Nerve growth factor acutely potentiates synaptic transmission in vitro and induces dendritic growth in vivo on adult neurons in airway parasympathetic ganglia

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Submitted 13 June 2006; accepted in final form 1 December 2006

Hazari MS, Pan JH, Myers AC. Nerve growth factor acutely potentiates synaptic transmission in vitro and induces dendritic growth in vivo on adult neurons in airway parasympathetic ganglia. Am J Physiol Lung Cell Mol Physiol 292: L992–L1001, 2007. First published December 28, 2006; doi:10.1152/ajplung.00216.2006 — Elevated levels of nerve growth factor (NGF) and NGF-mediated neural plasticity may have a role in airway diseases such as asthma and chronic obstructive pulmonary disease (COPD). Although NGF is known to affect sensory and sympathetic nerves, especially during development, little is known regarding its effect on parasympathetic nerves, especially on adult neurons. The purpose of this study was to analyze the acute and chronic effects of NGF on the electrophysiological and anatomical properties of neurons in airway parasympathetic ganglia from adult guinea pigs. Using single cell recording, direct application of NGF caused a lasting decrease in the cumulative action potential afterhyperpolarization (AHP) and increased the amplitude of vagus nerve-stimulated nicotinic fast excitatory postsynaptic potentials. Neuronal responsiveness to nicotinic receptor stimulation was increased by NGF, which was blocked by the tyrosine kinase inhibitor, K-252a, implicating neurotrophin-specific (Trk) receptors. Neurotrophin-3 and brain-derived neurotrophic factor had no effect on the synaptic potentials, AHP, or nicotinic response; inhibition of cyclooxygenase with indomethacin inhibited the effect of NGF on the cumulative AHP. Forty-eight hours after in vivo application of NGF to the trachealis muscle caused an increase in dendritic length on innervating neurons. These results are the first to demonstrate that NGF increases the excitability of lower airway parasympathetic neurons, primarily through enhanced synaptic efficacy and changes to intrinsic neuron properties. NGF also had dramatic effects on the growth of dendrites in vivo. Such effects may indicate a new role for NGF in the regulation of parasympathetic tone in the diseased or inflamed lower airways.

asthma; remodeling; bronchus; trachea; neurotrophins; synapse; autonomic

NEUROTROPHINS ARE USUALLY associated with the development, differentiation, and survival of embryonic and neonatal parasympathetic neurons, but there is a recent interest in how these molecules affect neurons in adult animals, especially in the airways where neurotrophins may have a role in modulating airway and allergic diseases (12, 37, 49). The first-discovered and prototypical neurotrophin is nerve growth factor (NGF); the other known neurotrophins are brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and NT-4/5. Neurotrophins act on a family of receptor tyrosine kinases first identified as tropomyosin-related kinase (Trk) receptors associated with tyrosine kinase activity, NGF preferentially activates TrkA receptors, BDNF and NT-4/5 are selective for TrkB receptors, and NT-3 is selective for TrkC receptors (reviewed in Ref. 18). NGF is elevated in the serum of allergic individuals (3) and can also be found in bronchial lavage fluid during allergic reactions in adults (47) and viral infection in infants (45). The sources of NGF in the airway include nerves (48), infiltrating cells, fibroblasts, smooth muscle, epithelium (42), and glands (48), most of which are near neuronal processes (41). Although it is known that transgenic mice overexpressing NGF during development have altered sensory and sympathetic innervation in the lower airways (13; reviewed in Ref. 12), and NGF enhances lower airway cholinergic contractile response in vitro (2), no studies have been directed at determining the cellular mechanism by which NGF affects parasympathetic nerve excitability in adult animals.

Parasympathetic cholinergic nerve fibers located in tracheal and bronchial smooth muscle have their cell bodies in nearby clusters referred to as ganglia. An important property of neurons in lower airway parasympathetic ganglia is to regulate signals emanating from the central nervous system (CNS) (17, 26, 35), and alterations in this regulation can greatly affect the tone of airway smooth muscle (5, 33). It is well known that airway caliber is regulated by parasympathetic nerves, but it has only recently become apparent that alterations in these nerves may be associated with changes in diseased or damaged airways (50, 53): such alteration may be caused by neurotrophins (14) or related neurokines (51). Although several studies have reported increases in neurotrophins in the airway that may mediate such plasticity (45, 47), little is known about the role neurotrophins may have in the manifestation of airway diseases such as asthma (6, 38) and, in particular, their effects on the activity of airway parasympathetic neurons that mediate airway smooth muscle contraction. Changes in structure and function of these neurons, known as neural plasticity, may therefore represent an important facet of airway disease pathophysiology associated with neurotrophins. In the present study, we provide evidence that NGF contributes to enhanced excitability of parasympathetic neurons and affects anatomical properties of neurons in the airways of adult animals.

MATERIALS AND METHODS

Animals. The methods for animal use were approved by the Johns Hopkins Animal Care and Use Committee, The Johns Hopkins Uni-
versity, Baltimore, MD. Male Hartley albino guinea pigs (Hilltop, Scottsdale, PA), weighing 200–300 g, were used in this study.

**Immunohistochemical staining.** For immunofluorescent staining of the high affinity receptor for NGF (TrkA) receptors, we used tyramide signal amplification. Guinea pigs were killed by an overdose of pentobarbital (150 mg/kg ip) and transcardially perfused with phosphate-buffered saline (PBS; pH 7.4) containing heparin (100 U/ml) and procaine (0.2%) followed by fixative (4% formaldehyde in PBS) and post-fixed for 2 h (4°C). After rinsing in PBS (24 h, 4°C), 3-mm squares of dorsal trachealis muscle containing a parasympathetic ganglion was cryoprotected in 18% sucrose in PBS (24 h, 4°C), frozen in optimum cutting temperature (OCT) mounting medium, and alternating serial transverse sections (10 μm) were collected on lysole-coated slides and air-dried. Endogenous peroxidase activity was blocked with hydrogen peroxide (0.5% in 50% methanol in PBS, 30 min). To minimize nonspecific binding of the secondary antibody (raised in goat), blocking solution containing 10% goat serum (and 1% BSA and 0.1% Tween 20) in PBS was applied for 1 h at room temperature. The sections were then incubated overnight (4°C) in a mixture of mouse antibody recognizing choline acetyltransferase (ChAT; 1:25 dilution; Chemicon, Temecula, CA) and rabbit antibody recognizing NH2 terminus of TrkA (1:50; Santa Cruz Biotechnology, CA) and rabbit antibody recognizing cysteine-rich repeats of the low affinity 75-kDa neurotrophin receptor (p75ntr; 1:200; Sigma Chemical, St. Louis, MO) diluted in PBS containing 1% BSA, 0.5% Triton X-100. For negative control, separate sections were processed similarly, but the primary antibody was replaced with rabbit IgG to evaluate nonspecific staining; positive controls were guinea pig dorsal root ganglia and superior cervical ganglia. After rinsing, a tyramide signal amplification kit (Molecular Probes, Eugene, OR) was used; briefly, sections were incubated with peroxidase-conjugated goat antirabbit or goat anti-mouse immunoglobulin (1 μg/ml) and then with Alexa Fluor 568-conjugated tyramide in amplification solution. Washed slides were coversoned with Tris-buffered glycerol (pH 8.6), viewed, and photographed with an epifluorescence microscope (Olympus BX50; Olympus America, Melville, NY).

**Electrophysiology tissue preparation and recording.** Guinea pigs were euthanized with an overdose of pentobarbital (150 mg/kg ip), exsanguinated, and transcardially perfused with Krebs bicarbonate buffer (60 ml, 20–22°C); composition of this buffer was (in mM): NaCl, 136; KCl, 5.6; MgCl2, 1.2; CaCl2, 2.2; NaH2PO4, 1.2; NaHCO3, 14.3; and dextrose, 11; equilibrated with 95% O2/5% CO2 (pH 7.4). The trachea was separated from the right mainstem bronchus (pH 7.4). The trachea was separated from the mainstem bronchus medially. The mainstem bronchus was then separated from the secondary bronchus laterally. The bronchus was separated and dissected, keeping the right vagus nerve intact and connected to the bronchus by the peribranchial nerves to maintain preganglionic innervation. The trachea or bronchus were then cut longitudinally along the ventral midline, opened, and tightly pinned as a sheet, with the dorsal surface of the tissue facing upwards, to Sylgard (Dow Corning, Midland, MI), which lined the floor of a Petri dish. The tissue was dissected in flowing (5 ml/min) Krebs bicarbonate buffer at 20–22°C for up to 1 h before equilibration and electrophysiological experimentation. Unstained ganglia were exposed in the trachea and bronchus by fine dissection with the aid of a stereomicroscope at magnifications of ×25–80, using reflected transmitted light as previously described (30). Tracheal ganglia were located on or within the trachealis smooth muscle by following inlet nerves arising from the recurrent laryngeal nerves. Any overlying connective tissue was gently dissected away to allow unhindered penetration by the microelectrode.

After identifying several ganglia, the trachea or bronchus were pinned to the Sylgard-coated floor of a recording chamber with Z-shaped pins. The chamber contained a channel running along its length with a widened area (0.2 ml volume) in the center for pinning out the tissue. The recording chamber was then transferred to the fixed stage of a compound microscope equipped with a long working-

distance objective (×20). Krebs bicarbonate buffer (above) was superfused over the tissue continuously, being introduced at one end of the chamber and suctioned away at the other. Using a suction electrode, the vagus nerve was pulled into a capillary tube containing, and surrounded by, chloridized silver wires, which were connected to a stimulator (Grass, Astro-Med, West Warwick, RI) for preganglionic nerve stimulation and generation of fast excitatory postsynaptic potentials (fEPSPs). Ganglia could then be visualized (×200) for impalement of neurons with a microelectrode. The tissue was allowed to equilibrate for at least 1 h in flowing (5–8 ml/min) Krebs buffer at 36–37°C in the recording chamber before experimentation.

Intracellular recording techniques were similar to those previously reported (5, 29). Changes in the resting membrane potential and input resistance (Ri) were noted in the presence of vehicle (as control) before drug application and during and after application of NGF-β (rat recombinant) or application of the nicotinic receptor agonist, 1,1-diethyl-4-phenylpiperazinium (DMPP). Vehicle controls were performed using PBS diluted ≥1,000-fold or 50% DMPP diluted 10,000-fold. Control active membrane property measurements were recorded before NGF-β application as follows: both tracheal and bronchial neurons were classified as either tonic or phasic as previously described (29). Tonic neurons responded to suprathreshold (0.5–2.0 nA) current steps (500 ms) with continuous action potentials throughout the stimulus, whereas phasic neurons responded with either one or a burst of action potentials in the initial 50 ms of the stimulus but then accommodated. The duration (from end of last action potential spike to return to prestimulus resting potential) and amplitude (from prestimulus resting potential to peak) of the action potential and the afterhyperpolarization (AHP) were monitored for a single action potential (2 ms, 2 nA stimulus) and four consecutive (2 ms, 4 nA, 40-Hz stimuli) action potentials (cumulative AHP). The neurons were then exposed to NGF-β (1, 10, or 100 ng/ml) for 2 min, and active membrane properties were determined on cessation of exposure (time 0 in figures) and at 5-min intervals thereafter. In all experiments, only one concentration of NGF (or other neurotrophins) and one neurotrophin was applied to a single neuron.

fEPSPs were elicited by 1-Hz square pulses delivered to the rostral end of the vagus nerve, 10–30 mm from the ganglion. These stimuli ranged from 5–40 V and 0.02–0.8 ms in duration (voltage and duration adjusted to obtain subthreshold fEPSPs if necessary). One hundred consecutive vagus nerve-evoked fEPSPs were averaged for the control response, and the neurons were then exposed to NGF-β (1, 10, or 100 ng/ml) for 2 min, and the same vagus nerve stimulation was repeated on cessation of exposure to NGF-β. In most preparations, atropine (0.1 μM) was added to the buffer to prevent the effects of muscarinic receptor stimulation on neuronal properties (34) and smooth muscle contraction (46). The effect of NGF-β on fEPSPs was analyzed for as long as the recording was maintained, up to 30 min.

The nicotinic responsiveness of these neurons was assessed by superfusion of the neurons with DMPP, diverted from an inline reservoir for a period of 10 s, and the resulting brief depolarization was monitored on a chart recorder and digital readout of the membrane potential. Neurons depolarized from resting potential and returned to rest within 30 s. Increasing concentrations of DMPP (10, 50, and 100 μM) were administered to each cell with 5-min intervals between concentrations for generation of a control DMPP concentration–response curve. The neurons were then exposed to NGF-β (1, 10, or 100 ng/ml) for 2 min, and the DMPP concentration response was repeated on cessation of exposure to NGF-β.

To ensure that NGF-β was not cross-reacting with other Trk receptors, nicotinic responsiveness was repeated with other neurotrophins. Involvement of tyrosine kinase in mediating changes in nicotinic responsiveness was evaluated by measuring responses to 50 μM DMPP before and after NGF-β (10 ng/ml) in the presence of the tyrosine kinase inhibitor K-252a (200 nM; Ref. 16). Involvement of cyclooxygenase in NGF-mediated changes in AHP, synaptic potentials, and nicotinic responsiveness was evaluated by measuring these
responses before and after NGF-β (10 ng/ml) in the presence of the nonspecific cyclooxygenase inhibitor, indomethacin (30 μM), which was added to the Krebs buffer during dissection, equilibration, and experimentation.

**Dendritic morphology.** To expose parasympathetic ganglionic neurons to NGF in vivo, a method used for dye injection was employed as previously described (32). The ventral surface of the cervical trachea was exposed in an anesthetized (50 mg/kg ketamine hydrochloride, 2.5 mg/kg xylazine hydrochloride im) guinea pig, and a fine needle was passed through the tracheal lumen into the trachealis muscle. NGF-β (100 ng/ml in sterile saline) was micro-injected into the cervical and thoracic trachealis muscle (5 sites, 2 μl per site) on the dorsal wall of the trachea. In initial experiments (n = 3 animals), 1% rhodamine-dextran (Molecular Probes) was included with NGF-β to demonstrate that neurons that take up the dye most likely were also exposed to NGF-β; dye injection without NGF-β was also performed as a control (n = 3). Previously, we analyzed the distribution of dye using this method (32) and were satisfied that we were injecting predominantly into the smooth muscle. Based on results from experiments using dye plus NGF-β injections, all neurons were equally affected, and the remaining experiments (n = 4 animals) were performed without the dye. After 48 h, guinea pigs were killed by an overdose of pentobarbital (150 mg/kg ip) and transcardially perfused with oxygenated Krebs buffer (20°C, above). The trachea was exposed in an anesthetized (50 mg/kg ketamine hydrochloride, 2.5 mg/kg xylazine hydrochloride im) guinea pig, and a fine needle was passed through the tracheal lumen into the trachealis muscle. NGF-β (100 ng/ml in sterile saline) was micro-injected into the cervical and thoracic trachealis muscle (5 sights, 2 μl per sight) on the dorsal wall of the trachea. In initial experiments (n = 3 animals), 1% rhodamine-dextran (Molecular Probes) was included with NGF-β to demonstrate that neurons that take up the dye most likely were also exposed to NGF-β; dye injection without NGF-β was also performed as a control (n = 3). Previously, we analyzed the distribution of dye using this method (32) and were satisfied that we were injecting predominantly into the smooth muscle. Based on results from experiments using dye plus NGF-β injections, all neurons were equally affected, and the remaining experiments (n = 4 animals) were performed without the dye. After 48 h, guinea pigs were killed by an overdose of pentobarbital (150 mg/kg ip) and transcardially perfused with oxygenated Krebs buffer (20°C, above). The trachea was prepared for electrophysiological recording as described above. In initial experiments, tracheal ganglionic neurons that project axons to the tracheal muscle were identified by the accumulation of rhodamine-dextran in their cell bodies. The method used for ionophoresis of dye into identified neurons and analysis of dendrites is similar to those previously described (30) and as described above for intracellular recordings, except the microelectrodes were filled with Neurobiotin (2% in 1 M KCl; Vector Laboratories, Burlingame, CA). Once electrophysiological membrane properties were characterized (see above), Neurobiotin was iontophoretically injected into the neuron by passing 3-nA anodal current steps, 150 ms in duration, at a rate of 3 Hz for 2 or more minutes. The electrode was withdrawn, and the tissue perfused in the recording chamber for an additional hour. The tissue was then fixed in 4% formaldehyde in PBS for 2 h at 4°C and rinsed in PBS. Once injected, the Neurobiotin was either developed for histochemical (avidin-biotin peroxidase complex and diaminobenzidine kits, Vector Laboratories) or fluorescence (avidin-D fluorescine, Vector Laboratories) staining, and the somal size, length of dendritic processes, and dendritic branching and “sprouting” were measured (30). No differences in dendritic length or number for control neurons was observed using these two methods of Neurobiotin development (not shown). Previously (30), sprouting of dendritic terminals (dendrites that terminate with 3 or more short, <5 μm branches) was not seen in control neurons. Three-dimensional measurements of dendrites were made from whole mount preparations of injected neurons at a magnification of ×200 using deconvoluted images with length- and volume-rendering analysis program (IPLab, Biovision Technologies, Exton, PA).

**RESULTS**

**TrkA immunofluorescent staining.** Ganglia (n = 6) containing 6–11 neurons (8.5 ± 0.4 neurons per ganglion; n = 51 neurons) were located on the serosal surface from four guinea pig tracheas. These ganglia were in a similar location as ganglia in guinea pig tracheas containing neurons reported to be cholinergic, based on their expression of ChAT (Fig. 1, A and C; Ref. 4). Sections of neuronal cell bodies in these tracheal ganglia had varying levels of TrkA-immunoreactivity (Fig. 1B) and p75ntr (Fig. 1D). Neurons in sections of similar ganglia used for negative

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**Fig. 1.** p75ntr and tropomyosin-related kinase A (TrkA) receptors on neurons in guinea pig tracheal parasympathetic ganglia. **A:** guinea pig bronchial neurons (n) show positive choline acetyltransferase immunoreactivity (ChAT-ir). **B:** in the same section as A, there is TrkA immunoreactivity (Trk-ir; at arrows) in several neurons. **C:** the neighboring section of the same ganglion used for A shows ChAT-ir. **D:** the same section as C shows scant p75ntr immunoreactivity (p75ntr-ir; at arrows). Scale bar in D is for all images.
controls (no specific primary antibody) had no or sparse staining dispersed outside of the neuronal cell bodies.

**Electrophysiological recording.** Intracellular recordings of passive and active membrane properties were made from the cell bodies of neurons located in tracheal and bronchial parasympathetic ganglia. Consistent with previous studies of guinea pig airway parasympathetic neurons (5, 30), resting membrane potential and membrane resistance ranged from -38 to -70 mV and 38 to 66 MΩ, respectively, and did not change significantly during the course of experimentation or in the presence of or following NGF-β (1–100 ng/ml, n = 5–11) exposure. In control phasic neurons (29, 30), a prolonged, suprathreshold depolarizing pulse (500 ms, 1.0 nA) elicited 3.3 ± 0.7 (n = 6) action potentials, followed by accommodation to the stimulus. There was no significant change in the accommodation properties of these neurons during a 2-min application of 1, 10, or 100 ng/ml of NGF-β (4.3 ± 0.9 action potentials; n = 4–6 for each concentration) or following exposure (up to 30 min). Control tonic neurons responded to a similar suprathreshold stimulus by generating 19 ± 2 action potentials (n = 6) and, after treatment with NGF-β (1, 10, or 100 ng/ml, n = 3–6), 18 ± 3 action potentials. As tonic and phasic neurons have similar single action potential characteristics (29, 35), and NGF-β had no effect on accommodation, the following data were pooled and averaged from both tonic (n = 6) and phasic (n = 5) neurons: there was no significant change in the control single action potential spike duration (5.7 ± 0.3 ms), single spike AHP duration (67 ± 11 ms), or AHP amplitude (17 ± 2 mV) during or following 100 ng/ml NGF-β (5.8 ± 0.3 ms, 51 ± 13 ms, and 19 ± 3 mV, respectively; not shown). However, following four action potentials (cumulative AHP), there was a significant decrease in cumulative AHP duration from 186 ± 14 ms (control) to 148 ± 6 ms after NGF-β (100 ng/ml), a decrease of 22% (n = 8; P < 0.05; no difference in tonic and phasic neurons). This decrease was not immediate, i.e., no change on cessation of NGF-β application (time 0 in Fig. 2C), peaked at 10 min post-NGF-β (Fig. 2, A and C), and lasted as long as the cell remained impaled (up to 30 min). The lowest concentration of NGF-β tested (1 ng/ml) decreased AHP duration by 19% (P < 0.05; n = 4), and indomethacin (30 μM) inhibited the decrease in cumulative AHP duration evoked by NGF-β (1 ng/ml, n = 5; Fig. 2, B and D). The traces in Fig. 2, A and B, are representative traces from two different populations that were analyzed. NT-3 (100 ng/ml, n = 4) or BDNF (100 ng/ml, n = 4) had no effect on active or passive electrophysiological properties (not shown); at this concentration, these neurotrophins would most likely activate both low (p75ntr) and high affinity receptors, but, as this concentration had no effect, we did not investigate the effects of these neurotrophins at lower concentrations.

To determine whether NGF-β affected synaptic transmission, fEPSPs were measured before and after a 2-min exposure to NGF-β. In control neurons, vagus nerve stimulation elicited fEPSPs that were subthreshold for action potential formation and had an amplitude of 6.2 ± 1.1 mV (n = 6; Fig. 3A). After

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**Fig. 2.** Nerve growth factor (NGF)-β decreases the duration of the cumulative spike afterhyperpolarization (AHP). A: in left trace from a control neuron, the cumulative AHP of 184 ms is generated following 4 action potentials stimulated at 40-Hz, 3-nA pulses; dashed line represents the level of the prestimulus resting potential, and the downward arrow represents the endpoint were the AHP returns to the resting potential. In A, right trace, in the same neuron, the AHP is 150 ms, 10 min after NGF-β (100 ng/ml) exposure; dashed line and arrow as in left trace. B: in a different neuron, left trace shows the control AHP in the presence of indomethacin (Indo.; 30 μM) is 190 ms, and, in the right trace, 10 min after NGF-β (100 ng/ml) exposure in the presence of indomethacin (30 μM), the AHP duration (192 ms) is unaffected. Peaks of action potentials are cut off in all traces; scale bars are for all traces. C: summary of decrease in AHP duration before and after NGF-β (100 ng/ml) over time (0–10 min; n = 6); 0-min time point measurement was taken at the end of the 2-min NGF-β exposure. * P < 0.05 compared with vehicle control. D: NGF-β (100 ng/ml) had no effect on AHP duration at any time point in the presence of indomethacin (30 μM; n = 4); 0-min time point measurement was taken at the end of the 2-min exposure to NGF.
Fig. 3. NGF potentiates synaptic transmission. A: vagus nerve-stimulated (at shock artifact, upward arrow) fast excitatory postsynaptic potentials (fEPSPs; downward arrow) recorded in a control bronchial parasympathetic ganglionic neuron, trace shows 10 superimposed consecutive traces. B: in the same neuron as A, vagus nerve-stimulated fEPSP (downward arrow) amplitude is potentiated following application of NGF-β (100 ng/ml). Scale bars in B are for traces in A and B. C: summary of the effect of exogenous application of NGF-β on fEPSP amplitude; *P < 0.05 compared with controls. D: vagus nerve stimulation (shock artifact, upward arrow) produces 2 subthreshold fEPSP populations (downward arrows). E: in the same cell as D, NGF-β (100 ng/ml) causes vagus nerve-stimulated fEPSP to be potentiated to action potential threshold (arrow) and a new population appears (at asterisk). Scale bars in E are for traces in D and E.

Exposure to NGF-β (100 ng/ml), the amplitude of fEPSPs was significantly increased to 11.8 ± 4.8 mV in all neurons (n = 5; P < 0.05; Fig. 3, B and C). Lower concentrations of NGF-β, 1 ng/ml (n = 4) and 10 ng/ml (n = 4), potentiated fEPSPs by 31% and 67%, respectively. The increase in fEPSP amplitude (90%, compared with control) following exposure to 100 ng/ml NGF-β caused fEPSPs to reach action potential threshold (e.g., Fig. 3, D and E) in two of six neurons. We also observed that treatment with NGF-β (100 ng/ml) initiated temporally distinct fEPSPs that were not present before treatment (Fig. 3E) in two of five neurons. Indomethacin (30 μM) had no effect on the NGF-β-induced potentiation of fEPSP amplitude evoked by two different concentrations of NGF-β (1 ng/ml, n = 3; 100 ng/ml, n = 3; not shown). NT-3 (100 ng/ml, n = 4) and BDNF (100 ng/ml, n = 4) had no effect fEPSP amplitude (not shown); for reasons stated above, we did not investigate the effects of these neurotrophins at lower concentrations. The effect of NGF (10 or 100 ng/ml) on synaptic potentials was not different in tonic (n = 4) and phasic neurons (n = 6; not shown).

To determine if NGF was affecting nicotinic receptor channels, depolarization evoked by the nicotinic agonist DMPP (10, 50, or 100 μM) was also assessed before and after administration of NGF-β; 10 μM DMPP was fivefold above the threshold concentration for depolarization (35). For 100 ng/ml NGF-β, the responses to 10 μM, 50 μM, or 100 μM DMPP were increased by 11–46% (Fig. 4B) compared with control responses (P < 0.05 for all DMPP concentrations; Fig. 4, A and C). Lower concentrations of NGF-β (1 or 10 ng/ml) also increased the DMPP response. For example, DMPP elicited control responses of 6.0 ± 2.0 mV (10 μM), 10 ± 1 mV (50 μM), and 17.5 ± 1.5 mV (100 μM), and immediately following application of NGF-β (1 ng/ml), the response to 10 μM DMPP was 9.5 ± 0.5 mV, 50 μM DMPP was 17 ± 1 mV, and 100 μM DMPP was 31.5 ± 3.5 mV (n = 4; P < 0.05 for all DMPP concentrations; Fig. 4D). A concentration of 10 ng/ml NGF-β potentiated DMPP responsiveness to a similar level (n = 4). To examine whether the effects of NGF-β were mediated by TrkA receptor-associated tyrosine kinases, nicotinic responsiveness to DMPP was measured before and after NGF-β in the presence of the tyrosine kinase inhibitor, K-252a. K-252a (200 nm) blocked the increase in responsiveness to 50 μM DMPP observed following NGF-β treatment (Fig. 5, B and C). NT-3 (100 ng/ml, n = 4) and BDNF (100 ng/ml, n = 4) had no effect on nicotinic receptor stimulation (not shown); as stated above, at this concentration, these neurotrophins would most likely activate both low and high affinity receptors.

Effects of in vivo NGF on cell structure. NGF-β (100 ng/ml) or vehicle control were injected into the trachealis muscle with or without rhodamine-dextran, and, 24 or 48 h later, tracheal ganglionic neurons that project axons to that region of the trachealis muscle were filled with Neurobiotin, developed, and analyzed for changes in cell structure. There was a trend...
toward increased dendritic growth 24 h after NGF-β injection, but dendritic lengths of NGF-β-treated neurons were not significantly different than controls (n = 6; not shown). In 48 h-vehicle control-treated animals, tracheal neurons (n = 6) were oval in shape with minimum and maximum axes of 20 ± 2 and 32 ± 2 μm, respectively; each neuron had, on average, 4 ± 1 dendrites and average total dendritic length of 878 ± 104 μm (range of 405–1,088 μm) per neuron; an example of a control neuron is shown in Fig. 6A. There were no differences in cell body size or the average total dendritic length for neurons exposed to vehicle with rhodamine-dextran 48 h before (948 ± 78 μm; n = 3) or for vehicle only (809 ± 207 μm; n = 3). Forty-eight hours after NGF-β (100 ng/ml) exposure, there was no change in cell body size (22 ± 3 by 34 ± 4 μm; n = 6), and the number of dendrites remained the same (5 ± 1), but there was a near doubling in dendritic length (1,525 ± 190 μm per neuron; P < 0.05) and a >10-fold increase in sprouting of dendritic endings (2.75 per neuron for the NGF-treated cells, Fig. 6B, compared with 0.17 per neuron for controls). The number of dendritic branch points on each neuron was the same in the two groups (3.7 ± 1.1 for control, 4.9 ± 1.4 for NGF-β treated; P > 0.05).

DISCUSSION

In the present study, we provide evidence that the effects of NGF-β include acute changes in fully developed parasympathetic ganglionic neurons. Such effects are most likely due to activation of specific receptors for NGF, and, based on immunohistochemical staining, we provide anatomical evidence that adult parasympathetic neurons express TrkA, the high affinity receptor for NGF. The acute effects discussed below include changes in electrophysiological properties, increased synaptic efficacy and nicotinic responsiveness; furthermore, we provide evidence that prior exposure to NGF-β induces change in neuroanatomy of ganglionic neurons.

The active membrane properties of neurons are associated with action potential threshold, duration, accommodation, and AHP; current carried by various ion channels regulate these properties and thus determine whether and how the neuron fires action potentials (reviewed in Ref. 31). Many studies have demonstrated that neurotrophins can alter the expression of specific ion channel proteins associated with action potential characteristics (e.g., Ref. 10) but relatively few studies have reported immediate effects of neurotrophins on electrophysiological properties, and these are usually on developing or cultured neurons. For example, NGF prolongs the action potential duration of cultured sensory neurons from mature mice (43) and BDNF rapidly depolarizes neonatal CNS neurons (15). In our study, NGF-β had immediate effects on the cumulative action potential AHP duration but did not affect other measured action potential or passive membrane properties (resting potential and resistance). The NGF-induced decrease in action potential AHP duration may be due to a decrease in calcium ion current or a direct effect on potassium and possibly chloride channels. As NGF-β had no effect on action potential duration, alteration of calcium current is unlikely (29); this result is similar to that observed in a previous study with bronchial ganglionic neurons where prostaglandin E2 inhibited the AHP following repetitive action potentials with no effect on action potential duration or accommodation (17). Such a decrease in AHP duration most likely indicates an inhibition of the calcium-activated potassium current that is not associated with accommodation in these cells (29). Because of the relative refractory period associated with the AHP, NGF-induced shorter AHPs may increase the likelihood for generating succeeding synaptic and action potentials and thus decrease filtering of preganglionic input (31).

Synaptic efficacy in airway parasympathetic ganglionic neurons can be affected by changes in electrophysiological and/or anatomical properties of both preganglionic and postganglionic elements. As an impulse is conducted along the preganglionic fiber, the possibility of generating an above-threshold fEPSP in the ganglionic cell depends on the number of activated synapses as well as the quantity of acetylcholine released at those
synapses. At the postganglionic site, synapses on guinea pig airway parasympathetic ganglionic neurons are predominantly located on dendritic processes (30), and thus the number and length of dendrites affect how the cell integrates the preganglionic impulse into an action potential (reviewed in Ref. 24). In addition to anatomical characteristics, active and passive membrane properties, response of neuronal nicotinic receptors, and recent activity of the neuron also affect neuronal integration of preganglionic synaptic input (reviewed in Ref. 31). Several mediators have been shown to increase synaptic efficacy in lower airway parasympathetic ganglionic neurons. Inflammatory mediators such as prostaglandin F2α potentiate fEPSP amplitude (17); similar elevations in fEPSP amplitude are reported for bronchial parasympathetic ganglionic neurons following tachykinin neurokinin-3 receptor stimulation, most likely by a preganglionic mechanism (5). As it has been reported that NGF enhances neurotransmitter release from sympathetic nerve fibers by TrkA receptor activation (52), in the present study, elevation of acetylcholine release from preganglionic fibers induced by NGF-β may be a mechanism for higher fEPSP amplitudes observed in airway parasympathetic ganglionic neurons. Stimulating the vagus nerve with a single pulse can produce one to multiple distinct fEPSP populations in guinea pig (35) and human (16) airway parasympathetic neurons, indicating that multiple preganglionic axons are converging on the same neuron (reviewed in Ref. 31). In this study, NGF-β not only increased the amplitude of existing fEPSPs, but, in some neurons, initiated the generation of new fEPSP populations that were not obvious before treatment (Fig. 3), possibly indicating a preganglionic mechanism (52). NT-3 and BDNF also modulate the efficacy of synaptic transmission at a presynaptic site via the Trk receptors on CNS neurons (reviewed in Ref. 39). However, NT-3 and BDNF had no effect on membrane properties or synaptic transmission in the present study.
concentrations of NGF-nicotinic agonist, DMPP, was determined. We observed that all investigate postganglionic mechanisms, sensitivity to the nicotinic cholinergic preganglionic axon transduce the impulse from the cholinergic preganglionic axon to postsynaptic current in hippocampal neurons (22), most likely by phosphorylation of specific N-methyl-D-aspartate (NMDA) receptor subunits (23), which increases probability of opening NMDA receptor channels (21). It is possible that TrkA activation by NGF mediates phosphorylation of nicotinic acetylcholine receptors (nAChRs) in ganglionic neurons and elevates cation influx through these channels, as has been reported for TrkB receptors in CNS neurons (15). Intrinsically to the neurons in parasympathetic ganglia are ionotropic nicotinic receptors that transduce the impulse from the cholinergic preganglionic axon to the dendrites and cell body of the postganglionic neuron. To investigate postganglionic mechanisms, sensitivity to the nicotinic agonist, DMPP, was determined. We observed that all concentrations of NGF-β tested increased nicotinic agonist-induced depolarization of the ganglionic neurons. That the tyrosine kinase inhibitor, K-252a, inhibited the nicotinic hyperresponsiveness indicates there may be an interaction between the intracellular domains of the TrkA receptors, which possess tyrosine kinase activity, and the nAChR cation channel. It could also be due to secondary activation of related kinases such as Fyn (27). It is important to note that we ruled out activation of other Trk receptors or the p75 receptors, as the nicotinic responsiveness was potentiated by a low concentration of NGF-β (1 ng/ml), which should only activate TrkA receptors and not p75 low affinity receptors, as well as the lack of effect of BDNF or NT-3, which, at the concentration used, should activate specific Trk receptors as well as p75 receptors. NGF may acutely increase the probability of opening nAChRs and amplify depolarization in the airway ganglionic neuron. Increased responsiveness to nicotinic agonists may contribute to the elevation of fEPSP amplitude in ganglionic neurons, a response that is observed following treatment with NGF-β.

As many of the effects of NGF were similar to those induced by prostaglandins (17), and NGF is reportedly capable of activating the release of prostaglandins from mast cells (28) and structural cells (9) in rats, we attempted to block the effects of NGF-β with the nonspecific cyclooxygenase inhibitor, indomethacin. Indomethacin had no effect on the ability of NGF-β to alter synaptic transmission or on the potentiated nicotinic response. However, indomethacin reduced the effect of NGF-β on the cumulative AHP, indicating that NGF-β may be acting indirectly by activation of cyclooxygenase. It is most likely that this secondary effect of NGF-β is not due to mast cell activation: cyclooxygenase stimulation due to specific antigen challenge in the guinea pig bronchus is associated with the release of high levels of prostaglandin D₂ (17), a prostaglandin that causes hyperpolarization of the resting potential, an increase in membrane resistance, and dramatically reduces accommodation by neurons in guinea pig bronchial parasympathetic ganglia (36). That NGF-β did not cause any of these effects but did decrease the cumulative AHP duration, similar to prostaglandin E₂, indicates that NGF-β may be causing activation of a specific tissue/cell subtype, such as fibroblasts, that selectively produces prostaglandin E₂ (9).

It is well known that neurotrophins play a major role in regulating the development, maintenance, and growth of neuronal dendrites and axons in the CNS; however, little is known about the function of these molecules on principal neurons in parasympathetic ganglia in adult animals. Several studies have shown that NGF increases the length of dendrites on parasympathetic neurons in vitro (25) and on sympathetic neurons in vivo as well as enhancing neurite extension in vitro (20, 40, 44). Like neurons in rodent and guinea pig sympathetic ganglia (11), neurons in guinea pig lower airway parasympathetic ganglia have multiple and branching dendritic processes (19, 30, 35). We injected NGF-β into the trachealis muscle, a primary site for innervation by cholinergic neurons located in the tracheal ganglia (4) and, after 48 h, filled the ganglionic neurons with an intracellular dye. Control tracheal neurons had a similar size and dendritic complexity as previously reported (19). Neurons exposed to NGF-β showed an increase in dendritic length after 48 h compared with neurons exposed to vehicle and/or rhodamine-dextran alone (Fig. 6). We also noted an increase in the number of dendrites that displayed sprouting. Sprouting (dendrites that terminate with 3 or more short, <5 μm branches) was only observed on one dendrite on one of six control neurons, which is in dramatic contrast to neurons treated with NGF-β. Dendritic sprouting precedes dendritic growth or division in the mammalian nervous system (e.g., Ref. 8). Such growth in dendritic process may be trophic for preganglionic fibers to increase input to individual ganglionic neurons (7). Keeping in mind that neuronal plasticity due to NGF in the airways thus far has primarily focused on sensory and sympathetic nerves (13, 14), our findings are unique in that NGF-β also affects neuronal growth of efferent ganglionic neurons, which are associated with controlling airway caliber.

In conclusion, we have demonstrated a heretofore unrealized role for NGF in regulation of adult airway parasympathetic nerves. That NGF-β induces changes in excitability of airway parasympathetic neurons, possibly at both presynaptic and postsynaptic sites, indicates a unique function for this neurotrophin in regulation of parasympathetic tone in the lower airways. Mechanistically, these changes may involve enhanced neurotransmitter release, which supports the concept that neurotrophins can act as both trophic factors and regulatory molecules. Furthermore, we provide convincing evidence that NGF can alter the dendritic architecture of adult parasympathetic neurons in vivo; whether this translates to increased synaptic input, or possibly inhibition of synaptic activity, remains to be determined. Given the possibility that NGF may play a role in airway diseases such as asthma and viral infection, the data presented here demonstrate that such elevations of NGF may affect the nerves that directly regulate airway caliber and thus airflow to the lungs.
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