were constructed and probed following procedures identical to those employed for the CNTF receptor α Northerns. The cells were exposed to a saturating concentration (25 ng/ml) of CNTF or vehicle for 30 min prior to harvest.

2.4. Phosphotyrosine immunoprecipitation and Western blot analysis

Cells were treated with CNTF, lysed, immunoprecipitated with anti-phosphotyrosine antibodies and assayed by Western blot analysis for proteins containing phosphotyrosine. Previously published methods (Boulton et al., 1994) were employed for the entire procedure.

3. Results

In our initial experiments we treated NB41A3 mouse neuroblastoma cells for 2 h or 24 h with the cell-permeable cAMP analogue, dibutyryl cAMP, at a concentration of 1.0 mM. This treatment produces a reliable increase in process formation by NB41A3 cells that is apparent by 24 h of treatment (Fig. 1a). Poly A RNA extracted from the treated cells and controls was used to construct a Northern blot which was subsequently probed with a rat CNTF receptor α cDNA (MacLennan et al., 1994). In agreement with our previous work

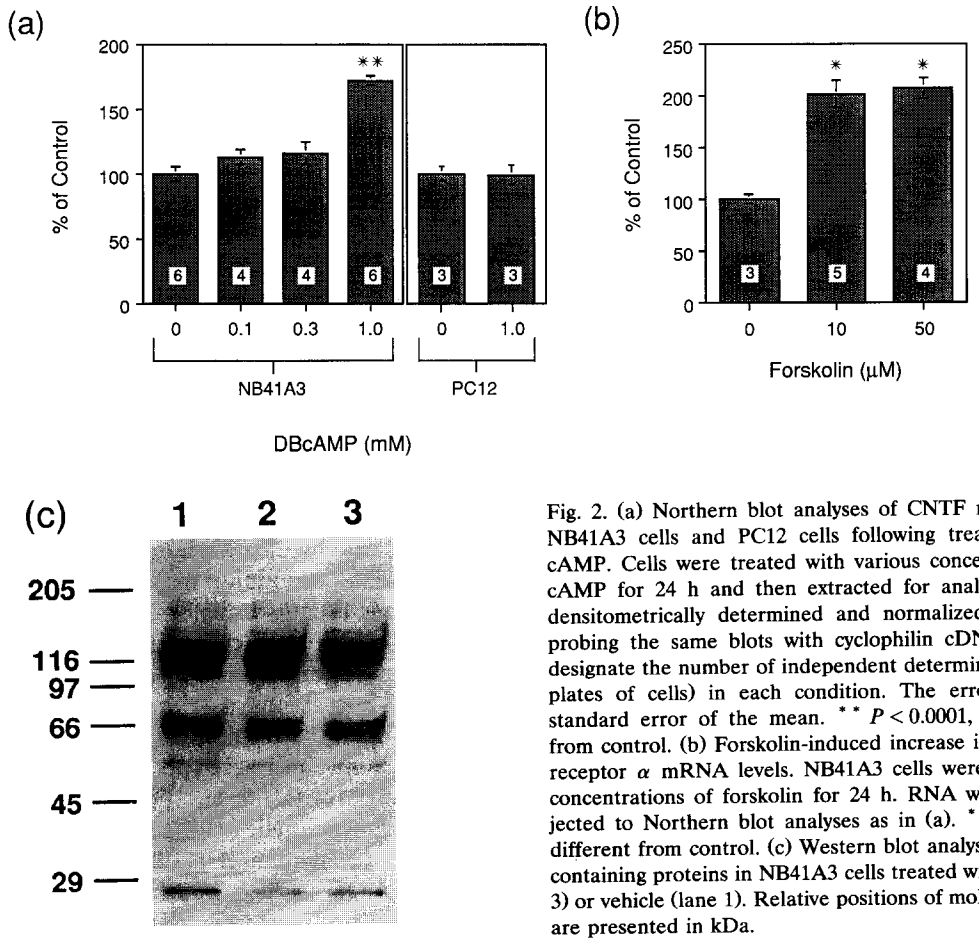

Fig. 2. (a) Northern blot analyses of CNTF receptor α mRNA in NB41A3 cells and PC12 cells following treatment with dibutyryl cAMP. Cells were treated with various concentrations of dibutyryl cAMP for 24 h and then extracted for analyses. All values were densitometrically determined and normalized with the results of probing the same blots with cyclophilin cDNA. Numbers in bars designate the number of independent determinations (from separate plates of cells) in each condition. The error bars illustrate the standard error of the mean. ** $P < 0.0001$, significantly different from control. (b) Forskolin-induced increase in NB41A3 cell CNTF receptor α mRNA levels. NB41A3 cells were treated with various concentrations of forskolin for 24 h. RNA was extracted and subjected to Northern blot analyses as in (a). * $P < 0.02$, significantly different from control. (c) Western blot analyses of phosphotyrosine containing proteins in NB41A3 cells treated with CNTF (lanes 2 and 3) or vehicle (lane 1). Relative positions of molecular weight markers are presented in kDa.

(MacLennan et al., 1994), the CNTF receptor α cDNA detected a single ~2.1 kb CNTF receptor α transcript in NB41A3 cell RNA (Fig. 1b). Densitometric analysis of the results indicated that, in the two independent determinations conducted, 2 h of dibutyryl cAMP exposure led to a 28–38% increase in CNTF receptor α mRNA concentrations over control values while 24 h of exposure led to a 72–82% increase (Fig. 1b). In additional experiments NB41A3 CNTF receptor α mRNA concentrations returned to control levels by 3 days of continuous dibutyryl cAMP treatment (data not shown).

Like NB41A3 cells, PC12 cells are a neuronal model line that express CNTF receptor α mRNA (MacLennan et al., 1994) and display several dibutyryl cAMP-elicited responses including increased process outgrowth (Schubert and Whitlock, 1977; Gunning et al., 1981). We confirmed that our PC12 cells display the increased process outgrowth response with a similar time course to that observed with NB41A3 cells (data not shown). CNTF receptor α mRNA expression was then examined in PC12 cells treated with dibutyryl cAMP. The procedures employed were identical to those utilized in the NB41A3 cell experiment. The results indicate that the two cell lines display contrasting regulation of CNTF receptor α mRNA, in that dibutyryl cAMP treatment did not affect CNTF receptor α mRNA expression in PC12 cells (Fig. 2a).

The effect of dibutyryl cAMP on NB41A3 cell CNTF receptor α mRNA levels was further characterized by exposing the cells to various concentrations of dibutyryl cAMP or vehicle for 24 h. RNA was extracted and Northern blots were constructed and probed as in our other experiments. Both 0.1 mM and 0.3 mM dibutyryl cAMP treatments elicited 10–20%, statistically insignificant, increases in CNTF receptor α mRNA whereas 1.0 mM dibutyryl cAMP produced a significant 72% increase (Fig. 2a).

CNTF receptor α mRNA levels were also measured in NB41A3 cells challenged with forskolin, a direct activator of adenylate cyclase. The cells were treated for 24 h with either vehicle or forskolin at either 10 μM or 50 μM. Northern blot analyses indicated that forskolin at both 10 μM and 50 μM produced a greater than 100% increase in NB41A3 cell CNTF receptor α mRNA levels over control values (Fig. 2b).

Neither NB41A3 cells nor PC12 cells displayed a CNTF-elicited induction in c-fos mRNA expression (data not shown). CNTF treatment also failed to alter the population of NB41A3 cell proteins that contain phosphotyrosine (Fig. 2c).

4. Discussion

The increase in NB41A3 cell CNTF receptor α mRNA levels observed following treatment with the cAMP analogue, dibutyryl cAMP, strongly suggests that CNTF receptor α mRNA levels can be regulated by intracellular cAMP concentrations. This possibility is further supported by the data demonstrating that NB41A3 CNTF receptor α mRNA concentrations are similarly increased by forskolin, a potent activator of the intracellular enzyme that synthesizes cAMP.

In addition to its effect on CNTF receptor α mRNA, dibutyryl cAMP treatment elicits an increase in NB41A3 cell process outgrowth. However, our PC12 cell data indicate that CNTF receptor α mRNA levels do not rise as a general consequence of cAMP-induced neuronal process outgrowth. Thus, PC12 cell CNTF receptor α mRNA levels are unaffected during a similar dibutyryl cAMP-induced differentiation. Instead, it appears that cAMP regulation of CNTF receptor α mRNA is a cell type-dependent phenomenon as has been reported for other effects of cAMP (Dumont et al., 1989). As such this regulation is unlikely to directly involve transcription initiated by the constitutively expressed cAMP response element binding protein (CREB). The gradual nature of the CNTF receptor α mRNA induction is also consistent with an indirect regulation of CNTF receptor α mRNA by cAMP given that gradual induction is typical of cAMP-induced gene expression that is not the direct consequence of cAMP response element (CRE)-dependent transcription (Lemke, 1992).

The presently reported PC12 cell data also relate to our previous work demonstrating that CNTF receptor α mRNA levels are reduced in PC12 cells as these cells grow processes in response to nerve growth factor (NGF) (MacLennan et al., 1994). The data indicating that dibutyryl cAMP does not affect PC12 cell CNTF receptor α mRNA levels while it elicits process outgrowth similar to that observed with NGF treatment suggest that the NGF effect on PC12 cell CNTF receptor α mRNA is not a secondary result of PC12 cell process outgrowth. These data also suggest that NGF, which has been reported to increase PC12 cell cAMP levels (Schubert and Whitlock, 1977), regulates PC12 cell CNTF receptor α mRNA levels through signal transduction events independent of cAMP. In summary, CNTF receptor α mRNA levels can be upregulated, as in the case of cAMP treatment of NB41A3 cells, and downregulated, as with NGF treatment of PC12 cells, through mechanisms that appear to be dependent on the cell type and differentiating agent involved and independent of neuronal process outgrowth in general.

Our Northern and Western blot studies indicate that NB41A3 cells do not contain increased concentrations of c-fos mRNA or tyrosine phosphorylated proteins following CNTF exposure, thereby suggesting that these cells lack some component of currently recognized CNTF signal transduction mechanisms. How-

ever, the present data demonstrate that changes in intracellular cAMP concentrations can lead to changes in CNTF receptor α mRNA concentrations. This interaction raises the possibility that a similar in vivo interaction in CNTF-responsive cells may provide a means by which signal transduction processes that involve intracellular cAMP can 'crosstalk' with CNTF receptor α-mediated signal transduction by regulating the synthesis of CNTF receptor α. Furthermore, as mentioned in the Introduction, several recent reports indicate that, following its enzymatic cleavage from the outer surface of cell membranes, CNTF receptor α can combine with the LIFRβ and gp130 of cells that do not express CNTF receptor α, and by doing so make the cells responsive to CNTF (Gimble et al., 1994; Panayotatos et al., 1994; Stahl and Yancopoulos, 1994). Therefore, in vivo cells, analogous to NB41A3 cells, which express CNTF receptor α but do not themselves respond to CNTF, could nevertheless play an important role in CNTF signal transduction by supplying soluble CNTF receptor α which functionally interacts with other cells. Thus, cAMP regulation of CNTF receptor α expression in such cells may play a significant role in determining the in vivo effects of CNTF.

Finally, it is interesting to note that the differentiating effects of CNTF on noradrenergic (Louis et al., 1993) and dopaminergic (Magal et al., 1993) neurons in primary cultures are modulated by noradrenaline and dopamine respectively. Given that both of these neurotransmitters can regulate intracellular cAMP concentrations, it is tempting to speculate that they may at least partially modulate the effects of CNTF by regulating CNTF receptor α expression through cAMP.

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References

Boulton, T.G., N. Stahl and G.D. Yancopoulos, 1994, Ciliary neurotrophic factor/leukemia inhibitory factor/interleukin 6/oncostatin M family of cytokines induces tyrosine phosphorylation of a common set of proteins overlapping those induced by other cytokines and growth factors, J. Biol. Chem. 269, 11648.

Danielson, P.E., S. Forss-Petter, M.A. Brow, L. Calavetta and J. Douglass, 1988, p1B15: a cDNA clone of the rat mRNA encoding cyclophilin, DNA 7, 261.

Dumont, J.E., J.-C. Jauniaux and P.P. Roger, 1989, The cyclic AMP-mediated stimulation of cell proliferation, Trends Biochem. Sci. 14, 67.

Gimble, J.M., F. Wanker, C.-S. Wang, H. Bass, X. Wu, K. Kelly, G.D. Yancopoulos and M.R. Hill, 1994, Regulation of bone marrow stromal cell differentiation by cytokines whose receptors share the gp130 protein, J. Cell. Biochem. 54, 122.

Gunning, P.W., G.E. Landreth, M.A. Bothwell and E.M. Shooter, 1981, Differential and synergistic actions of nerve growth factor and cyclic AMP in PC12 cells, J. Cell Biol. 89, 240.

Lemke, G., 1992, Gene regulation in the nervous system, in: An Introduction to Molecular Neurobiology, ed. Z.W. Hall (Sinauer Associates, Sunderland, MA) p. 313.

Lindsay, R.M., 1994, Neurotrophic growth factors and neurodegenerative diseases: therapeutic potential of the neurotrophins and ciliary neurotrophic factor, Neurobiol. Aging 15, 249.

Louis, J.-C., E. Magal, P. Burnham and S. Varon, 1993, Cooperative effects of ciliary neurotrophic factor and norepinephrine on tyrosine hydroxylase expression in cultured rat locus coeruleus neurons, Dev. Biol. 155, 1.

MacLennan, A.J., A.A. Gaskin and D.C. Lado, 1994, CNTF receptor α mRNA expression in rodent cell lines and developing rat, Mol. Brain Res. 25, 251.

Magal, E., P. Burnham, S. Varon and J.-C. Louis, 1993, Convergent regulation by ciliary neurotrophic factor and dopamine of tyrosine hydroxylase expression in cultures of rat substantia nigra, Neuroscience 52, 867.

Mitsumoto, H., K. Ikeda, B. Klinkosz, J.M. Cedarbaum, V. Wong and R.M. Lindsay, 1994, Arrest of motor neuron disease in wobbler mice cotreated with CNTF and BDNF, Science 265, 1107.

Panayotatos, N., D. Everdeen, A. Liten, R. Somogyi and A. Acheson, 1994, Recombinant human CNTF receptor α: production, binding stoichiometry, and characterization of its activity as a diffusible factor, Biochemistry 33, 5813.

Richardson, P.M., 1994, Ciliary neurotrophic factor: a review, Pharmacol. Ther. 63, 187.

Schubert, D. and C. Whitlock, 1977, Alteration of cellular adhesion by nerve growth factor, Proc. Natl. Acad. Sci. USA 74, 4055.

Stahl, N. and G.D. Yancopoulos, 1994, The tripartite CNTF receptor complex: activation and signaling involves components shared with other cytokines, J. Neurobiol. 25, 1454.