Nerve Growth Factor in the Hippocamptoseptal System: Evidence for Activity-Dependent Anterograde Delivery and Modulation of Synaptic Activity

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Neurotrophins have been implicated in regulating neuronal differentiation, promoting neuronal survival, and modulating synaptic efficacy and plasticity. The prevailing view is that, depending on the target and mode of action, most neurotrophins can be trafficked and released either anterogradely or retrogradely in an activity-dependent manner. However, the prototypic neurotrophin, nerve growth factor (NGF), is not thought to be anterogradely delivered. Here we provide the neuroanatomical substrate for an anterograde hippocamptoseptal transport of NGF by demonstrating its presence in mouse hippocampal GABAergic neurons and in their hippocamptoseptal axons that ramify densely and abut neurons in the medial septum/diagonal band of Broca (MS/DB). We also demonstrate an activity-dependent increase in septal NGF levels that is dependent on the pattern of intrahippocampal stimulation. In addition, we show that acute exposure to NGF, via activation of TrkA, attenuates GABA_A receptor-mediated inhibitory synaptic currents and reduces sensitivity to exogenously applied GABA. These acute actions of NGF display cell type and functional selectivity insofar as (1) they were found in cholinergic, but not GABAergic, MS/DB neurons, and (2) glutamate-mediated excitatory synaptic activity as well as AMPA-activated current responses were unaffected. Our results advocate a novel anterograde, TrkA-mediated NGF signaling in the CNS.

Introduction

Neurotrophins have been implicated in myriad processes in the developing and adult nervous system. The traditional view is that neurotrophins exert influences via target-derived retrograde signaling. That is, they are produced and released by cells in target tissues, taken up by axon terminals of neurons that project there, transported retrogradely, and, within the soma, influence the expression of genes that regulate neuronal differentiation, specification, and survival (Lewin and Barde, 1996; Huang and Reichardt, 2001; Segal, 2003; Chen et al., 2005).

Nerve growth factor (NGF) is a prototypic neurotrophin in the nervous system. In the hippocamptoseptal (HS) system, NGF is synthesized in hippocampal neurons and retrogradely delivered to the medial septum/diagonal band of Broca (MS/DB) (Schwab et al., 1979; Seiler and Schwab, 1984; Korsching et al., 1985; Conner and Varon, 1992; DiStefano et al., 1992; Lapchak et

al., 1993; Lauterborn et al., 1993; Takeda et al., 1993; Pascual et al., 1999; Acsády et al., 2000). There, it promotes the survival of cholinergic neurons (Johnston et al., 1987; Fusco et al., 1989; Gibbs and Martynowski, 1997; Kawaja et al., 1998; Gustilo et al., 1999) and protects them from atrophic insults (Williams et al., 1986; Montero and Hefti, 1988; Junard et al., 1990; Williams, 1991). More recent studies point to an anterograde signaling scheme for neurotrophin action (Altar and DiStefano, 1998). Specifically, brain-derived neurotrophic factor (BDNF) was shown to modulate synaptic transmission as well as regulate synaptic plasticity in a fashion and time course that could best be accounted for by an anterograde signaling mechanism. In this light, current literature is replete with demonstrations of BDNF modulating, in the short term, voltage-gated ion channels and ligand-gated ionotropic receptors (Levine et al., 1996, 1998; Tanaka et al., 1997; Kafitz et al., 1999; Brünig et al., 2001; Blum et al., 2002; Cheng and Yeh, 2003; Mizoguchi et al., 2003a,b; Wardle and Poo, 2003; Fernandes et al., 2008), and, in the longer term, synaptic strength and plasticity (Kang and Schuman, 1995; Korte et al., 1998; Pozzo-Miller et al., 1999; Xu et al., 2000; Minichiello et al., 2002; Du and Poo, 2004; Pang and Lu, 2004; Bramham and Massouidi, 2005). Importantly, the signaling pathways downstream from Trk receptor activation that confer the modulatory effects on synaptic efficacy appear to be distinct from those subserving the classic trophic actions of neurotrophins (Segal, 2003; Bramham and Massouidi, 2005). However, for NGF, a case for it exerting neuronal effects via anterograde signaling has not been

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as compelling. Indeed, the prevailing concept is that NGF is not anterogradely transported (Altar and DiStefano, 1998).

Nonetheless, early neuroanatomical studies demonstrated that NGF mRNA is present in hippocampal GABAergic neurons, notably in the subpopulation of somatostatin-containing GABAergic neurons that projects to the MS/DB (Pascual et al., 1999; Acsády et al., 2000). It was postulated then that hippocampal GABAergic projection neurons might deliver NGF anterogradely to the MS/DB (Acsády et al., 2000). Here, we present neuroanatomical, biochemical, and electrophysiological evidence that, in toto, implicate a functional scheme for an activity-dependent anterograde mode of NGF signaling in the HS system.

Materials and Methods

All procedures involving animals were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the Geisel School of Medicine Institutional Animal Care and Use Committee. The experiments used mice of either sex from three transgenic mouse lines: (1) the FVB-GIN (Friend virus B-type/GFP-expressing inhibitory neurons) line (Oliva et al., 2000; Jackson Laboratory); (2) the BAC-*ChAT* line, generated by the NINDS Gene Expression Nervous System Atlas Bacterial Artificial Chromosome (BAC) Transgenics Project (Gong et al., 2003) using a BAC vector in which the choline acetyltransferase (*ChAT*) gene drives the expression of EGFP (obtained from Dr. Robert Brownstone, Dalhousie University, Halifax, Canada), and (3) the *GAD67*-EGFP(Δ neo) line (Tamamaki et al., 2003). In the MS/DB of the BAC-*ChAT* mouse, ~90% or more of the EGFP-fluorescent neurons were immunopositive for ChAT (Fig. 1A1–A3), as they were for TrkA (Fig. 1B1–B3) and p75NTR (Fig. 1C1–C3). These observations are consistent with TrkA and p75NTR being predominantly expressed in cholinergic MS/DB neurons (Sobrievila et al., 1994; Wu and Yeh, 2005). The *GAD67*-EGFP(Δ neo) mouse line expresses EGFP regulated by the *GAD67* promoter; in the mature cortex, >80% of all GABAergic interneurons are EGFP-fluorescent (Tamamaki et al., 2003). Consistent with this, we found EGFP expressed in 86% of the parvalbumin-immunoreactive subpopulation of GABAergic MS/DB neurons (data not shown). The EGFP-fluorescent cells in the BAC-*ChAT* and *GAD67*-EGFP(Δ neo) mice greatly facilitated identification of cholinergic and GABAergic MS/DB neurons in the acute brain slices used in our electrophysiological experiments. Figure 5A2 illustrates two EGFP-fluorescent neurons in the MS/DB of an acute brain slice obtained from the BAC-*ChAT* mouse.

Immunohistochemistry. Postnatal day 21–28 (P21–P28) mice were asphyxiated with carbon dioxide and immediately perfused intra-aortically with heparinized PBS followed by 4% paraformaldehyde/0.1 M PBS. Brains were isolated, postfixed overnight in 4% paraformaldehyde, and then immersed sequentially in 0.1 M PBS containing 15 and 30% sucrose. Cryosections (40 μ m) containing the MS/DB or hippocampus were blocked in 10% normal goat serum/0.05% Triton X-100/PBS before incubation with primary or secondary antibodies. The primary antibodies used were mouse anti-GFP (1:1000; Invitrogen), rabbit anti-NGF (1:1000; Sigma-Aldrich), mouse anti-parvalbumin (1:1000; Millipore), rabbit anti-ChAT (1:1000; Millipore), rabbit anti-TrkA (1:1000; Millipore), and rabbit anti-p75NTR

Figure 1. EGFP-immunopositive MS/DB neurons in the BAC-*ChAT* mice are cholinergic and express TrkA and p75NTR. **A**, ChAT immunoreactivity (**A1**) in EGFP-immunopositive (**A2**) MS/DB neurons. The overlay (**A3**) and cell counts indicate that $\geq 90\%$ of the EGFP-expressing cells in the MS/DB are also immunoreactive to ChAT. **B**, TrkA expression (**B1**) in EGFP-immunopositive (**B2**) MS/DB neurons. Colabeling is evident in 93% of the cases (**B3**). **C**, p75NTR expression (**C1**) in EGFP-immunopositive (**C2**) MS/DB neurons. The overlay (**C3**) and cell counts indicate that colabeling is evident in $\geq 87\%$ of the EGFP-expressing cells. Scale bar: (in **C3**) **C3**, **B**, **C**, 50 μ m.

(1:1000; Millipore). Secondary antibodies used were Alexa Fluor 350, Alexa Fluor 488, Alexa Fluor 555, and Alexa Fluor 568 (Invitrogen) diluted 1:800 in PBS. Sections were coverslipped in FluorSave Reagent (Calbiochem). In one series, the HS system was microdissected intact and 40 μ m cryosections with contiguous hippocampus, HS pathway, and MS/DB were processed for immunohistochemistry (see Fig. 2A). Immunofluorescent images were captured using a spinning disc confocal microscope (BX61, Olympus), some images were deconvoluted and edited using Photoshop CS (Adobe). Negative control sections with the primary antibody omitted were routinely run in parallel.

Intact HS preparation. Assessing septal NGF levels following hippocampal stimulation called for a preparation in which the connection between the two structures is preserved. To this end, the hippocampus, the HS pathway, and the septum were dissected intact from brains isolated from P21–P28 mice and placed in a custom-made two-compartment chamber, immersed in oxygenated artificial CSF (aCSF) identical to that used for electrophysiological recording (see below), and maintained at 35–37°C (see Fig. 4A). The hippocampus was placed in one compartment and the septum in the other. Vaseline placed between the compartments and embedding the HS pathway served as a barrier so that solution exchanges and electrical stimulation could be achieved in one compartment without affecting the adjoining one. Routinely, the hippocampus of the HS preparation obtained from one side of the brain was either exposed to KCl (25 mM) or stimulated using a pair of bipolar electrodes for 15 min, after which the septum was detached and processed for NGF-ELISA (see below). As control, the HS preparation from the contralateral side of the brain was incubated in aCSF for the same length of time without electrical stimulation or KCl-induced depolarization.

Figure 2. Hippocamptoseptal projecting GABAergic neurons contain NGF. **A**, Cryosection containing contiguous hippocampus and MS/DB derived from the FVB-GIN mouse brain illustrating EGFP-fluorescent hippocampal neurons (arrows) and axons emanating from the hippocampus (HP) and running along the hippocamptoseptal pathway to ramify densely in the MS/DB. Scale bar, 1 mm. **B**, Representative field taken from the CA3 region of FVB-GIN hippocampus showing EGFP-immunopositive (**B1**, arrows) and NGF-immunopositive (**B2**, arrows) neurons. The overlay (**B3**) reveals that many of the EGFP-containing neurons are immunoreactive for NGF (arrows). Scale bar, 50 μ m. **C**, Higher magnification image showing overlay of EGFP (green) and NGF (red) immunoreactivity in CA3 stratum oriens (**B3**, boxed area). EGFP-expressing neurons (arrows) are a subset of those that are NGF-immunoreactive as some NGF-immunoreactive neurons do not express EGFP (arrowheads). **D**, EGFP-immunopositive HS fibers in the FVB-GIN mice are decorated by punctate NGF-immunoreactive profiles. **E**, In MS/DB, EGFP-immunoreactive fibers (green) course around and come into close contact with a p75NTR-immunoreactive cholinergic neuron (red, white arrow). Negative controls (parallel processing without primary antibody) revealed no nonspecific immunostaining. HP, hippocampus; SR, CA3 stratum radiatum; SP, CA3 stratum pyramidale; SO, CA3 stratum oriens. Scale bar: (in **D**) **C–E**, 20 μ m.

NGF-ELISA. NGF sandwich ELISA was performed using NGF Emax ImmunoAssay System (Promega). Briefly, ELISA plates (Co-star) coated with anti-NGF polyclonal antibody were incubated overnight at 4°C and then blocked at room temperature for 1 h using blocking and sampling buffer. Supernatant from homogenate of dissected septum containing the MS/DB and hippocampus were diluted in blocking and sampling buffer, added into the wells of ELISA plates along with NGF standards, and incubated at room temperature for 6 h. Anti-NGF monoclonal antibody was then added and the samples were allowed to incubate overnight at 4°C. Anti-rat IgG HRP conjugate was then added at room temperature for 2.5 h. Color development was achieved by adding TMB One Solution and terminated by adding 1N HCl. The plates were read at 450 nm (Bio-Rad), and a standard curve was constructed using murine NGF standards to derive estimates of NGF content in hippocampal and MS/DB samples. Data were reported as mean $\pm$ SEM. Comparative analysis of NGF levels in the septum and hippocampus without and with hippocampal stimulation was performed using Student's *t* test.

Electrophysiology. Mice (P10–P22) were asphyxiated by carbon dioxide and decapitated. The brain was quickly isolated, blocked by removing the cerebellum and hindbrain, and immersed en bloc in ice-cold oxygenated aCSF containing (in mM) the following: 125 NaCl, 2.5 KCl, 2.0 CaCl₂, 1.0 MgCl₂, 1.25 NaH₂PO₄, 26 NaHCO₃, and 10 glucose. All solutions were bubbled continuously with 5% CO₂ and 95% O₂. Coronal brain slices (200 μ m) containing MS/DB were obtained using a vibrating tissue slicer (Model OTS-4000, Electron Microscopy Sciences). The slices were incubated in aCSF at room temperature for at least 60 min before electrophysiological recording.

During electrophysiological recording, the brain slices were maintained at 32–33°C on a heated stage fit onto an upright microscope (BXWI50, Olympus) and continuously perfused with oxygenated aCSF at rate of 0.5 ml/min. A CCD camera attached to a video frame grabber board (Integral Technologies) displayed real-time images to facilitate visualization of cells under Hoffman Modulation Optics and placement of recording electrodes and drug pipettes. In slices derived from wild-type mice, putative cholinergic and noncholinergic MS/DB neurons were identified in the cell-attached mode by firing rate (Wu and Yeh, 2005). In slices derived from the BAC-*Chat* mice, cholinergic neurons were readily identifiable by their expression of fluorescent EGFP (Fig. 1).

Patch electrodes (2–6 M Ω in recording solution) were pulled from borosilicate glass capillary tubes (outer diameter, 1.5 mm; inner diameter, 0.86 mm). The internal (recording) solution used consisted of the following (in mM): 140 KCl, 4 NaCl, 2 MgCl₂, 10 EGTA, 10 HEPES, 2 Mg-ATP, and 3 Mg-GTP, pH 7.3, with KOH. Some experiments used an internal solution consisting of the following (in mM): 144 K-gluconate, 3 MgCl₂, 0.2 EGTA, 10 HEPES, 3 Mg-ATP, 3 Na-GTP, and 1 leupeptin, pH 7.3, with KOH. For recordings of inhibitory synaptic currents, the NMDA receptor antagonist 2-amino-5-phosphonopivalic acid (APV; 10 μ M; Tocris Bioscience) and AMPA receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 10 μ M; Tocris Bioscience) were added to the aCSF perfusion solution to block excitatory synaptic currents. Under these conditions, bicuculline methiodide (10 μ M; Tocris Bioscience) reversibly abolished all synaptic currents (data not shown). For recordings of excitatory synaptic currents, the GABA_A receptor antagonist bicuculline methiodide (10 μ M) or gabazine (10 μ M, Sigma-Aldrich) was added to the perfusion solution.

Membrane currents were monitored using a multiclamp 700B amplifier (Molecular Devices), filtered at 5 kHz, digitized using Pulse and analyzed off-line using PulseFit (HEKA Instruments), Mini Analysis software (Version 6.0.7, Synaptosoft), or Clampfit software (Molecular Devices). Statistical analysis was performed using SigmaPlot 8.02 (SPSS) and Prism (Version 5, GraphPad Software). Mean peak current and frequency of GABA-mediated spontaneous synaptic events were analyzed using paired Student's *t* test. Repeated-measures ANOVA and Bonferroni's multiple-comparison test were applied when comparing NGF treatment to baseline and washout in the same cell. Data were reported as mean $\pm$ SEM.

Applications of drugs and extracellular stimulation. Unless indicated otherwise, all chemicals were purchased from Sigma-Aldrich. NGF (2.5S NGF, Harlan), bicuculline methiodide, gabazine, APV, CNQX, GABA, and AMPA were dissolved in aCSF, stored as frozen stock and diluted to working concentrations with aCSF immediately before each recording session.

For focal application, the drug solutions were loaded into separate barrels of a six-barrel pipette assembly mounted onto a micromanipulator and navigated under microscopic control to be within 10 μ m of the soma of the MS/DB neuron under study (Wu and Yeh, 2005; see Fig. 5A1). Drug solutions were ejected by regulated pulses of pressure ($\leq$ 3 psi; Picospritzer, General Valve Company), with timing and duration controlled by a digital timing unit (Pulsemaster A300, World Precision Instruments). One barrel was routinely filled with external solution, which was applied between drug applications to clear drugs from the

vicinity of the cell and to control for mechanical artifacts due to bulk flow.

Extracellular stimulation was performed with a concentric bipolar electrode (Frederick Haer) inserted into one aCSF-filled barrel of the drug pipette assembly, placed $\geq 50 \mu\text{m}$ from the soma under study. The A360 Stimulus Isolator (World Precision Instruments) was used to generate the current pulses triggered by the digitizer. Stimulus intensity was adjusted in each case to evoke stable postsynaptic currents in the cell recorded and then kept constant throughout the recording.

Results

We addressed three questions: (1) Is NGF localized to GABAergic hippocampal neurons and their axons that project to the MS/DB? (2) Does stimulation of the hippocampus increase NGF levels in the MS/DB? (3) Does NGF modulate inhibitory and/or excitatory synaptic activity in the MS/DB?

NGF is present in hippocampal GABAergic neurons that project to the MS/DB

In the hippocampus and neocortex of the FVB-GIN (Friend virus B-type/GFP-expressing inhibitory neurons) mouse, a subpopulation of somatostatin-containing GABAergic neurons express EGFP (Oliva et al., 2000). As illustrated in Figure 2*A*, EGFP-immunopositive fibers course along the HS pathway and ramify densely in the MS/DB. These fibers are axons of GABAergic neurons in the hippocampus (Fig. 2*A*, arrows) since GABAergic neurons native to the MS/DB do not express EGFP (Oliva et al., 2000). NGF-like immunoreactive profiles could be found in all strata and cornu ammonis (CA) regions of the hippocampus. Figure 2*B1* shows EGFP immunoreactive GABAergic neurons in the CA3 region of the hippocampus (arrows), many of which coexpress NGF (Fig. 2*B2,B3*, arrows). The boxed area in Figure 2*B3* is shown at higher magnification in Figure 2*C* to illustrate that NGF immunoreactivity in the stratum oriens was not limited to the EGFP-immunofluorescent GABAergic subpopulation (Fig. 2*C*, arrows) but was also found in non-EGFP-fluorescent cells (Fig. 2*C*, arrowheads). These likely represent other subtypes of GABAergic neurons that express NGF mRNA in the hippocampus (Pascual et al., 1999). In addition, punctate NGF-immunoreactive profiles could be found along the length of the EGFP-containing axons in the HS pathway (Fig. 2*D*) and in the MS/DB (data not shown). In the MS/DB, these axons ramified densely, frequently outlining closely the contours of neurons, including virtually all cholinergic neurons identified by their expression of p75NTR immunoreactivity (Fig. 2*E*, arrow; Heckers et al., 1994; Sobreviela et al., 1994). This is in agreement with an earlier report that GABAergic hippocampal neurons innervate cholinergic MS/DB neurons, albeit to a lesser extent than their GABAergic counterpart (Tóth et al., 1993). Overall, these findings established the neuroanatomical substrate for an anterograde mode of NGF delivery in the HS system. We also noted many NGF-immunopositive cellular profiles in the MS/DB (Fig. 3). Presumably, many are GABAergic MS/DB neurons (Conner and Varon, 1992; Lauterborn et al., 1995), as indicated by colocalization of NGF with parvalbumin-immunoreactivity (Fig. 3*A1,A3,B*, arrowheads), or ChAT-immunoreactive cholinergic neurons, with retrogradely transported NGF sequestered in the soma (Fig. 3*A1,A2,B*, arrow), and others may be nonneuronal elements, such as astrocytes.

Stimulation of the hippocampus increases NGF levels in the MS/DB in a pattern-dependent manner

Using an intact HS preparation (Fig. 4*A*, inset), we asked whether stimulation of the hippocampus could increase the level of NGF

Figure 3. MS/DB neurons display NGF-like immunoreactivity. **A1**, NGF immunostaining in the MS/DB. Arrow and arrowheads point to three representative cells that display NGF-like immunoreactivity. **A2**, ChAT immunostaining in the same field as **A1**. Arrow points to a ChAT-immunoreactive cholinergic MS/DB neuron. **A3**, Parvalbumin immunostaining in the same field as **A1**. Arrowheads point to two parvalbumin-immunoreactive GABAergic MS/DB neurons. **B**, Overlay of **A1–A3** showing colocalization of NGF-like immunoreactivity in cholinergic (arrow) and GABAergic (arrowheads) MS/DB neurons. Scale bar: **B**, 40 μm .

in the MS/DB. Since the boundaries of the medial and lateral septa and diagonal band of Broca were not sufficiently demarcated to allow further microdissection in this preparation, the septum was isolated en bloc (Fig. 4*A*, black arrow; Fig. 4*A* inset, black arrowhead). The hippocampal portion of the HS preparation was subjected to either constant depolarization with KCl (25 mM) or, in an attempt to simulate firing patterns of different hippocampal neuronal subpopulations, one of three patterns of electrical stimulation: (1) a burst-type pattern (24 Hz pulses delivered as trains of eight pulses at 100 Hz at 300 ms intervals; 500 μA), simulating the fast firing pattern of somatostatin-immunopositive GABAergic neurons in the hippocampus and cortex (Freund and Buzsáki, 1996; McBain and Fisahn, 2001), which frequently occurred in bursts, as reported in the frontal cortex (Kawaguchi and Kubota, 1996); (2) an evenly spaced barrage of the same number of single pulses delivered at 42 ms intervals (24 Hz; 500 μA), simulating the general firing characteristics of hippocampal GABAergic interneurons (Freund and Buzsáki, 1996; McBain and Fisahn, 2001), and (3) evenly spaced pulses (500 μA) delivered at 3 Hz, simulating the activity of other neuronal types in the hippocampus with low rates of spontaneous firing. Following 15 min of stimulation, the hippocampus and septum were detached and processed separately to determine NGF levels by ELISA. The HS preparation obtained from the left hemisphere was routinely used for stimulation; that obtained from the right hemisphere was used as no-stimulation control.

The scatter plot in Figure 4*B* illustrates ELISA data from 30 separate experiments in which septal NGF levels were assessed following various forms of hippocampal stimulation. Each point represents an individual experiment and denotes the NGF level in the septum following hippocampal stimulation relative to that obtained in the control counterpart. The equivalence line (dashed line) represents the result expected if NGF levels in the septum remained unchanged following hippocampal stimulation. All of the points representing the individual experiments in which the hippocampus was stimulated using a burst pattern (gray diamonds) were positioned above the equivalence line, in-

Figure 4. Intact hippocamptoseptal preparation and experimental setup for demonstrating stimulus-evoked NGF levels in hippocamptoseptal system. **A**, Custom-made temperature-controlled two-compartment incubating chamber holding the intact septohippocampal preparation (inset). The hippocampus was placed in one compartment and the septum in the other. A strip of Vaseline positioned between the compartments and embedding the hippocamptoseptal pathway permitted solution exchange and stimulation in individual compartments. To demonstrate that the two chambers are isolated, methyl green (1%) added to the compartment holding the hippocampus (right) does not leak into the left compartment holding the septum. Three bipolar stimulating electrodes (triple arrowheads) aligned in parallel are shown inserted into the hippocampus. **B**, Scatterplot summarizing septal NGF levels determined by ELISA. In experiments with the intact septohippocampal preparation, the hippocampus was stimulated for 15 min with a train of eight pulses at 100 Hz delivered at 300 ms intervals (Burst, gray diamond), single regularly spaced pulses at 24 Hz (open circle) and 3 Hz, filled circle), and KCl (25 mM, gray square). In experiments with the fimbria/fornix severed, either the septum (FF cut sep stim, open triangle) or the hippocampus (FF cut hip stim, filled triangle) was stimulated with the burst-type pattern stimulation for 15 min. Each symbol represents data obtained from one experiment. The 45° equivalence line (dotted line) represents the result expected if the NGF level remains unchanged after stimulation compared with no-stimulation control. **C**, Mean percentage change from control of septal NGF levels using stimulation paradigms as indicated. Data expressed as mean $\pm$ SEM. HP, hippocampus; SEP, septum.

indicating consistently increased levels of NGF in the septum. As summarized in Figure 4C, patterned burst stimulation of the hippocampus increased NGF levels in the septum by $23.3 \pm 4.6\%$ ($N = 7$; $p < 0.05$).

By contrast, electrical stimulation with single, evenly spaced 24 or 3 Hz pulses (Fig. 4B, open and filled circles, respectively), or with constant KCl-induced depolarization (Fig. 4B, gray squares), were ineffective in increasing NGF levels in the septum; the data points tended to cluster around the equivalence line. As summarized in Figure 4C, stimulation with single, evenly spaced pulses at 24 Hz ($-4.4 \pm 24.8\%$; $N = 4$; $p = 0.82$) and at 3 Hz ($-3.1 \pm 8.6\%$; $N = 3$; $p = 0.54$) yielded statistically insignificant outcomes. In the hippocampi of the same preparations, there was

a trend toward elevated NGF levels following the three patterns of electrical stimulation, but this turned out to be statistically insignificant (data not shown).

We asked whether the stimulus-evoked increase in septal NGF levels was dependent on an intact HS pathway. To address this, we severed the fimbria/fornix in the HS preparation and found that intrahippocampal stimulation with the burst-type pattern no longer elevated septal NGF levels (Fig. 4C). In addition, despite demonstration of NGF endogenous to the MS/DB (Fig. 3), septal stimulation failed to elevate NGF levels in the septum (Fig. 4C). These results point to increased NGF levels in the septum following patterned stimulation of the hippocampus.

NGF modulates inhibitory but not excitatory neurotransmission in cholinergic MS/DB neurons

Our findings that NGF is present in axons of HS GABAergic neurons and that intrahippocampal stimulation increases septal NGF levels are consistent with there being an anterograde route of NGF delivery to the MS/DB neurons. Since extracellularly applied NGF excites cholinergic MS/DB neurons (Wu and Yeh, 2005), we hypothesized that the neurotrophin could do so by modulating GABA and/or glutamate receptor function in cholinergic MS/DB neurons, GABAergic MS/DB neurons, or both.

We first assessed whether NGF affected GABA-activated current responses in cholinergic neurons in acute brain slices derived from wild-type, BAC-*ChAT*, and *GAD67-EGFP*(Δ neo) mice. In Figure 5A1, the recording pipette (R) is pointed at an EGFP-expressing MS/DB neuron (Fig. 5A2) in a brain slice from a BAC-*ChAT* mouse. The multibarrel drug pipette assembly for focal application of drugs (P) is positioned near the neuron under recording. Pressure-regulated pulses of GABA (20 μ M; 200–600 ms duration) were applied at 20 s intervals. With aCSF applied throughout the length of the interpulse interval, the GABA responses remained stable and did not run down for the duration of the recording session. Whole-cell current responses to GABA were monitored continuously before, during, and after NGF (100 ng/ml) application (Fig. 5B1–B3). For each cell, the peak amplitude of six consecutive responses recorded under each condition were averaged and used for quantitative comparison. Figure 5C illustrates superimposed examples of such averaged responses before, during, and after NGF exposure obtained from the same cell. Repeated-measures ANOVA revealed a significant effect of acute NGF exposure on the peak amplitude of the GABA response (Fig. 5D; $n = 12$; $**p = 0.003$); *post hoc* Bonferroni's multiple-comparison test indicated that the peak amplitude of the GABA response during NGF application (81.02 ± 17.52 pA) was significantly reduced compared with control period (110.4 ± 23.36 pA; $*p < 0.05$). In two cells, focal application of K252a (10 min) prevented NGF-induced suppression of GABA_A receptor-mediated current responses (data not shown), consistent with the involvement of TrkA signaling in mediating the NGF-induced suppression of GABA responses.

We then asked whether NGF alters GABAergic synaptic transmission in the MS/DB. We recorded spontaneous GABA_A-mediated IPSCs (sIPSCs) from cholinergic MS/DB neurons in acute coronal slices derived from the BAC-*ChAT* mice. Representative sIPSCs recorded from a cholinergic MS/DB neuron before and during exposure to focally applied NGF are illustrated in Figure 6A. Before NGF exposure, the mean amplitude of the sIPSCs was 50.3 ± 6.7 pA (Fig. 6B, open bar). Upon a 2 min exposure to NGF, there was a significant, albeit modest, decrease

in the mean amplitude of the sIPSCs (Fig. 6*B*, striped bar; 46.7 ± 5.8 pA; $n = 12$; paired t test, $p = 0.03$). Application of NGF did not alter the mean frequency of IPSCs (Fig. 6*C*; $n = 12$; paired t test, $p = 0.29$).

In another set of experiments, electrical stimuli (0.5 ms duration, 500 ms intervals) were delivered to the vicinity of cholinergic MS/DB neurons. The mean amplitude of 20 evoked IPSCs (eIPSCs) obtained during the control (pre-NGF) period was compared with that obtained during a 2 min focal application of NGF and that following a 5 min clearing of the neurotrophin with continuous perfusion of aCSF. Representative averaged traces obtained from a cell under each of the three conditions are superimposed and illustrated in Figure 6*G*. NGF application significantly decreased the mean amplitude of eIPSCs (Fig. 6*H*; $n = 9$, repeated-measures ANOVA, $p < 0.01$). Specifically, the mean amplitude of eIPSCs during NGF application (40.64 ± 9.85 pA) was significantly lower compared with the control (47.26 ± 10.04 pA) and wash periods (44.29 ± 10.36 pA) (Fig. 6*H*; Bonferroni's multiple-comparison tests, $p < 0.05$). The above results suggest that NGF-TrkA signaling suppresses GABA_A-receptor-mediated neurotransmission in cholinergic MS/DB neurons.

Unlike its suppression of GABA-activated responses, NGF did not affect the amplitude of AMPA ($20 \mu\text{M}$)-activated current responses in cholinergic MS/DB neurons ($n = 9$, repeated-measures ANOVA, $p > 0.05$). The mean amplitude of the whole-cell current responses to AMPA before NGF exposure (control; 53.06 ± 8.39 pA) was not statistically different from that during NGF application (60.92 ± 11.68 pA) nor that following washout (52.51 ± 7.83 pA). Focal application of NGF also did not alter spontaneous EPSCs (sEPSCs) in cholinergic MS/DB neurons. In 20 cholinergic neurons, the mean sEPSC amplitude and frequency during the control period were 18.07 ± 1.31 pA and 1.50 ± 0.72 Hz, respectively. These values remained unchanged upon NGF application (mean amplitude, 17.25 ± 1.24 pA; mean frequency, 1.45 ± 0.66 Hz; paired t test, $p > 0.05$). Similarly, NGF had no effect on evoked EPSCs (eEPSCs) in cholinergic MS/DB neurons. The mean eEPSC amplitude during NGF application was 40.16 ± 5.90 pA, which was not significantly different from that recorded before or after NGF exposure (41.45 ± 5.64 pA and 39.90 ± 6.51 pA, respectively; $n = 6$, repeated-measures ANOVA, $p > 0.05$). Together, these results indicate that NGF preferentially modulates inhibitory vis-à-vis excitatory neurotransmission in the MS/DB.

Is the modulatory effect of NGF specific to the cholinergic neurons in the MS/DB? To answer this question, we examined sIPSCs (Fig. 6*D–F*) and sEPSCs (data not shown) in EGFP-fluorescent MS/DB neurons from the *GAD67-EGFP(Δneo)* as well as non-EGFP-fluorescent MS/DB neurons from the *BAC-ChAT* mice. These cells typically displayed a fast rate of firing consistent with them being noncholinergic NS/DB neurons, as reported previously (Wu and Yeh, 2005). Acutely exposing these neurons to NGF did not affect the amplitude or frequency of

Figure 5. NGF decreases GABA-evoked whole-cell current responses in cholinergic MS/DB neurons. *A*, EGFP-fluorescent MS/DB neurons viewed under Hoffman Modulation Optics (*A1*) and epifluorescence (*A2*) in an acute coronal brain slice containing the MS/DB from a *BAC-ChAT* mouse. The tip of the patch-clamp recording pipette (*A1*, R) is placed on an EGFP-expressing cholinergic MS/DB neuron from the right and a six-barrel drug pipette assembly (*A1*, P) is positioned to the left of the neuron. Scale bar: *A2*, 20 μm . *B*, Whole-cell current responses to pressure-regulated pulses of GABA ($20 \mu\text{M}$; 200–600 ms duration; arrowheads) were monitored before (*B1*), during (*B2*), and after (*B3*) NGF application in a cholinergic MS/DB neuron. *C*, Averaged GABA-evoked current responses during control (red), NGF (black), and wash (blue) periods from the same cell are superimposed. *D*, NGF reversibly attenuates responses to exogenously applied GABA as reflected in a decrease in the peak amplitude of the current responses during NGF application (repeated-measures ANOVA, $**p < 0.01$; Bonferroni's multiple-comparison test, $*p < 0.05$).

sIPSCs (Fig. 6*E,F*; $n = 9$) nor of sEPSCs ($n = 6$) (paired t test, $p > 0.05$). These results indicate that NGF not only preferentially modulates postsynaptic GABA_A receptor function, but also does so selectively in the TrkA-expressing cholinergic MS/DB neurons (see Fig. 1*B1–B3*).

Discussion

The mode of neurotrophin transport bears important functional implications. Thus, the traditional scheme of retrogradely transported target-derived NGF being sequestered in the soma and enhancing the expression of genes and factors that promote neuronal differentiation, phenotypic specification, and survival provided the framework for marshaling the classical “neurotrophin hypothesis” (Davies, 1996). Along the same line, the demonstration that certain neurotrophins, notably BDNF and NT3, are in synaptic terminals of brain regions where the corresponding mRNAs are absent prompted the notion of anterograde transport, which in turn provided a scheme for their activity-dependent release and extracellular accumulation in modulating synaptic efficacy and plasticity (Altar et al., 1997; Conner et al., 1997; Yan et al., 1997; Schinder and Poo, 2000). To date, evidence for activity-dependent release of NGF and its effect on synaptic plasticity is piecemeal at best (Blöchl and Thoenen, 1995, 1996; Wu et al., 2004).

Our study combined immunohistochemical, ELISA, and electrophysiological analyses to determine whether NGF synthesized in the hippocampus could signal in an anterograde fashion to modulate synaptic transmission in target MS/DB neurons. The data presented here indicate for the first time that NGF in the HS system fulfills many of the prerequisite criteria for a functional anterograde mode of delivery and activity-dependent release, placing NGF in a favorable position to modulate synaptic transmission. The major findings are as follows: (1) HS GABAergic neurons display NGF-like immunoreactivity, which can be traced along their axons en route to and within the MS/DB; (2) hippocampal stimulation rapidly elevates septal NGF levels in a stimulus pattern-dependent fashion; and (3) NGF selectively attenuates inhibitory neurotransmission in TrkA receptor-expressing cholinergic MS/DB neurons.

A Cholinergic

B Cholinergic

C Cholinergic

D Non-cholinergic

E Non-cholinergic

F Non-cholinergic

G Evoked IPSCs

H Evoked IPSCs

Figure 6. NGF attenuates inhibitory synaptic activity in cholinergic MS/DB neurons. **A**, sIPSCs recorded in the presence of CNQX and APV ($10 \mu\text{M}$ each) in a cholinergic MS/DB neuron before and during focal application of NGF. **B**, Histogram summarizing the amplitude of sIPSCs before NGF (open bars) and during NGF (striped bars) in cholinergic MS/DB neurons. The mean amplitude of sIPSCs was significantly decreased upon NGF application (paired *t* test, $p < 0.005$). **C**, Summary of the frequency of sIPSCs before NGF (open bars) and during NGF (striped bars) obtained from cholinergic MS/DB neurons. NGF did not significantly alter sIPSC frequency (paired *t* test, $p > 0.005$). **D–F**, The same sets of experiments performed to assess NGF effects

NGF expression in the HS system

The FVB-GIN mouse line (Oliva et al., 2000) facilitated the examination of NGF expression in the EGFP-fluorescent subpopulation of somatostatin-containing GABAergic neurons that project from the hippocampus to the MS/DB. Beside being localized to EGFP-expressing cells, NGF-immunoreactivity in the hippocampus proper was also localized to other nonpyramidal cells, presumably GABAergic interneurons expressing other peptidergic or calcium-binding protein markers (Freund and Buzsáki, 1996). Our findings corroborate earlier reports localizing NGF mRNA to hippocampal neurons immunoreactive for a number of neuropeptides, including somatostatin, neuropeptide-Y, vasoactive intestinal polypeptide, and cholecystokinin (Pascual et al., 1999).

Oliva et al. (2000) speculated that the EGFP-expressing neurons projected extrahippocampally. Here, as revealed in cryosections with the HS system intact, we demonstrate unequivocally such a projection from the hippocampus proper via the fornix to the MS/DB. Since we never observed EGFP-expressing cell bodies in the MS/DB of FVB-GIN mice, the dense EGFP-fluorescent fibers seen along the HS pathway and in the MS/DB must have been axons of GABAergic neurons residing in the hippocampus. Furthermore, punctate NGF-immunoreactive profiles were present within these axons through the entire extent of the projection. In line with an earlier report that GABAergic hippocampal neurons innervate the cholinergic MS/DB neurons (Tóth et al., 1993), we found in the FVB-GIN mice EGFP-expressing axons outlining the contours of many neurons in the MS/DB, including the cholinergic neurons immunolabeled by p75NTR. Overall, these observations provided the prerequisite neuroanatomical basis to assess stimulus-dependent release and modulation of synaptic activity in the MS/DB.

NGF levels in the MS/DB: dependence on intrahippocampal stimulation

Intrahippocampal stimulation elevated NGF levels in the MS/DB in an acute intact HS preparation. The hippocampus was the source of the increased NGF level since, with the fimbria/fornix severed, the intrahippocampal stimulus-evoked effect was abolished, and stimulation of the septum itself did not increase NGF levels. Employing a similar intact preparation, Manseau et al. (2008) recently reported that oscillatory activity in the hippocampus could be relayed via the HS pathway to phase the firing of GABAergic neurons in the MS/DB. Hippocampal rhythmic activity of this nature would be in a position to drive NGF delivery to and release in the MS/DB.

An intriguing and striking finding in the present study was that the stimulus-evoked increase in NGF levels was stimulus pattern-dependent. In the cortex and hippocampus, GABAergic neurons are typically fast-spiking cells (Kawaguchi and Kubota, 1997; McBain and Fisahn, 2001). Among them, the majority of the fast-spiking somatostatin-containing subpopulation tends to fire in 3 Hz bursts of several spikes (Kawaguchi and Kubota, 1996), within the range of theta oscillations. Indeed, when simulated and applied as stimulation paradigm to the intact HS prep-

on sIPSCs in noncholinergic MS/DB neurons (**D**) revealed no changes in either the amplitude (**E**) or frequency (**F**). **G**, Representative traces of averaged evoked IPSCs in a cholinergic MS/DB neuron during control (red), NGF (black), and wash (blue) epochs. **H**, Histogram summarizing the mean amplitude of eIPSCs recorded in cholinergic neurons during each of the three epochs. NGF decreased the mean amplitude of eIPSCs (repeated-measures ANOVA, $**p < 0.01$); the mean amplitude during NGF application was significantly lower compared with control and wash periods (Bonferroni's multiple-comparison tests, $*p < 0.05$).

aration, it was this type of burst-spiking pattern of stimulation of the hippocampus that elevated NGF levels in the MS/DB. We note that our stimulation paradigm does not discriminate as to whether the observed increase in NGF levels is due to translocation or release, be it via either anterograde or retrograde mechanisms. We also note that our NGF ELISA measurements presumably included both intracellular and extracellular NGF. Future studies will continue to explore in the intact HS preparation whether stimulation of the hippocampus or fornix causes release of endogenous NGF and whether this results in modulation GABAergic neurotransmission in the MS/DB.

NGF modulation of inhibitory synaptic activity in cholinergic MS/DB neurons

Assuming that release from HS GABAergic axons into the extracellular milieu contributes to the stimulus-evoked increase in NGF levels in the MS/DB, we applied NGF exogenously to simulate stimulus-evoked increase in extracellular NGF. We found that NGF selectively suppressed inhibitory, vis-à-vis excitatory, synaptic transmission in the MS/DB in the rapid time scale that has been shown for BDNF (Levine et al., 1996, 1998; Blum and Konnerth, 2005; Cheng and Yeh, 2003, 2005). Our results point to the neurotrophin favoring a postsynaptic mechanism; NGF attenuated GABA-evoked current responses and decreased the amplitude of sIPSCs and eIPSCs without altering the frequency of sIPSCs. Exogenously applied GABA most likely activates both synaptic and extrasynaptic GABA_A receptors, and it is possible for NGF signaling to modulate the function of both GABA_A receptor pools. In this light, ongoing studies are investigating whether NGF modulates a recently uncovered GABA_A receptor-mediated tonic conductance in the MS/DB. We are also assessing the existence of an ambient presence of NGF in the MS/DB, and whether it tonically dampens GABAergic neurotransmission in cholinergic MS/DB neurons. Consistent with this notion, results of pilot experiments suggest that applying the NGF scavenger protein TrkA-Fc alone (5 μ g/ml) enhanced GABA-mediated responses in cholinergic MS/DB neurons (L. Guo and H. H. Yeh, unpublished observation).

An issue not addressed in the present study is whether intrahippocampal stimulation induced the release of mature NGF, pro-NGF, or both. Although somewhat controversial, activity-dependent neuronal release of both mature and precursor forms of neurotrophins from central neurons has been reported (Lessmann et al., 2003; Bruno and Cuello, 2006; Matsumoto et al., 2008). Considering that our experiments were conducted using the 2.5S form of NGF, the involvement of mature NGF is likely. Nonetheless, this does not preclude a role for pro-NGF, as both are biologically active (Lu et al., 2005).

Functional implications of anterograde NGF action

What might be the physiological relevance of anterogradely transported NGF from the hippocampus to the MS/DB? The hippocampal GABAergic projection neurons constitute the major, if not the sole, feedback loop for the inflow of activity from the MS/DB to the hippocampus. As a result, these neurons are likely under the control of powerful convergent excitatory and disinhibitory synaptic influences within the hippocampal circuit that, in turn, exert influences through feed back to the MS/DB. Such HS flow of activity has been demonstrated to phase theta-like rhythmic discharges in the MS/DB (Manseau et al., 2008) and, conceivably, could promote anterograde delivery of NGF. Since NGF is present in the GABAergic HS axons, the neurotrophin may be coreleased with GABA, although how this might

occur and the underlying mechanisms remain to be demonstrated experimentally. By suppressing postsynaptic GABA_A receptor function, NGF could keep in check GABA-mediated inhibition in the MS/DB. This, in combination with the previously reported direct and rapid excitatory effect on the firing activity of cholinergic neurons (Wu and Yeh, 2005), serves to maintain an elevated muscarinic tone in the MS/DB and, thereby, heightened hippocampus-bound septal outflow that may underlie the long implicated role of the MS/DB in learning and mnemonic functions (Olton et al., 1978; Poucet and Herrmann, 1990; Dutar et al., 1995).

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