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Nerve Growth Factor Rapidly Induces Prolonged Acetylcholine Release from Cultured Basal Forebrain Neurons: Differentiation between Neuromodulatory and Neurotrophic Influences

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Long-term exposure to nerve growth factor (NGF) is well established to have neurotrophic effects on basal forebrain cholinergic neurons, but its potential actions as a fast-acting neuromodulator are not as well understood. We report that NGF (0.1–100 ng/ml) rapidly (<60 min) and robustly enhanced constitutive acetylcholine (ACh) release (148–384% of control) from basal forebrain cultures without immediate persistent increases in choline acetyltransferase activity. More ACh was released in response to NGF when exposure was coupled with a higher depolarization level, suggesting activity dependence. In a long-term potentiation-like manner, brief NGF exposure (10 ng/ml; 60 min) induced robust and prolonged increases in ACh release, a capacity that was shared with the other neurotrophins. K252a (10–100 nM), BAPTA-AM (25 μM), and Cd^{2+} (200 μM) prevented NGF enhancement of ACh release, suggesting the involvement of TrkA receptors, Ca^{2+}, and voltage-gated Ca^{2+} channels, respectively. Forskolin (10 μM), a cAMP generator, enhanced constitutive ACh release but did not interact synergistically with NGF. Tetrodotoxin (1 μM) and cycloheximide (2 μM) did not prevent NGF-induced ACh release, indicative of action at the level of the cholinergic nerve terminal and that new protein synthesis is not required for this neurotransmitter-like effect, respectively. In contrast, after a 24 hr NGF treatment, distinct protein synthesis-dependent and independent effects on choline acetyltransferase activity and ACh release were observed. These results indicate that neuromodulator/neurotransmitter-like (protein synthesis-independent) and neurotrophic (translation-dependent) actions likely make distinct contributions to the enhancement of cholinergic activity by NGF.

Key words: brain-derived neurotrophic factor; choline acetyltransferase; neurotransmitter release; neuromodulation; cholinergic; neurotrophin

Basal forebrain cholinergic neurons (BFCNs) innervate cortical and associated structures (Fibiger, 1982), are important for attention (Baxter and Chiba, 1999), and degenerate in Alzheimer's disease (Bartus, 2000). Rapid modulation of acetylcholine (ACh) release by physiological and pathological factors [e.g., neurotransmitters (Raiteri et al., 1984; Hersi et al., 1995), certain growth factors (Kar et al., 1997), β-amyloid (Auld et al., 1998), and interleukins (Hanisch et al., 1993)] is likely critical for the consequences of innervation.

Neurotrophins, including nerve growth factor (NGF), are crucial for the survival and function of certain neuronal populations (Levi-Montalcini, 1987). Regions of BFCN innervation (e.g., hippocampus, cortex) are enriched in NGF (Korsching et al., 1985; Large et al., 1986), and NGF is retrogradely transported by BFCNs (DiStefano et al., 1992), with these neurons expressing TrkA and p75NTR receptors (Koh and Loy, 1989; Holtzman et al., 1992). NGF and TrkA are important for BFCN development, maintenance, and function *in vivo* (Vantini et al., 1989; Li et al., 1995; Chen et al., 1997; Fagan et al., 1997; Molnar et al., 1998; Debeir et al., 1999; Ruberti et al., 2000), and NGF exposure (days to weeks) enhances cholinergic markers in BF cultures (Hartikka and Hefti, 1988; Takei et al., 1988, 1989; Svendsen et al., 1994; Nonner et al., 1996; Pongrac and Rylett, 1998; Oosawa et al., 1999; Auld et al., 2001). The neurotrophins brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4) also enhance cholinergic markers (Nonomura et al., 1995; Nonner et al., 1996; Auld et al., 2001). Interestingly, even a 30 min exposure to NGF, BDNF, NT-3, or NT-4 increases choline acetyltransferase (ChAT) activity 24 hr later (Nonner et al., 2000).

Neurotrophins rapidly increase intracellular Ca^{2+} in several neuronal phenotypes, including BFCNs (Wildering et al., 1995; Stoop and Poo, 1996; Jiang and Guroff, 1997; Li et al., 1998; Jia et al., 1999; Nonner et al., 2000), and acutely modulate neurotransmission (Lu and Chow, 1999; Schinder and Poo, 2000). BDNF and NT-3 rapidly and Ca^{2+}-dependently enhance neurotransmitter release from *Xenopus* motor neurons (Lohof et al., 1993; Stoop and Poo, 1996; He et al., 2000). BDNF can enhance hippocampal neurotransmission (Lessmann et al., 1994; Kang and Schuman, 1995; Li et al., 1998), inhibit high-frequency stimulation-associated fatigue (Gottschalk et al., 1998; Pozzo-Miller et al., 1999), and facilitate long-term potentiation (LTP) induction (Korte et al., 1995; Figurov et al., 1996; Patterson et al., 1996; Chen et al., 1999; Xu et al., 2000), with presynaptic TrkB typically being involved. Also, NGF rapidly modulates stimulated ACh release from hippocampal (Knipper et al., 1994) and visual-

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cortex (Sala et al., 1998) synaptosomes and likely influences visual-cortex LTP by modulating ACh release (Pesavento et al., 2000).

Using embryonic BF cultures, we report that NGF rapidly and potently enhanced ACh release in activity- and Ca^{2+}-dependent manners and that increases persisted after NGF removal. Dichotomous actions consisting of protein synthesis-dependent "neurotrophic" effects on ChAT activity and protein synthesis-independent "neuromodulator" increases of ACh release were identified. These distinct capacities may make complementary contributions to NGF enhancement of BFCN function.

MATERIALS AND METHODS

Culture. All experiments followed guidelines of the Canadian Council on Animal Care and McGill University policies. Cultures were prepared as described previously (Auld et al., 2000a). BF regions (septum, diagonal band of Broca, and substantia innominata) of day 17 rat embryos (Charles River, St. Constant, QC, Canada) were dissected in HBSS (Life Technologies, Burlington, ON, Canada) containing 0.65% D(+)-glucose (Sigma, St. Louis, MO), 15 mM HEPES, 10 U/ml penicillin, and 10 mg/ml streptomycin (Life Technologies). These were dissociated at 37°C with 0.08% trypsin (Life Technologies) and 0.1% DNase I (Sigma) for 18 min [terminated with 10% fetal bovine serum (FBS; Immunocorp, Montréal, QC, Canada)]. The dissociation was completed mechanically with a fire-polished Pasteur pipette. Cultures were plated at 700,000–750,000 cells per well [precoated with poly-L-ornithine (0.3 μg/ml); Life Technologies] in 500 μl of growth medium in four-well tissue culture plates (Nunc, Naperville, IL), and cultures were maintained at 37°C and 5% CO_2. The medium consisted of DMEM (#11965; Life Technologies) supplemented with KCl [20 mM; total, 25 mM; similar high-K^+ conditions are associated with improved viability in BF cultures (Nakamura et al., 1994)], sodium pyruvate (1 mM), D(+)-glucose (35 mM), HEPES (15 mM), and FBS (10%). Under similar culture conditions, which were optimized for the study of ACh release, both release and ChAT activity steadily increased between plating and 10 day *in vitro* (DIV 10) (Auld et al., 2000a).

Acetylcholine release. In most experiments, on DIV 7, the medium was removed, and cells were rinsed with Krebs' buffer [125 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 25 mM HEPES, 1.2 mM $MgSO_4$, 2.2 mM $CaCl_2$, 10 mM glucose, 10 μM choline, and 200 nM neostigmine (all from Sigma), pH adjusted to 7.4] containing 6 mM K^+. Unless described otherwise in Results, after a 60 min equilibration period at 37°C and 5% CO_2, this buffer was discarded and replaced for a 45 min period with fresh buffer containing rhβNGF (R & D Systems, Minneapolis, MN; lots HS178041 or HS189011) or vehicle, from which ACh release was measured. Other compounds were delivered during the 60 min equilibration period as well as simultaneously with NGF during the ACh release period [BAPTA-AM (RBI, Natick, MA), $CdCl_2$ (Sigma), cycloheximide (Sigma), forskolin (Sigma), K252a (Calbiochem, La Jolla, CA) Rp-cAMPS (RBI), tetrodotoxin (TTX; Tocris, Ballwin, MO)]. For the rhβNGF, rhNT-3, rhNT-4, or rhBDNF (R & D Systems; lots NG059091, OU02805, and OD048111) pretreatments (see Figs. 6, 7), medium with FBS was replaced with medium supplemented with B27 (2%; Life Technologies) containing the neurotrophin. After the indicated exposure period, culture wells were washed four times with NGF/neurotrophin-free buffer, and constitutive ACh release was then collected for 60 min periods in the buffer described above. In some experiments, p75NTR-IgG fusion protein (R & D Systems) was administered either concurrently with NGF or after NGF removal. All samples were kept at −80°C until ACh or ChAT activity quantification (<2 weeks).

The percentage of cholinergic neurons in BF cultures maintained under similar conditions is low (~1%) (Hartikka and Hefti, 1988; Svendsen et al., 1994). However, their unique ability to synthesize ACh makes quantification of supernatant ACh a reliable measure of their neurotransmitter release. To our knowledge, no report concerning an effect of NGF in BF cultures has indicated an indirect mechanism. BFCNs, but not GABAergic neurons, selectively express TrkA and p75NTR (Hartikka and Hefti, 1988; Koh and Loy, 1989; Holtzman et al., 1992; Svendsen et al., 1994). At a functional level, BFCNs, but not GABAergic neurons, respond to NGF and BDNF (Koliatsos et al., 1994). TrkB also appears to be selectively expressed on ChAT-immunoreactive somas in the BF (Molnar et al., 1998). Given that BFCNs selectively release ACh and respond to NGF, these cultures are an excellent model for studying their interactions.

Acetylcholine quantification. ACh was assayed by HPLC with electrochemical detection in conjunction with an enzyme reactor. Samples (100 μl) were injected manually via a 100 μl loop on a two-position valve (Valco, Houston, TX). ACh and choline, separated on a reverse-phase column (75 × 2.1 mm) pretreated with lauryl sulfate, passed through an enzyme reactor (10 × 2.1 mm) containing acetylcholinesterase (EC 3.1.1.7; Sigma, type VI-S) and choline oxidase (1.1.3.17; Sigma) covalently bound to glutaraldehyde-activated Lichrosorb NH_2 (10 μm; Merck, Darmstadt, Germany). All column hardware and packing materials were from Chrompack (Raritan, NJ). The resultant hydrogen peroxide was electrochemically detected at a platinum electrode at a potential of +500 mV versus an Ag/AgCl reference electrode (Antec VT-03/Decade, Leiden, The Netherlands). The mobile phase, 0.2 M aqueous potassium phosphate buffer, pH 8.0, with 1 mM tetramethylammonium hydroxide (Sigma), was delivered at 0.4–0.45 ml/min by a dual piston pump (ESA 580, Chelmsford, MA) connected to a degasser (CMA 260, Stockholm, Sweden). ACh eluted at ~4 min.

Choline acetyltransferase activity. Cultures were homogenized in 200 μl ice-cold buffer (40 mM sodium phosphate buffer, pH 7.4, 200 mM NaCl, and 0.5% Triton X-100). Aliquots in duplicate were assayed for ChAT activity using [^{14}C]-acetyl-CoA (New England Nuclear/DuPont, Markham, ON, Canada) and choline (Sigma) as substrate. After 60 min at 37°C, the reaction was stopped with ice-cold 10 mM sodium phosphate buffer, pH 7.4, containing 0.2 mM acetylcholine chloride (Sigma). Radioactive ACh was extracted using butyronitrile (Sigma) containing 15 mg/ml sodium tetraphenylborate (Sigma).

Statistical analysis. Data were statistically analyzed using either Student's *t* test (unpaired) or one- or two-way ANOVAs with Tukey's *post hoc* test, where appropriate. In all cases, $p < 0.05$ was considered statistically significant. The *n* represents individual culture wells evaluated in a given experiment, and unless indicated otherwise, data are expressed as mean ± SEM representing percentage of control wells receiving appropriate vehicle treatments.

RESULTS

NGF enhancement of ACh release is influenced by depolarization level

Exposure to NGF (100 ng/ml for 60 min in buffer with 6 mM K^+) resulted in increased ACh release (~192% of control) during an immediately subsequent 10 min period of K^+ (25 mM) depolarization (control, 168 ± 22 fmol per well per minute; NGF, 322 ± 16; $n = 4$; $p < 0.001$). Because some aspects of synaptic plasticity are influenced by the level of neuronal electrical activity, including neurotrophin modulation of neurotransmission (Gottschalk et al., 1998; Boulanger and Poo, 1999a), we examined whether NGF-associated increased ACh release could be modified by activity level. An identical NGF treatment in sister culture wells was associated with a greater increase in the amount of ACh released when subsequently paired with increased depolarization. Indeed, the same NGF treatment resulted in a ~2.4-fold greater increase of ACh release (femtomoles per well per minute) when followed by exposure to 25 mM K^+ compared with 6 mM K^+ (the increase in each K^+ condition was calculated vs ACh release from the same depolarization conditions in the absence of NGF) (Fig. 1).

We chose to further examine NGF enhancement of ACh release under conditions of constitutive ACh release associated with endogenous activity levels (in 6 mM K^+ buffer) because interpretation of mechanistic aspects of the NGF-associated increases would be complicated by the facts that K^+ (25 mM) depolarization was associated with a large Ca^{2+}-dependent induction of ACh release by itself, as well as the synergistic interaction between increased activity level and NGF action on ACh release. That most of the constitutive release was not sensitive to intracellular Ca^{2+} chelation (see below) enabled us to focus subsequent mechanistic studies directly on NGF-induced ACh

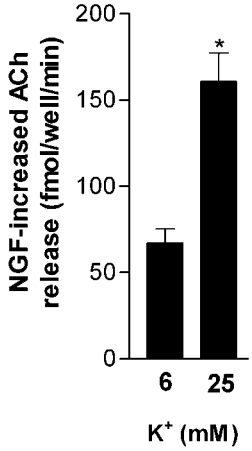

Figure 1. Activity-dependent enhancement of ACh release by NGF. Cultures were preexposed to NGF (10 ng/ml) for 60 min in low K^+ (6 mM) buffer. ACh release was then evaluated from low-activity (6 mM K^+) or high-depolarization (25 mM K^+) conditions for a 15 min period. *Columns* represent increased ACh (femtomoles per well per minute ± SEM; $n = 8$) associated with NGF preexposure versus the same depolarizing conditions without NGF preexposure. Significance was determined using Student's t test ($^*p < 0.001$ vs 6 mM K^+).

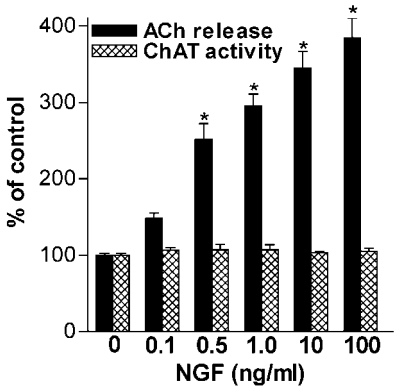

Figure 2. NGF acutely enhances constitutive ACh release from embryonic basal forebrain neurons in a concentration-dependent manner during a short-term exposure but does not induce persistent ChAT activity changes. Data are expressed as a percentage of release or ChAT activity in the absence of NGF [mean ± SEM; 0 ng/ml ($n = 59$), 0.1 ng/ml (14), 0.5 ng/ml (14), 1 ng/ml (37), 10 ng/ml (27), and 100 ng/ml (19); control ACh release was ~630 fmol/well for the 45 min exposure period, representing ~14 fmol per well per minute]. Significance was determined using a one-way ANOVA with Tukey's post-test ($^*p < 0.001$ vs control).

release. It should be pointed out that although there was a large relative effect of NGF compared with this spontaneous release (possibly representing nonspecific leakage), NGF actually induced more ACh release when coupled with a depolarizing stimulation (see above).

NGF enhances constitutive ACh release: contribution of TrkA and calcium

NGF (0.1–100 ng/ml) robustly enhanced constitutive ACh release from embryonic BF cultures during a 45 min exposure period without inducing an immediate, persistent increase in ChAT activity (Fig. 2). Furthermore, under these conditions, neither culture protein levels (control, 100 ± 1%, $n = 21$; NGF, 100 ng/ml, 103 ± 2%, $n = 8$) nor metabolic activity, indicated by MTT reduction (control, 100 ± 2%, $n = 7$; NGF 100 ng/ml, 99 ± 5%, $n = 4$), were altered; given the low percentage of cholinergic neurons in BF cultures, this was not unexpected (Hartikka and Hefti, 1988; Svendsen et al., 1994). The relative magnitude of NGF enhancement of ACh release was time dependent and during 15, 30, and 60 min exposure/release periods, NGF (1 ng/ml) enhanced constitutive ACh release to 127 ± 9% ($n = 4$; $p < 0.05$ vs control), 182 ± 11% ($n = 6$; $p < 0.001$), and 238 ± 25% ($n = 6$; $p < 0.001$) of control level, respectively. In the presence of TTX (1 μM), a 45 min exposure to NGF (1 ng/ml) still elicited robust ACh release (control, 100 ± 2%; TTX, 80 ± 6%; NGF, 287 ± 17%; NGF/TTX, 258 ± 27%; $n = 6$), suggesting an action at the level of the cholinergic nerve terminal.

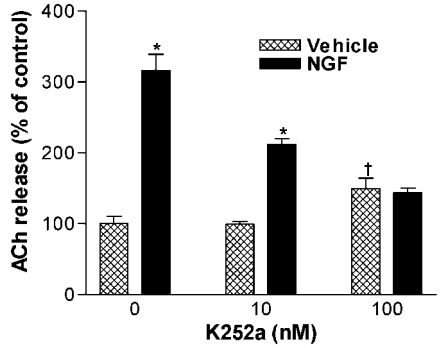

Figure 3. K252a (10–100 nM) prevents NGF (10 ng/ml) enhancement of ACh release during a short-term exposure. Data are expressed as a percentage of release in the absence of NGF and K252a (mean ± SEM; $n = 6$–8). Statistical analysis was performed using a two-way ANOVA with Tukey's post-test ($^*p < 0.001$ vs K252a only, at the same concentration; $^\dagger p = 0.0627$ vs cultures receiving neither K252a nor NGF).

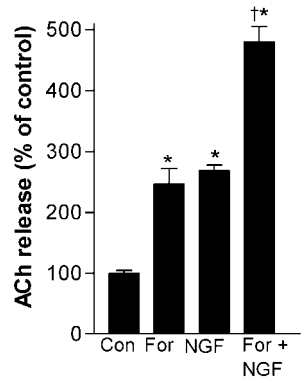

Figure 4. Forskolin (10 μM) alone increases ACh release but does not synergistically enhance NGF (1 ng/ml)-induced ACh release during a short-term exposure in low K^+ (6 mM) conditions. Data are expressed as a percentage of release in the absence of forskolin and NGF (mean ± SEM; $n = 6$). Significance was determined using a one-way ANOVA with Tukey's post-test ($^*p < 0.001$ vs control; $^\dagger p < 0.001$ vs both NGF and forskolin).

The tyrosine kinase inhibitor K252a blocked NGF (10 ng/ml)-induced increases in constitutive ACh release, suggesting the involvement of TrkA receptor signaling (Fig. 3). In the presence of 100 nM K252a, NGF did not increase ACh release beyond the control treated with K252a alone. Because cAMP signaling has been shown to enhance the effects of neurotrophins (Meyer-Franke et al., 1998; Boulanger and Poo, 1999b), we examined its actions on NGF-induced ACh release (Fig. 4). Forskolin, at a much higher concentration (10 μM), increased ACh release to a

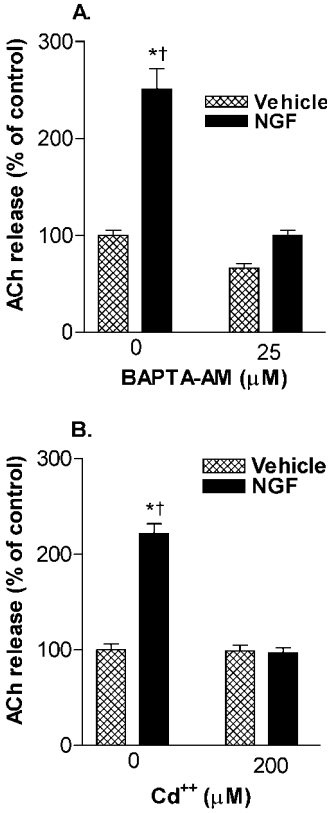

Figure 5. NGF (1 ng/ml)-enhanced ACh release involves Ca^{2+}. *A*, The intracellular Ca^{2+} chelator BAPTA-AM (25 μM) inhibits NGF-induced ACh release during a short-term exposure. Data are expressed as a percentage of release in the absence of BAPTA-AM and NGF (mean ± SEM). Significance was determined using a one-way ANOVA with Tukey's post-test (*$p < 0.001$ vs control; †$p < 0.001$ vs NGF/BAPTA-AM). *B*, NGF-induced ACh release is inhibited by the voltage-gated Ca^{2+} channel antagonist Cd^{2+} (200 μM). Data are expressed as a percentage of release in the absence of Cd^{2+} and NGF (mean ± SEM; $n = 6$). Significance was determined using a one-way ANOVA with Tukey's post-test (*$p < 0.001$ vs control; †$p < 0.001$ vs NGF/Cd^{2+}).

magnitude similar to NGF (1 ng/ml). The coapplication of NGF and forskolin, at these same concentrations, had only an additive effect without evidence of synergistic interaction. Furthermore, Rp-cAMPS (100 μM), a protein kinase A (PKA) antagonist, did not significantly decrease NGF (1 ng/ml)-induced increases in constitutive ACh release associated with a 45 min exposure (control, 100 ± 4%; Rp-cAMPS, 104 ± 1%; NGF, 329 ± 12%; NGF/Rp-cAMPS, 312 ± 12%; $n = 4$–9). Thus, the NGF increase of ACh release appears to not involve cAMP or PKA, at least under these low-K^+ conditions.

To determine whether Ca^{2+} was involved in the NGF-induced increases in constitutive ACh release, intracellular Ca^{2+} was chelated using BAPTA-AM (25 μM), and under these conditions NGF (1 ng/ml)-associated ACh release was prevented (Fig. 5*A*). We next investigated the involvement of voltage-gated Ca^{2+} channels (VGCC) using Cd^{2+}, a nonspecific antagonist. Cd^{2+} (200 μM) blocked NGF (1 ng/ml)-induced increases in ACh release, suggesting that voltage-gated Ca^{2+} channels were involved (Fig. 5*B*). Together with inhibiting ACh release increases caused by K^+ stimulation (25 mM; 10 min), Cd^{2+} blocked NGF enhancement of release under these conditions as well (data not shown).

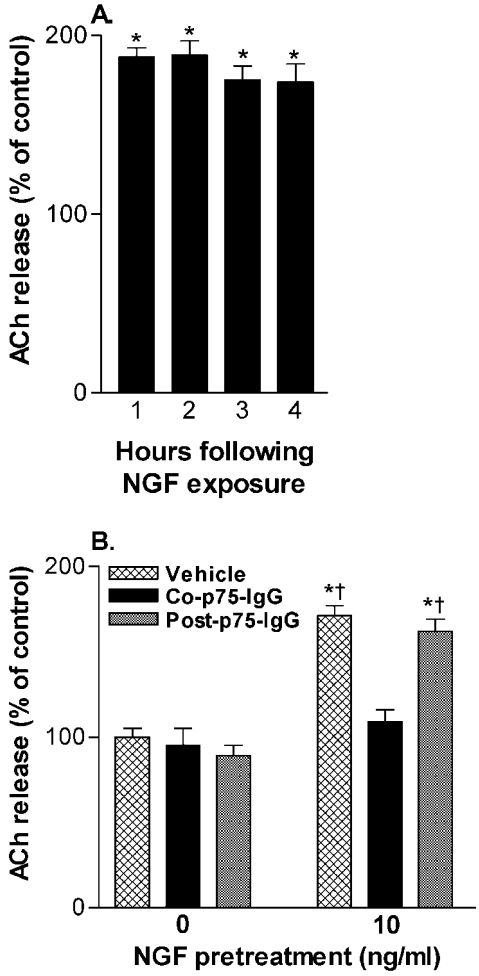

Figure 6. Brief exposure to NGF induces prolonged increase in ACh release. *A*, NGF (10 ng/ml for 60 min) treatment is associated with increased ACh release for at least 4 hr. Data are normalized according to release from control wells at the same hour and are expressed as mean ± SEM ($n = 17$–23). Statistical analysis was performed using repeated measure one-way ANOVA with Tukey's post-test; *$p < 0.001$ vs control. *B*, Treatment with NGF (10 ng/ml for 60 min) was associated with enhanced ACh release during the subsequent 60 min and was specific to availability during the defined exposure period, because a p75NTR-IgG fusion protein (5 μg/ml) only blocked the effect when coadministered with NGF. Data are normalized according to control wells and are expressed as mean ± SEM ($n = 4$–8). Statistical analysis was performed using a one-way ANOVA with Tukey's post-test: *$p < 0.001$ vs control, †$p < 0.001$ vs NGF/co-p75NTR-IgG fusion protein.

Brief exposure to neurotrophins induces prolonged ACh release

Treatment with NGF (10 ng/ml) for 60 min (followed by four rinses of culture plates) resulted in a robust increase of constitutive ACh release for at least the next 4 hr (Fig. 6*A*). Interestingly, comparable to the 60 min treatment, a 5 min exposure to NGF (10 ng/ml) also resulted in increased ACh release during the hour-long period subsequent to NGF removal (289 ± 31% of control; $n = 2$; $p < 0.05$). Thus, it is likely that increased time after initial NGF exposure, rather than the duration of NGF exposure, was important for the time-dependent effect noted previously. The specificity of the prolonged influence of NGF on ACh release for the defined exposure period, as opposed to a possible influence of potential residual NGF left after rinse with NGF-free buffer, was indicated by the capacity of a p75NTR-IgG

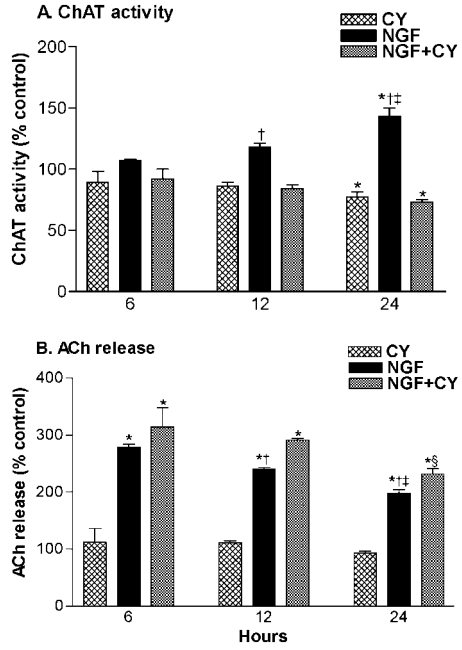

Figure 7. Evidence for protein synthesis-dependent and -independent effects of NGF on ChAT activity and ACh release, respectively. ChAT activity (*A*) and constitutive ACh release (*B*) were compared after 6, 12, and 24 hr exposures to NGF (100 ng/ml) and/or cycloheximide (*CY*; 2 μM). Data are normalized according to controls and are expressed as mean ± SEM (6 hr, $n = 4$; 12 hr, $n = 4–8$; 24 hr, $n = 20–24$). Statistical analysis was performed using two-way ANOVAs with Tukey's post-test: $^{*}p < 0.05$ vs vehicle-treated control and $^{\dagger}p < 0.05$ vs NGF/CY (within ChAT and ACh, same hour); $^{\ddagger}p < 0.05$ vs NGF at 6 and 12 hr (within ChAT and ACh); $^{\S}p < 0.05$ vs NGF/CY at 6 and 12 hr (within ACh). Furthermore, at 6, 12, and 24 hr, the percentage changes in ACh release and ChAT activity were different within NGF and NGF/CY groups ($p < 0.05$).

(5 μg/ml) fusion protein to block increases when coadministered with NGF (10 ng/ml), but not if given during the period of release determination immediately after NGF washout (Fig. 6*B*).

Considering that these neurons respond to NT-3, NT-4, and BDNF with increased ChAT activity (Nonomura et al., 1995; Nonner et al., 1996, 2000), as well as retrogradely transport them from target regions (DiStefano et al., 1992), we sought to determine whether they acutely induced a persistent increase in ACh release. Under the same conditions as with NGF, a 60 min pretreatment with the other neurotrophins (10 ng/ml) also enhanced ACh release in the hour subsequent to their removal: NT-3, 153 ± 6% of control ($n = 4$; $p < 0.001$ vs control); NT-4, 161 ± 16% ($n = 4$; $p < 0.01$); and BDNF, 177 ± 19% ($n = 4$; $p < 0.001$). Furthermore, NGF was also associated with increased K^+-stimulated ACh release that persisted after its removal (data not shown).

Differentiation between neurotrophic and neuromodulatory effects of NGF

Exposure to the protein synthesis inhibitor cycloheximide (2 μM) did not reduce NGF (1 ng/ml) enhancement of ACh release during a 45 min exposure period (control, 100 ± 3%; cycloheximide, 97 ± 5%; NGF, 329 ± 35%; NGF/cycloheximide, 362 ± 57%; $n = 6–8$), suggesting that new protein synthesis was not required for the rapid effect of NGF on ACh release. We next examined changes in ChAT activity and ACh release after 6–24 hr NGF (100 ng/ml) exposure and the contribution of protein synthesis to these effects (Fig. 7). After 6 and 12 hr of NGF exposure, ChAT activity was not significantly increased versus vehicle-treated control. Regardless of treatment with cycloheximide (2 μM), there was increased release of ACh from NGF-treated groups. After 24 hr, ChAT activity was significantly increased in the NGF condition but was not increased in the NGF/cycloheximide-exposed condition. Remarkably, the enhancement of ACh release associated with NGF/cycloheximide treatment was slightly larger than that associated with the NGF-alone condition, despite the markedly lower level of ChAT activity in the NGF/cycloheximide-treated cultures after 24 hr exposures (Fig. 7). Our preliminary data suggest that after a 24-hr treatment, enhanced ACh release associated with the NGF/cycloheximide treatment decays faster on removal of NGF compared with conditions in which protein synthesis is not prevented, which are associated with increased ChAT activity (Auld et al., 2000b).

DISCUSSION

We report that NGF influences BFCNs in a manner encompassing acute enhancement of ACh release in a neurotransmitter/neuromodulator-like manner. The concentrations of NGF enhancing ACh release indicate that it as one of the most potent ACh secretagogues ever recognized. More NGF-enhanced ACh release was associated with a greater depolarization, suggesting that this interaction could underlie some aspects of activity-dependent sculpting of BFCN synapses. Even brief exposures to NGF potentiated release for several hours after its removal, and this capacity was shared with BDNF, NT-4, and NT-3. After a 24 hr NGF treatment, distinct protein synthesis-dependent and -independent effects on ChAT activity and ACh release were observed. These findings imply that acute neurotransmitter-like as well as classical neurotrophic influences contribute to the effects of NGF on BFCN. These capabilities may make complementary contributions to the formation, maintenance, and activity of BFCN synapses.

The effective concentrations of NGF on ACh release suggest the involvement of TrkA receptors, which autophosphorylate at similarly low ligand concentrations (Kaplan et al., 1991). Inhibition of TrkA signaling with K252a prevented NGF-enhanced ACh release, consistent with K252a prevention of BDNF-enhanced neurotransmission in hippocampal cultures (Li et al., 1998). Considering that K252a (100 nM) increased ACh release to some extent in our model, it was not possible to reduce NGF-associated ACh release to vehicle-treated levels. Accordingly, a direct or modulatory role for p75NTR remains possible.

Although the steps linking TrkA to ACh release remain to be fully established, in our model, TTX-sensitive Na^+ channels were not critical. However, NGF-enhanced ACh release was prevented by BAPTA-AM and Cd^{2+}, suggesting that Ca^{2+} action after entry via VGCC was critical. In accord with these findings, NGF rapidly increases voltage-sensitive Ca^{2+} currents in molluscan neurons and PC12 cells (Wildering et al., 1995; Jia et al., 1999), as well as increasing intracellular Ca^{2+} in primary BF cultures (Nonner et al., 2000).

Depolarization augmented the quantity of ACh release associated with NGF exposure, implying a mechanism for preferentially maintaining more release at active synapses. This is in agreement with other reports of activity-dependent neurotrophin action on synaptic efficacy (Gottschalk et al., 1998; Boulanger and Poo, 1999a). Considering that synaptic fatigue may contribute to establishing ACh release levels during the high-K^+ exposure

period, it is interesting that fatigue accompanying high-frequency stimulation is prevented by BDNF (Gottschalk et al., 1998).

Our protocol involved a brief period of low activity (during and immediately before NGF exposure; 6 mM K^+) after sustained high-activity levels (growth conditions; 25 mM K^+). It is possible that depolarization during the maturation of BF cultures influenced NGF-associated signal transduction pathways and/or interacted with other BFCN characteristics to alter or facilitate NGF-induced ACh release. Indeed, short-term or multi-day K^+ depolarization has been shown to modulate features of neurotrophin signaling pathways in central (Meyer-Franke et al., 1998) or peripheral neurons (Vaillant et al., 1999), respectively. Moreover, in *Xenopus* motor neurons, K^+ depolarization rapidly increases the sensitivity of neurotransmission to enhancement by BDNF (Boulanger and Poo, 1999a). Nevertheless, our preliminary observations suggest that BF cultures grown under low-K^+ conditions also respond acutely to NGF (10 ng/ml) with increased ACh release (data not shown). Thus, NGF-induced ACh release is not unique to cultures grown under high-K^+ conditions, although more subtle differences could exist.

Activity upregulates synthesis and release of neurotrophins (Thoenen, 1995), with hippocampal expression and secretion of NGF being elevated by muscarinic and nicotinic receptor signaling (da Penha Berzaghi et al., 1993; Knipper et al., 1994; Blochl and Thoenen, 1995; French et al., 1999). The capacity of NGF to enhance ACh release and of ACh to increase NGF has been hypothesized to contribute to synaptic efficiency (Knipper et al., 1994). These characteristics suggest potential mechanisms for immediate (i.e., translation-independent secretagogue effects at the terminal level) and long-term (i.e., transcription/translation-dependent neurotrophic effects) strengthening of synaptic connectivity resulting from increased NGF availability. Moreover, the activity-dependent nature of NGF-enhanced ACh release implies that this feedback could be amplified at more active synapses.

TTX exposure did not prevent NGF-enhanced ACh release, and this is consistent with the selective expression of NGF receptors on BFCNs (Hartikka and Hefti, 1988; Koh and Loy, 1989; Holtzman et al., 1992; Svendsen et al., 1994). Because NGF is highly expressed in regions of BF innervation (Korsching et al., 1985; Large et al., 1986), action at the terminal agrees with the potential physiological modulation of ACh release by target-derived NGF. The TTX data also suggest that although increased depolarization is associated with greater NGF enhancement of ACh release, high levels of concurrent activity are not required. This may be significant for developing BFCNs first encountering target-derived NGF, which may increase ACh release from innervating fibers with low intrinsic activity, resulting in feedback between ACh release and NGF secretion and thereby increasing NGF available for retrograde transport. The translation-dependent actions of NGF likely include direct and indirect enhancement of action-potential generation probability. In an indirect neurotrophic manner, NGF increases BFCN excitability by altering properties of Ca^{2+} currents at the soma level (Levine et al., 1995) and increases excitability in other developing neurons by inducing expression of Na^+, K^+, and Ca^{2+} channels (Lesser and Lo, 1995; Toledo-Aral et al., 1995; Hilborn et al., 1998). At the soma level, NGF increases BFCN firing under some conditions, indicating that it can directly induce action potentials (Palmer et al., 1993; Albeck et al., 1999). Thus, target-derived, retrogradely transported NGF is likely to facilitate generation of action potentials, and this would be amplified by the synapse-strengthening feedback between ACh release and NGF secretion at the terminal level. This could subsequently interact with the activity-dependent nature of the NGF secretagogue action to further promote synapse consolidation.

A 60 min NGF exposure increased ACh release for at least 4 hr after its removal. This was not observed when ACh release was stimulated with high K^+ (without NGF), despite the ~10-fold increase (Auld et al., 2000a). The long-term enhancement of ACh release was dependent on NGF availability only during the exposure period, suggesting that critical signal transduction/effector processes were initiated quickly and remained activated for several hours. Even a 5 min exposure was associated with prolonged enhancement of release. These time frames are similar to NGF-induced increases in Ca^{2+}, TrkA phosphorylation, and downstream pathways in primary BF cultures (Knusel et al., 1992; Downen et al., 1993; Nonner et al., 2000). Thus, transitory target-derived NGF secretion may subsequently augment ACh release for several hours, greatly strengthening the synapse, although potential *in vivo* interactions with established circuitry could modify this response. Considering that NT-3, NT-4, and BDNF also induced prolonged ACh release, they could play a similar role during synaptic development and maintenance.

Complementary to our observations, a 30 min neurotrophin exposure increased ChAT activity measured 24 hr later in BFCNs (Nonner et al., 2000), and a 1 min exposure to NGF induced Na^+ channel expression in PC12 cells (Toledo-Aral et al., 1995). Thus, even short exposures to neurotrophins can cause lasting effects via both neurotrophic and secretagogue mechanisms. Regarding prolonged responses of BFCNs to brief neurotrophin exposures, it seems likely that increases in ChAT activity (Nonner et al., 2000) and translation-independent ACh release (this report) depend on common (e.g., TrkA, Ca^{2+}) and disparate (e.g., translation) mechanisms. Because Ca^{2+} is involved in NGF-induced ACh release, the mechanism(s) sustaining prolonged release likely involves Ca^{2+}-dependent elements. Ca^{2+} activates kinases that regulate neurotransmission and vesicle trafficking, such as Ca^{2+}/calmodulin-dependent kinase II (Llinas et al., 1985; Greengard et al., 1993). Interestingly, this kinase has been implicated in NT-3-induced neurotransmitter release from *Xenopus* motor neurons (He et al., 2000).

Rapid NGF enhancement of ACh release was not reduced by cycloheximide, suggesting that protein translation is not involved. In agreement with previous observations (Pongrac and Rylett, 1998), increased ChAT activity after 24 hr NGF exposure was dependent on new protein synthesis. Interestingly, even after 24 hr, and regardless of the lack of increase in ChAT activity, there was a large protein synthesis-independent enhancement of ACh release associated with the NGF/cycloheximide condition. Thus, NGF can enhance ACh release in a secretagogue manner after prolonged exposure, and this may have relevance for maintenance of ACh release levels at BFCN synapses that are exposed to target-derived NGF. It is also apparent that under some conditions, increased ChAT activity is not associated with—or required for—enhancement of ACh release after prolonged NGF exposure. Together, these data suggest that the translation-independent secretagogue action of NGF may contribute to a portion of increased ACh release, even after prolonged NGF exposures sufficient to induce transcription and translation-dependent increases in ACh synthesis capacity.

In summary, NGF rapidly enhanced ACh release from embryonic BF cultures. The NGF-associated increase was activity dependent and, unlike classical neurotransmitter modulation, per-

sisted for several hours after NGF removal in a manner akin to LTP. The NGF-induced increase was dependent on TrkA signaling, Ca^{2+}, and VGCCs, but not dependent on new protein synthesis. After a 24 hr treatment with NGF, distinct protein synthesis-dependent and -independent effects on ChAT activity and ACh release, respectively, were observed. Together, these data suggest that in addition to translation-dependent neurotrophic actions, NGF has strong influences on BFCN function via both rapid and prolonged modulation of ACh release in a neurotransmitter/neuromodulator-like manner.

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