Activation of TrkA by nerve growth factor upregulates expression of the cholinergic gene locus but attenuates the response to ciliary neurotrophic growth factor

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Nerve growth factor (NGF) stimulates the expression of the cholinergic gene locus, which encodes choline acetyltransferase (ChAT) and vesicular acetylcholine transporter (VAChT), the proteins necessary for the synthesis and storage of the neurotransmitter acetylcholine (ACh). To determine whether this action of NGF is mediated by the p140TrkA NGF receptor (a member of the Trk tyrosine kinase family) we used a murine basal forebrain cholinergic cell line, SN56, stably transfected with rat *trkA* cDNA. Treatment of these transfectants with NGF activated mitogen-activated protein kinase and increased cytosolic free calcium concentrations, confirming the reconstitution of TrkA-mediated signalling pathways. The expression of ChAT and VAChT mRNA, as well as ACh content, were coordinately up-regulated by NGF in SN56-*trkA* transfectants. None of these responses occurred in the parental SN56 cells, which do not express endogenous TrkA, indicating that these actions of NGF required TrkA. We previously reported that ciliary neurotrophic factor (CNTF) upregulates the expression of ChAT and VAChT, as well as ACh production, in SN56 cells. The combined treatment of SN56-*trk*A cells with CNTF and NGF revealed a complex interaction of these factors in the regulation of cholinergic gene locus expression. At low concentrations of CNTF (< 1 ng/ml), the upregulation of ACh synthesis evoked by these factors was additive. However, at higher concentrations of CNTF (> 1 ng/ml), NGF attenuated the stimulatory effect of CNTF on ChAT and VAChT mRNA and ACh content. This attenuation was not due to interference with early steps of CNTF receptor signalling, as pre-treatment of SN56-*trk*A cells with NGF did not affect the nuclear translocation of the transcription factor, Stat3, evoked by CNTF.

Key words: acetylcholine, choline acetyltransferase, mitogen-activated protein kinase, Stat3, vesicular acetylcholine transporter.

INTRODUCTION

The synthesis and storage of the neurotransmitter acetylcholine (ACh) depends upon the co-expression of two proteins, choline acetyltransferase (ChAT) [1] and vesicular acetylcholine transporter (VAChT), which transports ACh into secretory vesicles [2]. The genes coding for these two proteins are closely linked, with the VAChT open reading frame contained within the first intron of the ChAT gene [3–5]. The two genes share some regulatory promoter sequences and non-coding exons (reviewed by Eiden [6]). Of the two, ChAT has been studied more extensively, and most information on the regulation of the cholinergic phenotype is derived from studies on ChAT activity and expression [1,6]. In contrast, since molecular tools to study VAChT have only recently been developed, less is known about the regulation of VAChT expression and/or activity [6]. In the majority of experimental paradigms applied so far, the two genes are coordinately regulated [7–10].

Among extracellular factors that regulate the cholinergic phenotype and exert trophic effects on cholinergic neurons the best characterized are nerve growth factor (NGF), leukaemia inhibitory factor (LIF) and ciliary neurotrophic factor (CNTF). LIF and CNTF signal through the same pathway, involving the gp130 receptor subunit. We and others have shown that LIF and CNTF upregulate ChAT and VAChT expression in a coordinated fashion [7,8]. NGF was found to support cholinergic physiological functions both *in vitro* and *in vivo* [11–13] and recent studies have shown that NGF upregulates the expression of both ChAT and VAChT genes [14–17]. NGF interacts with two receptors, the low-affinity neurotrophin receptor p75, and the high-affinity receptor p140TrkA, which is a member of the Trk tyrosine kinase family. Binding of neurotrophins to Trks results in receptor dimerization, followed by a series of tyrosine phosphorylation events, including autophosphorylation of the receptor. Whereas the signal-transduction pathways activated by Trks have been well characterized [18,19], the biological role of, and the signalling pathways utilized by p75 are less clear. In some experimental paradigms this receptor was found to enhance TrkA signalling [20] and was also postulated to play a role in apoptosis [20]. There is a growing body of indirect evidence suggesting that the TrkA signalling pathway is responsible for the upregulation of ChAT and VAChT. Numerous studies have documented the co-localization of TrkA and ChAT/VAChT mRNAs and proteins in the brain, and specifically in basal forebrain cholinergic neurons [14,21]. Moreover, it has been shown that the elements of the TrkA signalling pathway can be activated by exogenous NGF in those cells [14]. Xu et al. [22] showed that viral transduction of *trk*A into spinal motor neurons

Abbreviations used: ACh, acetylcholine; AP-1, activator protein 1; $[Ca^{2+}]_i$, cytosolic free calcium concentration; ChAT, choline acetyltransferase; CNTF, ciliary neurotrophic factor; CNTFRE, CNTF response element; CRE, cAMP response element; CREB, cAMP response element-binding protein; DMEM, Dulbecco's modified Eagle's medium; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; JAK, Janus kinase; LIF, leukaemia inhibitory factor; MAP kinase, mitogen-activated protein kinase; NGF, nerve growth factor; NP40, Nonidet P40; N-Shc, neuronal Shc; PKA, cAMP-activated protein kinase; STAT, signal transducer and activator of transcription; VAChT, vesicular acetylcholine transporter.

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(which do not express endogenous TrkA and are not supported by NGF) allowed for NGF-mediated neuronal survival [22]. Moreover, the surviving cells expressed ChAT, suggesting the possibility that TrkA, in addition to its trophic actions, may regulate ChAT expression. More recently, Debeir et al. [23,24] demonstrated that a small peptide that blocks NGF–TrkA interaction is capable of decreasing NGF-induced ChAT activity in septal cultures and promoting the loss of VAChT-immunoreactive cholinergic boutons *in vivo*. There has been no direct evidence, however, that the upregulation of ChAT/VAChT gene expression is mediated by TrkA.

We have previously shown, using the mouse cholinergic cell line SN56, that the modulation of ChAT/VAChT expression by retinoids, glucocorticoids and cytokines (including LIF and CNTF), results in changes in ACh production [8,9]. SN56 cells are derived from basal forebrain and have many features characteristic of septal cholinergic neurons [25,26], including the expression of p75 (Dr. Bruce Wainer, personal communication). However, ChAT expression does not change in SN56 cells treated with NGF ([25], this paper), and immunoblotting analysis indicates that SN56 cells do not express the TrkA receptor (see below). In the present report, we describe the construction of SN56 derivatives expressing TrkA. The results demonstrate that these cell lines are suitable for studying NGF–TrkA signalling. We present evidence that signalling through TrkA is responsible for NGF-mediated upregulation of the cholinergic locus expression. Further, we analyse the NGF–CNTF interaction in regulating the cholinergic phenotype, and demonstrate that when both NGF and CNTF are present at maximally effective concentrations, their actions are antagonistic, rather than additive.

MATERIALS AND METHODS

Cell culture and treatments

Mouse septal neuron × neuroblastoma hybrids SN56 cells (a gift from Dr. Bruce Wainer, Department of Pathology and Laboratory Medicine, Emory University School of Medicine, Atlanta, GA, U.S.A.) were created by fusing N18TG2 neuroblastoma cells with murine (strain C57BL/6) neurons from postnatal day 21 septa [25,27]. SN56 cells were maintained at 37 °C in an atmosphere of 95% air/5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) containing 1 mM pyruvate and 10% (v/v) fetal bovine serum (FBS). For analysis of protein phosphorylation and nuclear translocation, the cells were temporarily (up to 12 h) maintained in the same medium without serum, and then stimulated briefly with test compounds, i.e. CNTF or NGF. When the cells were grown in the presence of test compounds (growth factors or forskolin) for extended periods of time (days), the medium contained the N2 supplement [28] (Gibco BRL) instead of FBS and was changed every 24 h.

Transfection and selection with G418

The plasmid pDM115, containing the rat *trk*A cDNA under the control of the murine sarcoma virus long terminal repeat promoter, was a gift from Dr. Moses Chao (Cornell University Medical College, New York, NY, U.S.A., now at Skirball Institute of Biomolecular Medicine, New York University Medical Center, New York, NY, U.S.A.). The plasmid contains the neomycin resistance gene under the control of the simian virus 40 promoter, for selection with geneticin (G418). SN56 cells were transfected with pDM115 using Lipofectamine reagent (Gibco BRL), according to the manufacturer's instructions, and then allowed to recover for 2 days before they were diluted 1:10 and subjected to selection in medium containing 300 μg/ml G418. The selective medium was replaced every 2 days and G418-resistant colonies were isolated 14–21 days after transfection. The cell lines were maintained in medium with G418 and screened for TrkA expression by Western blotting.

Northern analysis

Total RNA was extracted from cells using the acid guanidinium isothiocyanate–phenol–chloroform method [29,30]. RNA samples equalized for ribosomal RNA content (20 μg per lane) were size-fractionated on an agarose/formaldehyde gel and transferred to a Hybond-N+ nylon membrane (Amersham). Hybridization was carried out in the Rapid-hyb solution (Amersham), according to manufacturer's instructions. DNA probes derived from the coding regions of VAChT and ChAT were as described previously [8,9]. The probes were labelled with [α-^{32}P]dCTP (New England Nuclear) to a specific activity of $(1–2) \times 10^9$ c.p.m./μg of DNA using a Rediprime labelling kit (Amersham). Final washes were in 0.2 × SSC/0.1% SDS (where SSC is 0.15 M NaCl/0.015 M sodium citrate) at 60 °C for ChAT and at 65 °C for VAChT. The blots were visualized and band intensities were quantified with a PhosphorImager 400E using ImageQuant NT software (Molecular Dynamics).

Western blotting and immunoprecipitation

For Western blot analysis, protein from whole cells was extracted with lysis buffer [50 mM Tris, pH 7.5, 150 mM NaCl, 1% (v/v) Nonidet P40 (NP40), 2 mM 4-(2-aminoethyl)benzenesulphonyl fluoride, 1 μg/ml leupeptin, 2 μg/ml aprotinin, 2 μg/ml pepstatin and 1 mM sodium orthovanadate], vortex mixed vigorously, incubated on ice for 15 min and cleared by brief centrifugation. For analysis of Stat3 translocation to the nucleus, nuclear extracts were prepared by gentle lysis with 0.6% NP40 followed by high-salt extraction according to the method of Schreiber et al. [31]. The extracts, normalized for total protein content, were separated by SDS/PAGE and transferred to Immobilon P membrane (Millipore). The following primary antibodies were used: anti-Trk (sc-11; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), anti-phosphotyrosine (RC-20, Transduction Laboratories, Lexington, KY, U.S.A.), a polyclonal anti-Shc antibody (Upstate Biotechnology, Lake Placid, NY, U.S.A), a monoclonal anti-Shc antibody (Transduction Laboratories), anti-Stat3 (Santa Cruz Biotechnology) and an antibody specific for the phosphorylated forms of mitogen-activated protein kinase (MAP kinase) (New England Biolabs, Beverly, MA, U.S.A). The antibodies were diluted as recommended by the manufacturers. After the membrane was probed with the primary antibody, the antibody–antigen complexes were detected with either anti-rabbit IgG or anti-mouse IgG peroxidase conjugates, and visualized using the chemiluminescence method (Renaissance reagent, DuPont NEN) and Kodak X-Omat AR film.

For immunoprecipitation, whole cell extracts were prepared as described above and incubated overnight with the immunoprecipitating antibody (anti-Shc, at 5 μg/500 μg of extract) and with protein A–Sepharose (Oncogene Science, 3.5 mg per sample), centrifuged and washed three times with a buffer containing 0.1% (v/v) Triton X-100, 25 mM Tris pH 7.5, 250 mM NaCl and 1 mM sodium orthovanadate. Immunoprecipitates were then subjected to Western blotting as described above.

ACh measurements

Cells were grown to subconfluence in 35-mm culture dishes. After the desired treatment, the medium was removed and the

cells were incubated for 1 h at 37 °C in a physiological salt solution (pH 7.4) supplemented with 5 μM choline and 15 μM neostigmine and consisting of the following (in mM): NaCl, 135; KCl, 5; $CaCl_2$, 1; $MgCl_2$, 0.75; glucose, 10; Hepes, 10. The cells were washed once with ice-cold, choline-free physiological salt solution supplemented with 15 μM neostigmine. The cells were scraped into methanol and ACh was extracted using methanol/1 M formic acid/chloroform/water (1:0.1:2:1, by vol.). The samples were vortex mixed and centrifuged for 10 min at 6000 rev./min. (10000 ***g***) The aqueous phase (containing ACh) and the protein interphase were collected and dried under vacuum. ACh was determined by HPLC with an enzymic reactor containing acetylcholinesterase and choline oxidase and an electrochemical detector, using a commercial kit (Bioanalytical Systems, Inc.) based on the method of Potter et al. [32] as described previously [8]. Protein was determined by the method of Smith et al [33].

Cytosolic free calcium concentration ($[Ca^{2+}]_i$) measurements

Cells were grown on glass coverslips, loaded with 4 μM fura-2 acetoxymethyl ester (Calbiochem) dissolved in DMSO/10% pluronic acid F-127 (Molecular Probes), rinsed, and incubated in fresh medium for 10 min before the measurement. Fluorescence was measured at 510 nm for emission, with excitation wavelengths of 340 nm and 380 nm, on a Hitachi F2000 fluorescence spectrophotometer. Calibrations were performed with 10 μM ionomycin for the maximal fluorescent signal and with 10 mM EGTA for the minimal fluorescent signal. Calcium concentration was determined using the equation of Grynkiewicz et al. [34].

Statistical analyses

Two-way analysis of variance was used. If significant effects were found (at $P < 0.05$), both the simple effects and interaction terms were further analysed by Fisher least significant difference with the help of statistical software SYSTAT (SPSS Inc., Chicago, IL, U.S.A.) using Macintosh computers.

RESULTS

Construction and characterization of *trkA* transfectants of SN56 cells

We performed immunoblot analysis using an anti-TrkA antibody to determine if SN56 cells express TrkA. We found no immunoreactive 140 kDa band (the size of TrkA protein) in extracts of these cells (Figure 1). In rat brain extract, which was used as a positive control, the same antibody detected a 140 kDa immunoreactive protein. In order to study TrkA-mediated effects of NGF in SN56 cells, we constructed stable transfectants of SN56 cells with recombinant rat *trk*A cDNA and screened them for TrkA expression by Western blotting (Figure 1). Several G418-resistant clones showed immunoreactive bands of varying intensities with molecular masses of approx. 140 kDa. The TrkA-immunoreactive band appears as a doublet, consistent with previous studies by others reporting double bands in *trk*A-transfected PC12 cells [35]. The results indicate that the transfectants contain varying amounts of TrkA protein. Two independently isolated clones, designated T17 and T22, which expressed large amounts of TrkA, were used for further analysis. To exclude the possibility that the observed effects are due to clonal variation rather than TrkA expression, in the experiments described below both clones were analysed and compared with untransfected SN56 cells. All results reported here were duplicated in both TrkA-positive clones. TrkA-negative clone T1 served as a control in some experiments.

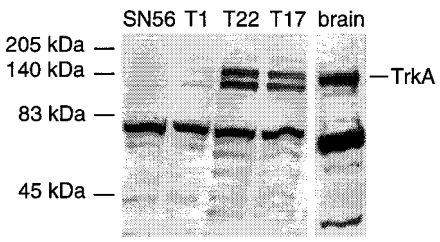

Figure 1 Analysis of TrkA expression in SN56 cells and transfected clones

Total cell lysates (100 μg per lane) were analysed by Western blotting with an anti-Trk antibody and visualized using the chemiluminescence method. Protein extract from rat brain (100 μg) served as a positive control. TrkA protein migrates as a doublet of approx. 140 kDa. The identity of the 75 kDa band, present both in native rat brain and in SN56 cells, is not known.

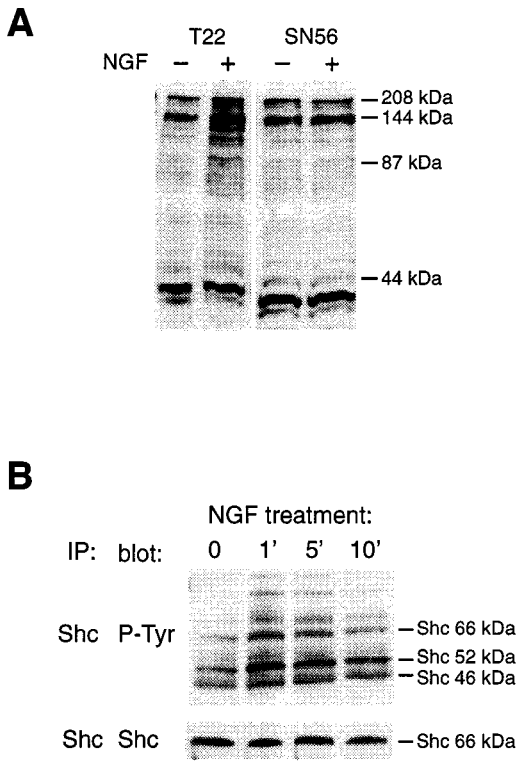

Figure 2 NGF-evoked tyrosine phosphorylation in SN56 cells and in transfectants expressing TrkA

(**A**) The cells were incubated with or without 100 ng/ml NGF for 5 min and quickly lysed. Cell lysates (40 μg per lane) were analysed by Western blotting with an anti-phosphotyrosine antibody. (**B**) T22 cells were treated with 100 ng/ml NGF for the indicated time periods. Cell lysates (200 μg per sample) were immunoprecipitated (IP) with a polyclonal anti-Shc antibody and then analysed by Western blotting with either an anti-phosphotyrosine antibody (upper panel) or a monoclonal anti-Shc antibody (lower panel).

NGF signalling evoked by TrkA in SN56-trkA cells

Activation of Trk receptor tyrosine kinases by neurotrophins is known to elicit a range of second-messenger responses [19]. In order to characterize the response to NGF in SN56-*trk*A transfectants, we first examined tyrosine phosphorylation events evoked by the growth factor. As demonstrated by immuno-

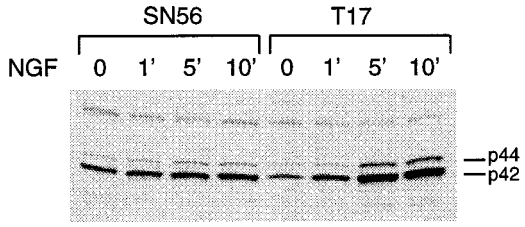

Figure 3 Activation of MAP kinase by NGF signalling in TrkA-positive transfectants

SN56 and T17 cells were treated with 100 ng/ml NGF for the indicated time periods. Cell lysates (80 μg per sample) were analysed by Western blotting with an antibody specific for the phosphorylated forms of MAP kinase.

blotting with an anti-phosphotyrosine antibody, no increase in tyrosine phosphorylation in response to NGF was observed in the parental SN56 cells, whereas addition of NGF to clone T22 rapidly increased tyrosine phosphorylation of numerous protein species, including the 140 kDa band presumed to be TrkA, and a 90 kDa band, which matches the size of SNT/FRS2, the specific intermediate in fibroblast growth factor and NGF signalling [36] (Figure 2A). Other TrkA-positive clones exhibited the same phosphorylation pattern upon NGF stimulation (results not shown). It has been documented that one of the major signalling proteins recruited to the Trk receptors immediately upon activation is the adapter protein Shc [19]. More recently it was demonstrated that the originally described Shc gene is not expressed in the central nervous system, where its function is assumed by a brain-specific homologue, neuronal Shc (N-Shc) [37]. In SN56-*trk*A cells stimulated with NGF, isoforms of a protein of the Shc family are among the tyrosine-phosphorylated protein species, as demonstrated by immunoprecipitation with an anti-Shc antibody, followed by anti-phosphotyrosine immunoblotting (Figure 2B). The anti-Shc antibody used here was raised against the C-terminus of the protein, which exhibits a high degree of homology between the original Shc and N-Shc. The antibody is therefore expected to recognize both Shc family members. Immunoblotting with an antibody directed against phosphorylated MAP kinase isoforms revealed that both extracellular signal-regulated kinase 1 p44 (ERK1 p44) and ERK2 p42 (known to be activated downstream from the TrkA–Shc interaction) are also rapidly phosphorylated in response to NGF (Figure 3). Only a slight increase of phosphorylation of MAP kinase occurred in the untransfected SN56 cells in response to NGF stimulation.

Since it has been reported that TrkA can also signal through elevation of the $[Ca^{2+}]_i$ [38,39], we assayed the effect of NGF on $[Ca^{2+}]_i$ in the *trk*A transfectants by fura-2 fluorescence (Figure 4). Basal $[Ca^{2+}]_i$ in the transfectants was approx. 100 nM. Application of NGF caused a small but sustained rise in $[Ca^{2+}]_i$ in clone T22 and a more transient response in T17. These responses appear to be mediated by TrkA, as no measurable changes in $[Ca^{2+}]_i$ were observed in TrkA-negative clone T1. As a positive control, we also determined the response of T1, T17 and T22 cells to activation by the muscarinic receptor agonist carbachol (1 mM), which caused a rapid and transient elevation in $[Ca^{2+}]_i$ in all three clones. The magnitude and the kinetics of the calcium response to carbachol were identical with those observed in SN56 cells [40].

Together these results demonstrate that we have reconstituted the functional TrkA signalling pathways in the *trk*A-transfected SN56 cells. We then used these stable transfectants as a model for studies on the regulation of the expression of the cholinergic gene locus by NGF. The comparison with the parental SN56 cells provides direct evidence for those effects of NGF that are mediated by TrkA.

NGF and CNTF action on the cholinergic locus expression

We determined the levels of ChAT and VAChT mRNA and intracellular ACh content in both the parental line SN56 and SN56-*trk*A cells after treatment with NGF (100 ng/ml) for 48 h (Figures 5 and 6). The cholinergic locus-specific probes were derived from ChAT and VAChT coding regions, in order to detect all known mRNA variants resulting from differential promoter use and alternative splicing. Under our experimental conditions, ChAT and VAChT transcripts migrate as single bands of approximately 3.8 kb and 3 kb respectively. As demonstrated by Northern blotting, NGF increased the abundance of both mRNA species in the transfected T17 cells (Figure 5A), but was more effective in increasing the abundance of ChAT mRNA (by approx, 2-fold) than that of VAChT mRNA (by approx. 50 %), consistent with its actions *in vivo* [15]. The increased levels of cholinergic mRNAs were paralleled by a 2.4-fold increase in ACh production in the transfectants treated with NGF (Figure 5). However, NGF failed to alter ChAT/VAChT expression and ACh content in parental SN56 cells (Figure 6), indicating that TrkA signalling is necessary for those effects.

Since LIF and CNTF and neurotrophins, like NGF, both influence the cholinergic properties of neurons *in vivo*, we

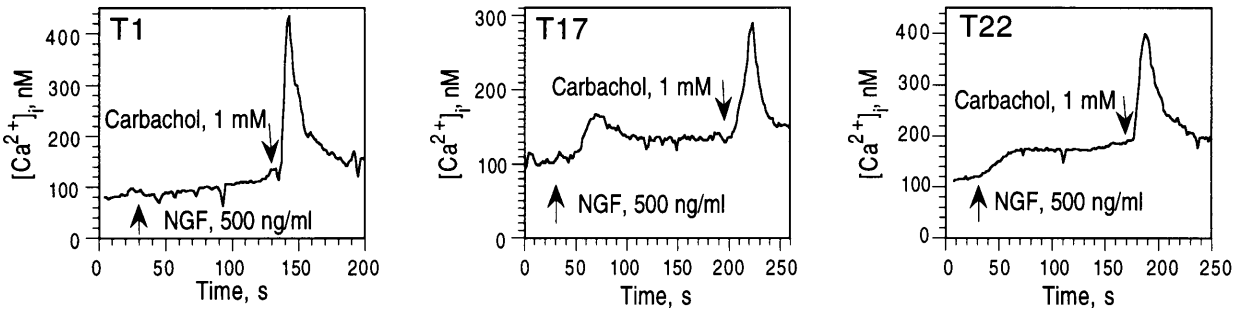

Figure 4 Effects of NGF and carbachol on calcium signals in SN56-derived clones

TrkA-negative clone T1 and two TrkA-expressing transfectants T17 and T22 were loaded with the fluorescent calcium indicator fura-2 and changes in $[Ca^{2+}]_i$ were measured as described in the Materials and methods section. Arrows indicate the time of application of NGF or carbachol.

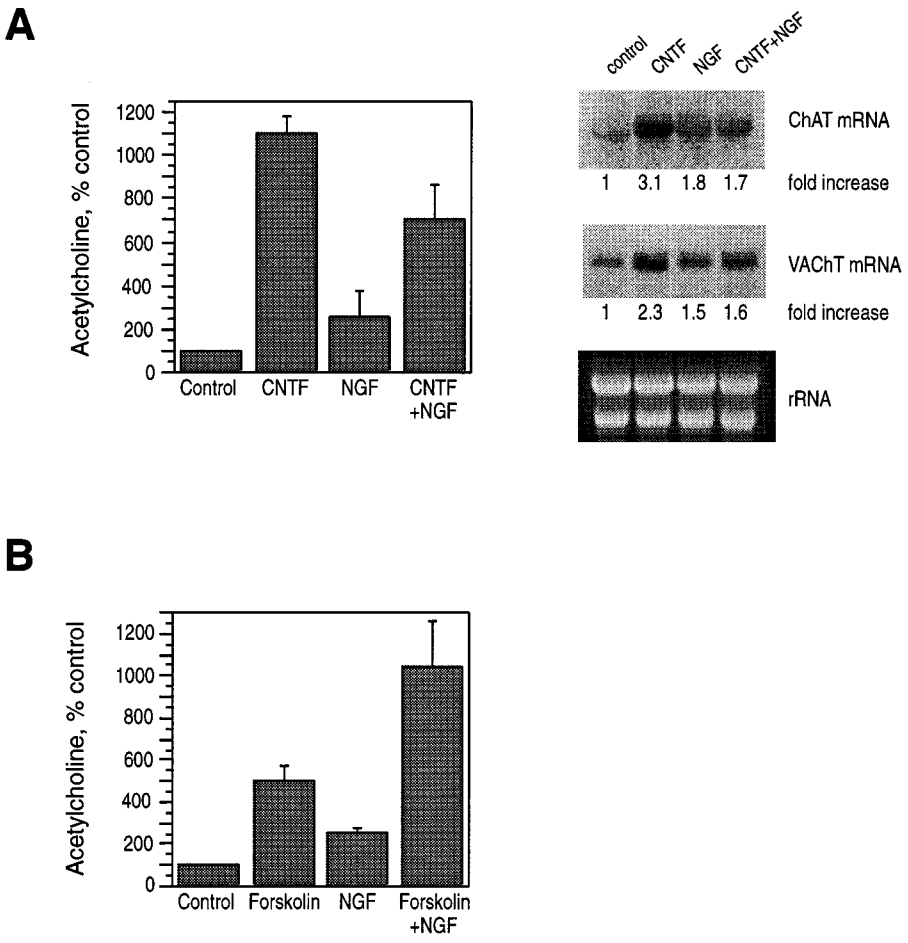

Figure 5 Regulation of the cholinergic properties of TrkA-positive T17 cells by NGF

(**A**) NGF attenuates the effect of CNTF on cholinergic gene expression. The cells were grown for 2 days in the presence or absence of 20 ng/ml CNTF, 100 ng/ml NGF, or a combination of the two. Intracellular ACh levels were measured as described in the Materials and methods section. The results are presented as means ± S.D. ($n = 5$). All comparisons are statistically significant. ChAT and VAChT mRNA levels were determined by Northern blotting with probes derived from their respective coding regions. Band intensities, quantified by phosphorimaging, are presented in comparison with control ('fold increase'). Each Northern blotting experiment was performed at least three times and representative blots are shown. rRNA, ribosomal RNA. (**B**) The effects of NGF and forskolin are additive. The cells were grown for 2 days in the presence or absence of 10 μM forskolin, 100 ng/ml NGF, or a combination of the two. ACh was measured as described above. The results are presented as means ± S.D. ($n = 3$). All comparisons are statistically significant.

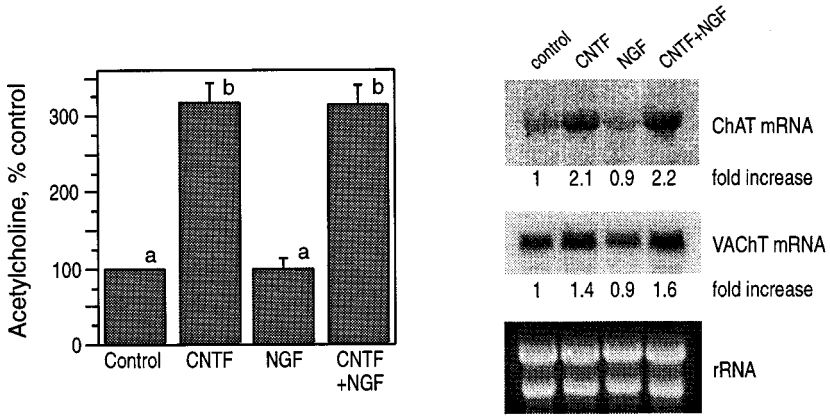

Figure 6 Lack of effect of NGF on the cholinergic properties of SN56 cells

Cell treatments, ACh measurements and Northern blotting were performed as described in Figure 5(A). ACh measurements are presented as means ± S.D. ($n = 5$). Bars with like letters are not statistically different from each other; all other comparisons are statistically different. Each Northern blotting experiment was performed at least three times and representative blots are shown. rRNA, ribosomal RNA.

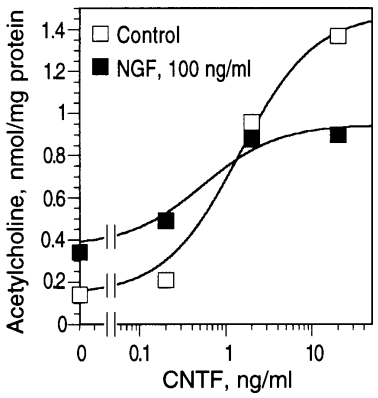

Figure 7 Concentration–response curves of the CNTF effect on intracellular ACh in the presence and absence of NGF

T17 cells were grown for 2 days in various concentrations of CNTF, in the presence or absence of 100 ng/ml NGF, as indicated. ACh levels were measured as described in the Materials and methods section. A two-way analysis of variance using NGF and CNTF as independent variables revealed a significant interaction ($P = 0.004$).

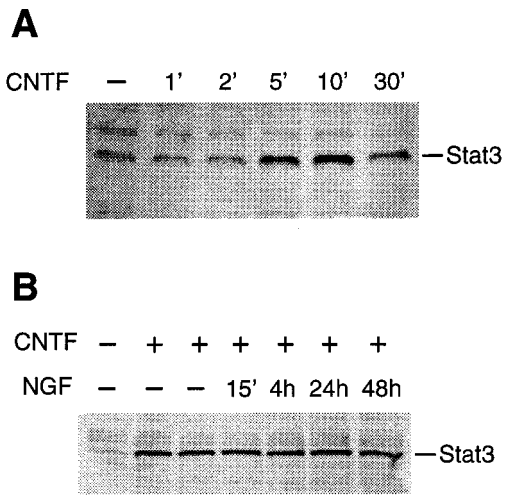

Figure 8 Lack of effect of NGF on Stat3 nuclear translocation evoked by CNTF

(**A**) Serum-starved T17 cells were treated with 20 ng/ml CNTF for the indicated time periods. Nuclear extracts were prepared as described in the Materials and methods section and analysed by Western blotting (12 μg of protein per lane) with an anti-Stat3 antibody. (**B**) The cells were treated with 100 ng/ml NGF for the time periods indicated. For all groups, for the last 4 h before harvest, the incubation was conducted in serum-free medium with no supplements. For the last 15 min of the incubation, 20 ng/ml CNTF was added (+), except for the control (–). Stat3 protein was detected in nuclear extracts as described in (**A**).

compared cells treated with NGF (100 ng/ml), CNTF (20 ng/ml) and a combination of the two factors. CNTF alone increased the expression of the cholinergic mRNAs significantly, in agreement with our previous results [8]. In T17 cells, the abundance of both ChAT and VAChT mRNA, as well as ACh production, was lower after treatment with the combination of NGF and CNTF than after treatment with CNTF alone (Figure 5A). The same attenuation of the CNTF effect by NGF was observed for the other independently isolated TrkA-expressing clone T22 (results not shown), but not in the parental SN56 cells (Figure 6). Thus, we conclude that the NGF–CNTF antagonism is mediated by TrkA. In order to examine whether this attenuation by NGF is specific for the CNTF signalling pathway, we treated the cells with the adenylate cyclase activator, forskolin, which has been shown to upregulate cholinergic gene expression [41]. Treatment of T17 cells with a maximally effective concentration of forskolin (10 μM) resulted in a 5-fold increase in ACh content. Unlike the CNTF effect, this response was fully additive with that evoked by NGF (Figure 5B).

Our previous experiments on the induction of ACh production by CNTF indicated that 20 ng/ml, used in the experiments described above, is the saturating concentration of the factor (results not shown). In order to better characterize the CNTF–NGF interaction in T17 cells, we measured ACh in cells treated for 48 h with various concentrations of CNTF, in the presence or absence of NGF (Figure 7). The results show that the concentration–response curve of the CNTF effect on intracellular ACh in TrkA-positive clone T17 changes in the presence of NGF. At low concentrations of CNTF, the effects of the two factors are additive, whereas at higher concentrations, the effect of CNTF is attenuated by NGF.

These results demonstrate that even though NGF by itself enhances the cholinergic properties of SN56-*trk*A cells, it interferes with CNTF-mediated upregulation of cholinergic gene expression. Since one of the best characterized elements of CNTF signalling is the activation of signal transducers and activators of transcription (STATs), and predominantly the transcription factor Stat3 [42], we investigated whether the interference by NGF involves down-regulation of the Stat3 signalling pathway. Immunoblotting of nuclear extracts with an anti-Stat3 antibody demonstrated that CNTF causes rapid (within minutes) translocation of Stat3 to the nucleus (Figure 8A). Pretreatment of the cells with NGF for various periods of time did not inhibit the translocation of Stat3, as shown in Figure 8(B). This suggests that, in the NGF-treated cells, Stat3 signalling is intact, although we cannot exclude NGF-mediated interference with Stat3 transcriptional activity in the nucleus.

DISCUSSION

Results presented in this study show that TrkA is necessary for the NGF-evoked upregulation of expression of the cholinergic gene locus and ACh production. SN56 cells lacking TrkA expression did not respond to NGF, whereas in *trk*A transfectants NGF upregulated the cholinergic phenotype. Our findings are in agreement with the work of Debeir et al [23,24] who showed the importance of TrkA for maintaining cholinergic nerve terminals *in vivo*. These results are also consistent with previous *in vivo* studies showing that *trk*A null mutant mice are characterized by a reduction of expression of cholinergic markers in brain, and with results on mutant mice lacking the p75 NGF receptor (but expressing TrkA) showing increased number and hypertrophy of cholinergic neurons in the basal forebrain [43]. Neurotrophins, including NGF, may enhance cholinergic neurotransmission by multiple mechanisms, including increases in the activities of ChAT [11,12,14,15] and high-affinity choline transport [44] and by increased ACh release [45]. Our results are consistent with previous studies of Pongrac and Rylett [46], showing that the principal reason for elevated production of ACh in NGF-treated cells is increased ChAT activity.

Enhanced expression of the cholinergic gene locus mediated by TrkA should depend on the activation both of the appropriate signalling pathways and of *cis*-acting elements within the gene. Our results show that *trk*A transfection of the basal forebrain-derived SN56 cells fully reconstitutes several known signalling pathways mediated by this receptor, including protein tyrosine

phosphorylation, activation of MAP kinase, and elevations of $[Ca^{2+}]_i$. In contrast, parental SN56 cells and clones that failed to express TrkA lacked these responses to NGF. By analogy with several other transcriptional effects of TrkA activation, it is likely that the activation of those signalling pathways is responsible for the observed upregulation of the cholinergic gene locus expression. The rat cholinergic gene locus contains a region located within 2 kb immediately 5′ of the R exon, which confers responsiveness to NGF in reporter gene assays [47]. The region contains two activator protein 1 (AP-1) sites as well as a putative cAMP response element (CRE). It has been suggested that these sites account for the response to NGF, because NGF activates the expression of the AP-1 complex [48], and the AP-1 component c-*fos* plays a role in the regulation of ChAT transcription by NGF [49]. Moreover, stimulation of TrkA by NGF causes phosphorylation and activation of the CRE binding protein (CREB) [50]. The same promoter region mediates the downregulation of cholinergic gene expression by cAMP-activated protein kinase (PKA) inhibitors [51]. PKA type II (PKA II) is apparently necessary for the coordinate regulation of ChAT and VAChT, because in PC12 cell mutants lacking PKA II, the expression of ChAT and VAChT mRNAs is markedly reduced. The expression can be restored by transfection with the catalytic subunit of PKA and can be enhanced in the wild-type PC12 cells by the use of selective PKA II agonists [51,52]. Upregulation of the cholinergic locus expression may be also mediated by additional *cis*-acting elements located downstream of VAChT. That area of the murine gene contains an AP-1 site and a CRE. The latter has been shown to be active in reporter gene assays in conferring inducibility by cAMP [53], but not by NGF [54]. Thus, activation of the cAMP pathway does not seem to account for the upregulation of ACh synthesis mediated by TrkA, and indeed, in our experiments, the direct stimulation of this pathway with forskolin was additive with the effect of NGF. The expression of the human cholinergic gene locus can be upregulated by additional transcription factors whose expression is induced by NGF (NGFI-A and NGFI-C), and the human gene apparently contains more than one region responsible for NGF inducibility [55,56]. Thus, the coordinated upregulation of ChAT and VAChT expression by activated TrkA may be mediated by several *trans*-acting factors and by *cis*-acting elements located in different parts of the cholinergic gene locus.

Consistent with previous studies, CNTF upregulated the expression of ChAT and VAChT in SN56 cells. This effect of CNTF is likely to be mediated by CNTF response elements (CNTFRE) within the gene. In other genes, *trans*-activation of those sequences follows the recruitment of Janus protein kinases (JAKs) to the gp130 subunit of the CNTF receptor complex and the subsequent tyrosine phosphorylation by JAKs of the STAT transcription factors which bind to CNTFRE. In the rodent cholinergic locus there are two consensus sequences for CNTFRE upstream of exon R, one of which is located within the NGF response region, and three additional consensus CNTFRE sequences located downstream of exon N. However, CNTF may also activate the ras-MAP kinase pathway and the AP-1 complex, as well as the members of the C/EBP family of proteins [57–59], indicating that the upregulation of the expression of the cholinergic locus may also involve these factors.

In SN56-*trk*A transfectants, CNTF-evoked upregulation of expression of the cholinergic gene locus and ACh production was modulated by NGF in a complex fashion. At low concentrations of CNTF (0.2 ng/ml) the actions of NGF and CNTF were additive. However, NGF significantly attenuated the response to higher concentrations of CNTF (20 ng/ml), affecting both ChAT and VAChT expression and ACh levels. The attenuation of the CNTF effect was not due to the downregulation of the initial events of CNTF signalling, because translocation of the transcription factor Stat3 to the nucleus in response to CNTF was not affected by the preincubation of T17 cells with NGF. Recently, there have been several reports on the interference between neurotrophin and cytokine pathways (see below); however, interpretation of the results is difficult and no molecular mechanism accounting for this interference has been proposed so far. In contrast with the results obtained in this paper, Ihara et al. [60] suggested direct inhibition of Stat3 phosphorylation by NGF in rat PC12 cells, whereas the recent report by Rajan et al. [61] demonstrated that prolonged (but not short-term) cultivation of rat superior cervical ganglia in the presence of NGF or brain-derived neurotrophic factor (but not neurotrophin-4) downregulated Stat1α and Stat3 binding to DNA. This decrease in binding was prevented by treatment with the serine kinase inhibitor H7, suggesting the involvement of a neurotrophin-activated serine kinase. It is not known what signalling proteins could be substrates for this putative kinase, and there are conflicting reports on the importance of direct serine phosphorylation of STATs for their DNA-binding ability [62–65]. In contrast, neurite formation was synergistically induced by a combination of NGF and CNTF in the human neuroblastoma cell line LA-N-2 [66]. Wu and Bradshaw [67] observed that neurite formation in PC12 cells was synergistically induced by a combination of NGF and interleukin-6, another gp130-utilizing cytokine. They described NGF-induced mobility changes of Stat3 on SDS/PAGE, which suggest induction of serine phosphorylation; at the same time, they detected no significant effect of NGF on Stat3 binding to DNA. Also, in a derivative of the PC12 cell line that responds poorly to NGF, the effects of NGF on neurite formation and ChAT expression were enhanced slightly by CNTF [68].

As noted above, some of the signalling pathways activated by NGF and CNTF are shared (i.e. MAP kinase pathway and AP-1 activation). Thus, one likely possibility is that the upregulation of expression of the cholinergic gene locus evoked by CNTF requires transcription factors and/or transcriptional co-activators which are also engaged in cells with activated TrkA. These factors might be sequestered in NGF-treated cells, preventing full expression of the actions of CNTF. This kind of competition for transcriptional co-activators has been observed previously. For example the CREB-binding protein, CBP/p300, acts as a co-activator in several signalling pathways (including JAK/STAT and Ras/AP-1), thus integrating them; however, competition for this factor can result in attenuated signalling [69–71].

Our results, obtained with an *in vitro* model, indicate that the cross-talk between signalling pathways mediated by NGF/TrkA and CNTF in regulating the expression of the cholinergic gene locus is complex, ranging from additive to apparently antagonistic. Regulation of expression of the cholinergic locus by the combined influence of NGF and CNTF/LIF may also vary *in vivo* during development and aging, as cells express different amounts and repertoires of neurotrophic factors and their receptors. Moreover, brain injury and disease can bring about alterations in expression of NGF and CNTF/LIF and their receptors, and regulation of expression of those receptors can be directly influenced by the trophic factors. In this respect, the results of our *in vitro* studies are consistent with a report that in NGF transgenic mice ChAT expression in brain is downregulated during development [72], suggesting that high concentrations of NGF can cause a reduction of cholinergic locus expression *in vivo*. Future investigations will determine how the interaction of neurotrophins that act via Trks (e.g. NGF) with the cytokines

that signal via gp130 (e.g. CNTF) influence the expression of the cholinergic gene locus *in vivo* under a variety of physiological and pathological conditions.

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