Responsiveness to nicotine of neurons of the caudal nucleus of the solitary tract correlates with the neuronal projection target

Lin Feng,* Evgeny A. Sametsky,* Alexander G. Gusev, and Victor V. Uteshev

Department of Pharmacology, Southern Illinois University School of Medicine, Springfield, Illinois

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Responsiveness to nicotine of neurons of the caudal nucleus of the solitary tract correlates with the neuronal projection target. J Neurophysiol 108: 1884–1894, 2012. First published July 18, 2012; doi:10.1152/jn.00296.2012.—The caudal nucleus of the solitary tract (NTS) is the key integrating center of visceral sensory-motor signaling supporting autonomic homeostasis. Two key projections of this nucleus are the parabrachial nucleus (PbN) and the dorsal motor nucleus of the vagus (DMV). The PbN integrates and relays viscerosensory information primarily to the forebrain, supporting emotional, and endocrine responses to visceral events, while the DMV contains parasympathetic preganglionic cholinergic motoneurons that support primarily gastrointestinal reflexes. Subsets of caudal NTS neurons express presynaptic and somatodendritic nicotinic acetylcholine receptors (nAChRs). However, the anatomical identification of nicotine-responsive caudal NTS neurons has not been determined. This study used in vivo and ex vivo fluorescent tracing and slice patch-clamp electrophysiological recordings from anatomically identified caudal NTS neurons to test the hypothesis that the responsiveness of these cells to nicotine correlates with the target of their axonal projections. The results demonstrate that the majority of glutamatergic terminals that synapse on PbN-projecting caudal NTS neurons are unaffected by nicotine. Moreover, only a fraction of these cells express somatodendritic nAChRs. In contrast, the majority of DMV-projecting caudal NTS neurons exhibit robust presynaptic and somatodendritic responsiveness to nicotine. However, PbN-projecting neurons also exhibit significantly lower background frequencies of glutamatergic miniature postsynaptic currents than DMV-projecting neurons. Therefore, presynaptic unresponsiveness to nicotine may result from deficient glutamatergic innervation of PbN-projecting neurons. Nevertheless, the caudal NTS contains function-specific subsets of cells with target-specific responsiveness to nicotine. These results may support development of therapeutic strategies for selective targeting of specific autonomic pathways and impaired autonomic homeostasis.

nicotinic receptors; parabrachial nucleus; presynaptic responsiveness; solitary tract; vagus

MANY AUTONOMIC VISERAL FUNCTIONS such as gastrointestinal and cardiorespiratory reflexes are controlled centrally via reciprocal connections among various brain stem nuclei. The caudal nucleus of the solitary tract (NTS) is the key recipient of primary afferents from peripheral visceral sensory receptors. Two distinct subsets of caudal NTS neurons can be defined on the basis of differences in projection sites (Hermes et al. 2006): 1) cells that relay visceral information to the forebrain, shaping behavioral and endocrine responses to visceral events, and 2) cells that project to preganglionic or premotor brain stem sites directly regulating parasympathetic and sympathetic visceral reflexes (Bailey et al. 2006a; Davis et al. 2004; Glatzer et al. 2003; Mendelowitz 1999; Ruggiero et al. 1994; Talman et al. 1992).

Glutamate is a key excitatory neurotransmitter in the brain stem, but many drugs can modulate glutamatergic neurotransmission in the dorsal-vagal complex via activation of presynaptic ligand-gated receptors (Bailey et al. 2002, 2006b; Browning et al. 2006) such as nicotinic acetylcholine receptors (nAChRs) (Kalappa et al. 2011; Smith and Uteshev 2008). nAChRs are expressed in most brain regions including the NTS and the dorsal motor nucleus of the vagus (DMV) (Ferreira et al. 2002; Kalappa et al. 2011; Smith and Uteshev 2008). Accordingly, nicotinic agonists exhibit potency for modulation of autonomic functions (Beleslin and Krstic 1987; Ferreira et al. 2000; Jo et al. 2002; Laffan and Borison 1957; Nagata and Osumi 1990). Although nAChRs and nicotine-sensitive glutamatergic synapses have been detected in the NTS, the anatomical identification of nicotine-responsive neurons has not been previously performed. In addition, it remains unclear whether the behavioral effects of nicotinic agonists reflect activation of nAChRs within a specific category of autonomic pathways and thus will affect some autonomic functions more than others. For example, increased fidelity of nicotine-sensitive glutamatergic neurotransmission in the caudal NTS (Kalappa et al. 2011; Smith and Uteshev 2008) may enhance autonomic sensory-motor and visceral-central integration and alter the impact of centrally originating versus viscerosensory-originating inputs.

Despite the NTS involvement in multiple autonomic functions, individual NTS neurons tend to project to individual targets (Hermes et al. 2006), and therefore anatomical identification of NTS neurons by retrograde labeling unambiguously links each neuron to its projection target. The present study took advantage of this property to investigate links among neuronal responsiveness (i.e., presynaptic and somatodendritic) to nicotine and projection targets of caudal NTS neurons in relation to projections to the parabrachial nucleus (PbN) and the DMV. The PbN is a critical target of ascending caudal NTS projections to the forebrain, supporting gastrointestinal, cardiovascular, respiratory, nociceptive, endocrine, and other functions related to autonomic visceral homeostasis (Benarroch 2006; Ezure 2004; Herbert et al. 1990; Karimnamazi et al. 2002; Loewy and McKellar 1980; Mifflin and Felder 1990; Rinaman 2010). Similarly, the DMV is a key NTS projection target containing parasympathetic preganglionic cholinergic motoneurons that innervate most subdiaphragmatic organs in support of primarily gastrointestinal reflexes (Travagl and Rogers 2001). The underlying hypothesis of this study is that...
the responsiveness of caudal NTS neurons to nicotine is projection specific and thus function specific.

**MATERIALS AND METHODS**

**Animals**

Young adult male Sprague-Dawley rats (P22–P30) were used in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 86-23, Bethesda, MD). The use of animals and the animal protocol were approved by the Animal Care and Use Committee of Southern Illinois University. In total, 37 animals were successfully used in experiments involving in vivo injections in the PbN and 19 animals were used in experiments involving ex vivo injections in the DMV.

**In Vivo Microinjections of Fluorescent Microspheres to PbN**

A motorized computer-controlled Stoelting stereotaxic instrument with rat brain atlas integration and real-time visualization of the injection pipette in the atlas space (Neurostar, Sindelfingen, Germany; [http://www.neurostar.de](http://www.neurostar.de)) was used in experiments with in vivo injections of a fluorescent tracer in the PSN. Rats were anesthetized with chloral hydrate (400–450 mg/kg ip) and placed into the stereotaxic instrument, which secures the skull with ear bars and a clamp system that tightens against the frontonasal bone and the palate. During several initial injections, the system was calibrated to reliably deliver injections to the target region within the PbN, and in all subsequent experiments, the injections were conducted with the identical injection parameters and animals of similar age/size. The target injection site was defined by the following stereotaxic coordinates: −9.0 mm (bregma; rostral-caudal axis), 2.0 mm (medial-lateral axis), and 7.2 mm (ventral-dorsal axis). Fluorescent tracer deposits (620-nm fluorescent latex microspheres, Lumafluor) were made by injections via a picospritzer from glass micropipettes (10- to 30-mM tip size, 1–10 puffs, 100–200 ms, 10–30 psi). Direct visual inspection of meniscus movement relative to a calibration grid on the pipette ensured that known volumes were pressure injected. Injections were always made in the left PbN. During recovery from surgery, animals were monitored and maintained on a heated pad (30°C). Injected rats were killed 2–3 days later, and coronal slices containing the PbN and horizontal slices containing the NTS were made with a Vibratome-1000+ slicer (Leica Microsystems), and horizontal or coronal brain stem slices (−250–350 μm thick) containing the region of interest were prepared. Slices were transferred to a storage chamber and incubated at 30°C for 30 min in an oxygenated artificial cerebrospinal fluid (ACSF) of the following composition (in mM): 125 NaCl, 1.5 KCl, 2.23 NaH2PO4, 1 MgCl2, 2 CaCl2, 26 NaHCO3, and 10 glucose (pH 7.4). Slices were then stored in an identical oxygenated ACSF at 24°C for up to 8 h.

**Ex Vivo Iontophoretic Injections of Fluorescent Tracer to DMV**

The DMV is located next to the NTS, and therefore blind in vivo injections of fluorescent tracers into the DMV would be impractical because of the high probability of contamination of the neighboring NTS region. Therefore, injections of fluorescent tracers into the DMV were done ex vivo. Coronal brain stem slices (−300–350 μm thick) were prepared as described above and transferred to the recording chamber mounted on an upright Olympus BX-51WI microscope (Olympus America, Center Valley, PA). The DMV and NTS regions and neurons were visually identified with ×4 and ×40 objectives. Dextran fluorescein (490 nm) tracer (mol wt 3,000, anionic, lysine fixable, micro-emerald; Invitrogen) was dissolved at 2.5% in 125 mM NaCl solution, and either bilateral or unilateral iontophoretic injections to the DMV were conducted with glass pipettes (5- to 10-MΩ resistance) by applying a series of short-duration (~5 s) iontophoretic current steps (20–50 μA). These steps were delivered every 20 s for up to 1 h by a Neurolog system stimulus isolator unit (NL800, Digitimer) driven by an Axon Digidata 1440A system (Molecular Devices, Sunnyvale, CA). The tip of the application pipette was positioned within the DMV, ~20 μm below the slice surface, with a ×4 objective. This protocol allowed the formation of a small (100–200 μm), fairly round injection site within the DMV, while retrogradely labeled NTS cells were clearly detected ipsilateral to the injection site within 3 h after the end of injection. In five control experiments, unilateral tracer injections in the DMV did not cause any detectable labeling in the contralateral NTS. The lack of detection of previously reported (Herman et al. 2009; Kalia and Sullivan 1982) contralateral labeling in our experiments most likely reflects insufficient time (~4 h) allowed for labeling. Another possibility is that some of the contralateral projections may have been damaged during slice preparation. To facilitate diffusion of the tracer, the perfusate temperature was maintained at 28°C. Although the injections of fluorescent tracers were conducted under direct visual control, the probability of contamination of NTS neurons from the DMV injections was higher than from the PbN injections because of the close proximity of the DMV and the NTS regions. To minimize this probability and avoid a drift of tracer molecules from the DMV to the NTS via extracellular space, slices were always positioned in the recording chamber in a manner that ensured that the NTS was in front of the DMV against the flow of ACSF. Moreover, during iontophoretic tracer injections the flow of ACSF was maintained at a higher rate (i.e., 2 ml/min vs. standard 1 ml/min). However, it was possible that some DMV-projecting NTS neurons did not accumulate enough tracers prior to recordings and may have appeared unlabeled. It was also possible that caudal NTS fibers traveling through the DMV to other brain destinations accumulated fluorescent tracer molecules injected into the DMV. Therefore, this study assumed that caudal NTS neurons labeled by foci fluorescent tracer injections into the DMV (i.e., DMV-labeled cells) were representative of the population of caudal NTS neurons projecting to the DMV. Within this assumption, DMV-labeled cells were identified as DMV-projecting cells.
tracer injection, slices remained in the recording chamber perfused with ACSF bubbled with carbogen for 3–4 h, allowing the retrograde transport of the tracer from the injection site to the NTS somata. To avoid photobleaching, all manipulations with fluorescent tracers were done with minimal or no illumination when possible.

**Electrophysiological Patch-Clamp Recordings**

For patch-clamp recordings, slices were secured in the recording chamber with a platinum ring insert with attached equally spaced nylon threads and perfused with oxygenated ACSF at a rate of 1 ml/min with a 2232 Microperpex S peristaltic pump (LKB, Uppsala, Sweden). Fluorescent signals from labeled NTS neurons were detected with a cooled Retiga-EXi digital camera using InVivo or QCapture software packages (Media Cybernetics, Bethesda, MD). Whole cell recordings were conducted at 24°C. The patch electrode solution contained (in mM) 140 K-gluconate, 1 NaCl, 2 MgCl₂, 2 Mg-ATP, 0.3 Na-GTP, 10 HEPES, and 0.42 KOH (pH 7.4). Membrane voltages were not corrected for the liquid junction potential: $V_{\text{LJ}} = 16.2$ mV. The electrophysiological data were recorded with a Multi-Clamp-700B patch-clamp amplifier (Molecular Devices). The seal resistance was $\geq 2 \Omega$. The access resistance was $<30$ MΩ and was not compensated. Patches with access resistances $>30$ MΩ were corrected by application of an additional negative suction or discarded. Data were sampled at 10–50 kHz and filtered at 5–10 kHz.

**RESULTS**

Caudal NTS neurons sending ascending projections to the PbN and descending projections to the DMV were identified by in vivo and ex vivo retrograde fluorescent tracing, respectively. Patch-clamp electrophysiological recordings were then conducted from labeled caudal NTS cells to investigate their responsiveness to nicotine. In these experiments, two types of responses to nicotine were detected: presynaptic and somatodendritic. Presynaptic responsiveness was defined by a greater than twofold increase in the frequency of mEPSCs upon brief puffs (100–200 ms, 0.5 psi) of 0.2 mM nicotine, as reported previously (Kalappa et al. 2011; Smith and Uteshev 2008). To define EPSCs as “miniature” and nAChRs as “presynaptic,” all experiments were conducted in the continuous presence of 0.3–1 μM TTX, sufficient to completely inhibit voltage-gated Na⁺ ion channels and action potentials. Somatodendritic responsiveness was defined by nAChR-mediated whole cell currents with amplitudes greater than $-20$ pA (i.e., the peak-to-peak baseline current noise $\times 2$). All recordings were conducted with a K-gluconate-based internal pipette solution and a holding membrane potential of $-60$ mV, which falls near the reversal potential for chloride ions under these experimental conditions. Therefore, chloride ionic conductances (e.g., GABA<sub>A</sub> receptor mediated) were not detected, as evidenced by a complete block of recorded mPSCs by 15–20 μM DNQX, a selective AMPA receptor antagonist, as reported previously (Kalappa et al. 2011; Smith and Uteshev 2008).

**Caudal NTS Neurons Identified as Projecting to PbN**

In total, 60 caudal NTS neurons retrogradely labeled in vivo by injection of fluorescent microspheres into the PbN (Fig. 1, A–E) were investigated. Only the left PbN was used for injections (Fig. 1, A–C), and brain stem slices prepared from the caudal NTS located ipsilateral to the injection site (Fig. 1, D and E) were then used for electrophysiological patch-clamp recordings (Fig. 1, F–I). The mean input resistance, whole cell capacitance, and resting potential of labeled cells were 831.4 ± 459.5 MΩ ($n = 60$), 30.6 ± 14.8 pF ($n = 60$), and $-51.8 \pm 8.3$ mV ($n = 41$), respectively. Thirty-four PbN-projecting neurons were classified as presynaptic using fluorescence microscopy. A total of 27 of these neurons were subsequently recorded using whole cell patch-clamp electrophysiology to confirm that they functioned as functional presynaptic cells. Therefore, we report here that nicotine puffs elicited a significant increase in the frequency of miniature EPSCs in 27/34 (79%) caudal NTS neurons identified as PbN-projecting neurons.
caudal NTS neurons were successfully tested for responsiveness to nicotine puffs. The majority of PbN-projecting neurons (33 of 34, ~97%) were presynaptically unresponsive to nicotine puffs with regard to the ratio of mEPSC frequency after and before nicotine puffs \( \left( \frac{f}{1.13 \pm 0.56 (n = 33)} \right) \), where \( f = (\text{frequency}_{\text{after puff}})/(\text{frequency}_{\text{before puff}}) \). As a control, in some experiments after recording from labeled caudal NTS neurons (i.e., identified as PBN projecting), neighboring unlabeled cells (i.e., undetermined targets) were also tested. In total, 20 unlabeled cells were examined, and 11 of these (i.e., ~55%) were found to be presynaptically responsive to nicotine puffs \( \left( \frac{f}{3.5 \pm 1.7; n = 11} \right) \). Each of the remaining 9 cells (i.e., 45%) exhibited \( f < 2 \) (mean \( f = 1.1 \pm 0.2; n = 9 \)), and thus these cells were defined as presynaptically unresponsive to nicotine.

**Fig. 1.** Responsiveness to nicotine of caudal nucleus of the solitary tract (NTS) neurons identified as projecting to the parabrachial nucleus (PbN). A and B: coronal PbN sections were used to confirm the location of the injection site of fluorescent microspheres. Images from a representative experiment are shown. Yellow dashed demarcation lines in A and B mark the same region located at ~9.0 mm, bregma. An ipsilateral PbN injection site of fluorescent microspheres (red) is shown in the infrared (A) and fluorescent 620-nm (B) light spectra. C: the same PbN region and injection site as in A and B are shown in a higher-resolution combined image with infrared and fluorescent images overlaid. D and E: for electrophysiological recordings, horizontal NTS sections were prepared from brain stem sections ipsilateral to the injection site shown in A–C. A nicotine unresponsive PbN-projecting (red dot, D and E) and a nicotine-responsive unlabeled (blue dot, D and E) caudal NTS cells located ipsilateral to the injection site are shown in infrared (D) and corresponding fluorescent (E) images of the caudal NTS that were taken immediately after the completion of electrophysiological recordings shown in F and G, bottom, and H and I, bottom, respectively. The recording electrode (marked “Patch” in D) and the application picospritzer pipette (marked “Pico” in D) can be seen near the red dot. The distance between the red and the blue dots in D is 79 \( \mu \text{m} \). F: infrared image of a nicotine-unresponsive PbN-projecting neuron whose location within the slice is marked by the red dot in D and E. G: fluorescent image of the same neuron as shown in F. White squares in F and G mark the same region. A strong somatic labeling can be observed (red beads within the white square). Current traces (F and G, bottom) define this PbN-projecting cell as presynaptically and somatodendritically unresponsive to nicotine. H: infrared image of a nicotine-responsive unlabeled neuron (i.e., undetermined projection target) whose location within the slice is marked by the blue dot in D and E. I: fluorescent image of the same neuron as shown in H. White circles in H and I mark the same region. Although the sensitivity of detection was enhanced (hence the greater fluorescent background in I), neuronal labeling was not detected (area within the white circle). Current traces (H and I, bottom) define this unlabeled cell as presynaptically responsive \( (f > 2, \text{where } f = (\text{frequency}_{\text{after puff}})/(\text{frequency}_{\text{before puff}})) \) but somatodendritically unresponsive to nicotine.
nicotine. The representative injection site of fluorescent tracers within the PbN (encircled by dashed yellow ellipses) in a coronal brain stem slice is shown in infrared (Fig. 1A), fluorescent 620-nm (Fig. 1B), and combined infrared + fluorescent (Fig. 1C) light spectra. The positions of two representative caudal NTS neurons within the horizontal brain stem slice used for electrophysiological recordings are illustrated in infrared (Fig. 1D) and fluorescent 620-nm (Fig. 1E) light spectra. The red dot in Fig. 1, D and E, marks the location of a presynaptically and somatodendritically unresponsive neuron retrogradely labeled by the PbN injection (Fig. 1, F and G). In contrast, the blue dot in Fig. 1, D and E, marks the location of a presynaptically responsive neuron not labeled by the PbN injection (Fig. 1, H and I). These two neurons are shown at a higher resolution in infrared and fluorescent light spectra in Fig. 1, F and G (top), and Fig. 1, H and I (top), respectively.

Somatodendritic responses to nicotine. Somatodendritic responses to nicotine puffs were detected in 29% (10 of 34) of labeled and 45% (9 of 20) of unlabeled caudal NTS neurons (not shown). The response amplitudes ranged from 31 pA to 812 pA. The mean response amplitudes for labeled (280 ± 246 pA; n = 10) and unlabeled (104 ± 59 pA; n = 9) cells were not significantly different (P > 0.05, t = 2.09, unpaired 2-tailed Student t-test). The amplitudes of somatodendritic responses were estimated in the absence of glutamatergic antagonists because the kinetics of glutamatergic mEPSCs is much faster than the kinetics of somatodendritic responses and nicotine-mediated increases in the mEPSC frequency were insufficient to conceal somatodendritic responses. However, it is possible that some somatodendritic responses may have been somewhat overestimated.

Only 1 of 34 tested PbN-projecting caudal NTS neurons exhibited presynaptic responsiveness to nicotine (f = 1.95 ± 1.58; n = 7, not shown). The value of f was averaged over seven responses to consecutive nicotine puffs applied every 3 min. This ratio may have been somewhat underestimated because the first 5 s after nicotine puffs was not analyzed because of the presence of somatodendritic nAChR-mediated responses (not shown) and thus the mEPSC frequency was evaluated between 5 s and 15 s after nicotine puffs.

Caudal NTS Neurons Identified as Projecting to DMV

In total, 23 caudal NTS neurons retrogradely labeled ex vivo by injection of a fluorescent tracer in the DMV were investigated. Electrophysiological patch-clamp recordings were conducted in acute coronal brain stem slices from fluorescently labeled caudal NTS neurons located ipsilateral to the DMV injection site (Fig. 2, A–C). The experimental parameters and analysis of data were identical to those used for recordings of PbN-projecting caudal NTS neurons. The mean input resistance, whole cell capacitance, and resting potential of DMV-projecting cells were 916 ± 486 MΩ (n = 23), 36.5 ± 11.4 pF (n = 23), and –54.4 ± 8.4 mV (n = 23), respectively. The majority (18 of 23, 78.3%) of caudal NTS neurons identified as projecting to the DMV were presynaptically responsive to nicotine puffs (f = 3.8 ± 1.9; n = 18). The remaining five cells were defined as presynaptically unresponsive (f = 1.5 ± 0.4; n = 5). These findings are illustrated in Fig. 2: infrared (Fig. 2A) and fluorescent 490-nm (Fig. 2B) images of the left half of a coronal brain stem slice were taken with a ×4 objective shortly after the tracer injection. Yellow dashed lines identify the location of the NTS, the DMV, the fourth ventricle, and the solitary tract (Fig. 2, A and B). The injection site is clearly seen as a green cloud localized within the DMV (Fig. 2B). To detect DMV-projecting caudal NTS neurons, a ×40 objective was used. One such labeled cell is marked by a white dashed circle in Fig. 2C. The approximate location of this neuron within the NTS is illustrated by a gray rectangle within the NTS in Fig. 2B. Patch-clamp recordings from this neuron identified it as both presynaptically (f = 3.0 ± 0.7) and somatodendritically responsive (current amplitude 81.1 ± 64.0 pA) to nicotine puffs (Fig. 2D). In this experiment, the values of f and current amplitude were averaged over five consecutive nicotine puffs applied every 3 min.

Typical examples of current traces obtained before (Fig. 2, E and F, top) and after (Fig. 2, E and F, bottom) brief pressure puffs (100 ms, 10 psi) of 0.2 mM nicotine from representative presynaptically responsive (f = 3.8 ± 1.1; Fig. 2E) and presynaptically unresponsive (f = 1.25 ± 0.3; Fig. 2F) DMV-projecting caudal NTS neurons are shown in Fig. 2, E and F. Images of these neurons are not shown. In these two representative experiments, the values of f were averaged over eight (Fig. 2E) and five (Fig. 2F) consecutive nicotine puffs applied every 3 min. Both neurons were somatodendritically unresponsive (i.e., current amplitude <20 pA; see MATERIALS AND METHODS).

Somatodendritic responses to nicotine. Somatodendritic responses to nicotine puffs were detected in 41%, 37%, and 35% of PbN-, DMV-, and MbA-projecting caudal NTS neurons, respectively. The mean amplitude of somatodendritic responses was 140.5 ± 141.1 pA (n = 17). A summary graph of presynaptic and somatodendritic responses to nicotine of PbN- and DMV-projecting caudal NTS neurons is shown in Fig. 3.

Comparison of Background mEPSC Frequencies Recorded from PbN- and DMV-Projecting Caudal NTS Neurons

The effects of nicotine on synaptic release may correlate with the level of spontaneous (i.e., asynchronous) background glutamatergic miniature synaptic activity. To quantify the background mEPSC activity, the mEPSC frequency was calculated over 10-s recordings before nicotine puffs in PbN- and DMV-projecting neurons. The mean background mEPSC frequency calculated for PbN-projecting cells (1.16 ± 1.19 Hz, n = 34) was significantly lower than the background mEPSC frequency recorded from DMV-projecting caudal NTS neurons (4.40 ± 3.96 Hz, n = 23). Therefore, the lack of presynaptic responsiveness (to nicotine) of PbN-projecting caudal NTS neurons may directly correlate to deficient glutamatergic innervation of these neurons.

Morphology of PbN- and DMV-Projecting Neurons

Examples of PbN- and DMV-projecting caudal NTS neurons are shown in Fig. 4, A and B, respectively. Seven recorded caudal NTS neurons identified as projecting to the PbN were successfully labeled with Neurobiotin, and their key morphological characteristics (i.e., somal area, form factor, and number of branches) were evaluated with a Neurulucida system.
RESPONSIVENESS OF CAUDAL NTS NEURONS TO NICOTINE

(see MATERIALS AND METHODS). These values [somal area 155.9 ± 82.3 μm² (n = 7); form factor 0.60 ± 0.11 (n = 7); number of branches 2.0 ± 1.2 (n = 7)] were not significantly different (P > 0.05, t = 0.41, t = 0.32, and t = 1.13, respectively, unpaired 2-tailed Student t-test) from the values reported previously for caudal NTS neurons with undetermined targets (Smith and Uteshev 2008); somal area 137.4 ± 105.7 μm² (n = 17), form factor 0.62 ± 0.15 (n = 19), and number of branches 2.6 ± 1.2 (n = 19).

Similarly, 10 neurons identified as projecting to the DMV were successfully labeled with Neurobiotin, and their morphology was analyzed with a Neurolucida system. The somal area, 216.6 ± 60.4 μm² (n = 10), and the number of branches, 2.2 ± 0.9 (n = 10), were not significantly different from the corresponding values estimated for caudal NTS neurons identified as PbN projecting (P > 0.05; t = 1.76 and t = 0.39, respectively, unpaired 2-tailed Student t-test). However, the form factor, 0.74 ± 0.06 (n = 10), of DMV-projecting neurons was significantly greater (P < 0.05; t = 3.40, unpaired 2-tailed Student t-test) than the average form factor of PbN-projecting cells (see above). Therefore, DMV-projecting caudal NTS neurons appear to be more round than PbN-projecting caudal NTS neurons.

DISCUSSION

There is considerable diversity among NTS neurons in the expression of presynaptic and somatodendritic nAChRs, neuronal morphology, cytochemical identity, and projection targeting associated with the multimodal role of the NTS in controlling autonomic homeostasis (Bailey et al. 2006a; Glatzer et al. 2003; Kalappa et al. 2011; Kawai and Senba 1999; Smith and Uteshev 2008; Ueno et al. 1993). However, the results of this study suggest that, despite its heterogeneity,
the caudal NTS contains discrete projection-specific subsets of cells with unique nAChR expression properties. In electrophysiological patch-clamp experiments in brain stem slices, two types of responses of caudal NTS neurons to brief nicotine puffs were detected: presynaptic (defined by a nicotine-mediated increase in the frequency of glutamatergic synaptic release in the presence of 0.3–1 μM TTX, i.e., mEPSCs) and somatodendritic (defined by nicotine-mediated whole cell responses). In the predominant majority of PbN-projecting caudal NTS neurons (~97%), nicotine did not alter glutamatergic synaptic input (Fig. 3A). As a control, several neighboring unlabeled caudal NTS neurons were tested for responsiveness to nicotine, and in 55% of those unlabeled cells (i.e., not identified as projecting to the PbN) nicotine increased the synaptic release of glutamate (Fig. 3A). This fraction is slightly greater than the proportion of presynaptically responsive (to nicotine) cells among randomly selected caudal NTS neurons (i.e., undetermined targets) reported previously (~40%; Kalappa et al. 2011) and may reflect the absence of nonresponsive (i.e., PbN projecting) neurons among cells evaluated in these control experiments. Therefore, caudal NTS neurons that were not identified as PbN-projecting cells exhibited an ~18.3-fold greater chance (i.e., 55% vs. ~3%) of responding presynaptically to nicotine than cells identified as PbN projecting. Some of those unlabeled caudal NTS cells may project to the DMV, the site of parasympathetic preganglionic cholinergic motoneurons that support primarily gastrointestinal reflexes. Indeed, retrograde labeling of caudal NTS neurons identified as projecting to the DMV demonstrated that the majority (~78.3%) of those neurons were presynaptically responsive to nicotine (Fig. 3A). These data indicate that glutamatergic synapses on DMV-projecting caudal NTS neurons exhibit an ~26-fold greater chance (i.e., ~78.3% vs. ~3%) to be potentiated by nicotine than similar synapses on PbN-projecting cells. Therefore, by activation of presynaptic nAChRs on glutamatergic terminals, nicotine and other nicotinic agents including endogenous ACh may selectively enhance the efficacy of visceral sensory glutamatergic circuits directed at visceral (specifically, gastrointestinal related) versus forebrain targets (Fig. 3A).

However, this study also demonstrates that the background mEPSC frequency recorded in PbN-projecting caudal NTS neurons is significantly lower than the corresponding frequency recorded in DMV-projecting cells. As the basic properties of unitary synaptic events underlying spontaneous (i.e., asynchronous) and evoked (i.e., synchronous) synaptic currents appear to be similar (Liu and Tsien 1995a, 1995b; Raastad et al. 1992; Taschenberger et al. 1995; Uteshev et al. 2000), these results suggest deficient glutamatergic innervation of PbN-projecting neurons (i.e., fewer glutamatergic synapses characterized by a lower probability of glutamate release) compared with DMV-projecting cells. Thus it is possible that the observed lack of presynaptic responsiveness (to nicotine) in PbN-projecting neurons is, at least in part, due to deficient glutamatergic innervation of these neurons. However, these data do not exclude a possibility of deficient expression of functional nAChRs on presynaptic glutamatergic terminals innervating PbN-projecting versus DMV-projecting caudal NTS neurons. Regardless of the exact mechanism (i.e., deficient expression of presynaptic nAChRs, deficient glutamatergic innervations, or both), it is evident from the presented data that nicotine has a significantly smaller impact on synaptic release of glutamate in PbN-projecting versus DMV-projecting caudal NTS neurons.

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<th>% Somatodendritic Responsiveness</th>
<th>PbN-labeled cells</th>
<th>DMV-labeled cells</th>
<th>Undetermined target cells</th>
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<td>Somatodendritically-responsive (&gt;20 pA)</td>
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<td>26.1%</td>
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Fig. 3. Summary of results: presynaptic (A) and somatodendritic (B) responsiveness to nicotine among caudal NTS neurons. A: only 1 caudal NTS neuron identified as PbN projecting was presynaptically responsive to nicotine (f > 2), but note that although several responses to nicotine puffs produced f > 2 (not shown), the mean increase over 7 responses gave f = 1.95, thus <2. The remaining 33 of 34 tested PbN-projecting caudal NTS cells were presynaptically unresponsive (f < 2) (A, left). In contrast, 11 of 20 unlabeled caudal NTS cells (i.e., undetermined projection target; A, right) as well as 18 of 23 DMV-projecting caudal NTS cells (A, center) were presynaptically responsive (f > 2). Presynaptic responsiveness was defined by a >2-fold increase in the mEPSC frequency in response to nicotine puffs (dashed line, A). B: only 29.4% of PbN-projecting caudal NTS cells exhibited somatodendritic responsiveness to nicotine (B, left). By contrast, 45% of unlabeled caudal NTS neurons (B, right) as well as 73.9% of DMV-projecting caudal NTS cells (B, center) were somatodendritically responsive. The somatodendritic responsiveness was defined by nicotine acetylcholine receptor (nAChR)-mediated whole cell currents with amplitudes greater than ~20 pA.
nAChRs play an important role in modulation of neurotransmitter release, gene expression, neuroprotection, and neurotoxicity (Dajas-Bailador and Wonnacott 2004; Meyer et al. 1998; Role and Berg 1996), the role of somatodendritic nAChRs in brain signaling remains poorly defined in the predominant absence of classical nicotinic synapses in the central nervous system (Miwa et al. 2011). Therefore, the physiological relevance of differences in the expression pattern of functional somatodendritic nAChRs in DMV- and PbN-projecting caudal NTS neurons is not clear. A stronger expression of functional somatodendritic nAChRs in DMV-projecting caudal NTS neurons may increase responsiveness of these cells, compared with PbN-projecting cells, to nicotinic agents and cholinergic inputs, if somatodendritic nAChRs indeed contribute to electrochemical signaling within the caudal NTS (e.g., via cholinergic volume transmission).

The input resistance of caudal NTS cells identified as PbN projecting was found to be significantly higher than that of random cells (Kalappa et al. 2011): $831 \pm 459 \text{ M}\Omega \ (n = 60)$ vs. $510 \pm 258 \text{ M}\Omega \ (n = 169) \ (P < 0.0001, t = 6.62, \text{unpaired 2-tailed Student } t\text{-test})$ but not significantly different from the input resistance of caudal NTS cells identified as DMV projecting ($P > 0.05, t = 0.74, \text{unpaired 2-tailed Student } t\text{-test}$). The whole cell capacitance of PbN-projecting cells was not significantly different from both random caudal NTS cells ($30.6 \pm 14.8 \text{ pF} \ (n = 60)$ vs. $33.6 \pm 17.8 \text{ pF} \ (n = 169); P > 0.05, t = 1.72, \text{unpaired 2-tailed Student } t\text{-test}$) and DMV-projecting cells ($P > 0.05, t = 1.72, \text{unpaired 2-tailed Student } t\text{-test}$). Similarly, the resting potentials of PbN- and DMV-projecting cells were not significantly different ($P > 0.05, t = 1.20, \text{unpaired 2-tailed Student } t\text{-test}$). Although the somal areas and the number of primary branches of PbN- and DMV-projecting cells were not significantly different ($P > 0.05, t = 1.13, \text{unpaired 2-tailed Student } t\text{-test}$), the somatic shapes defined by the form factor were significantly different: caudal NTS neurons identified as DMV projecting were significantly more round than cells identified as PbN projecting ($P < 0.05, t = 3.40, \text{unpaired 2-tailed Student } t\text{-test}$).

Therefore, the results of this study suggest that the responsiveness of caudal NTS neurons to nicotine depends on where the specific cell projects. This study was specifically focused on nicotinic properties of subsets of caudal NTS neurons identified as projecting to the PbN and the DMV. These brain stem nuclei play different roles in the maintenance of autonomic homeostasis: the PbN primarily relays viscerosensory information to the forebrain, supporting behavioral, emotional, and endocrine responses to visceral events, while the DMV contains parasympathetic preganglionic cholinergic motoneurons and projects to postganglionic cells controlling most organs of subdiaphragmatic viscera in support of gastrointestinal reflexes (Browning and Travagli 2010; Travagli and Rogers 2001). The present data suggest that most glutamatergic terminals that synapse on PbN-projecting caudal NTS neurons are unaffected by nicotine and that only a fraction of these cells express functional somatodendritic nAChRs. In contrast, most DMV-projecting caudal NTS cells are both presynaptically and somatodendritically responsive. The anatomical identification of NTS neurons by retrograde labeling unambiguously links each neuron to its unique projection site (Hermes et al. 2006) and suggests a specific function. Therefore, these results support the hypothesis of a target-specific profile of nicotine effects in the caudal NTS and suggest a reduced responsiveness to nicotine within the viscerosensory circuitry of the caudal NTS that is directed at central-forebrain (via the PbN) versus visceral gastrointestinal-related (via the DMV) autonomic motor targets. These data also suggest that nicotine and other nicotinic agents including endogenous ACh and various tobacco products may alter the impact of specific autonomic pathways on the integration of sensory-motor and visceral-central signaling within the caudal NTS (Ferreira et al. 2000, 2002; Jo et al. 2002; Nagata and Osumi 1990, 1991).

The NTS-DMV complex serves as the key integrating processing center of gastrointestinal visceral sensory and motor signaling supporting gastrointestinal autonomic homeostasis. Gastrointestinal disorders are thought to primarily associate with abnormalities in motor function (Canimilleri and Bharucha 1996). The nAChR-mediated modulation of glutamatergic signaling within the NTS-DMV complex may contribute to establishing and maintaining a delicate balance in the brain-gut interactions supporting gastrointestinal reflexes. Moreover, treatment of patients with therapeutic cholinergic compounds (e.g., donepezil, an anti-Alzheimer’s inhibitor of ACh hydrolysis) often results in centrally mediated nausea and emesis (Inglis 2002; McCain et al. 2007) typically associated with gastric hypomotility (Andrews and Horn 2006). Therefore, adequate cholinergic inputs and nAChR-mediated...
modulation of synaptic signaling within the NTS-DMV complex may alter the brain-gut interaction, impairing autonomic homeostasis and may ultimately contribute to autonomic disbalance leading to emesis, dysphagia, gastric stasis, and irritable bowel syndrome (Aggarwal et al. 1994; Camilleri 1990; Camilleri and Bharucha 1996; van Orshoven et al. 2006).

Food digestion and gastrointestinal motility are autonomic processes regulated by the vago-vagal reflex through specific nerve fibers originating in the NTS-DMV region. Strong links exist between neuronal signaling in the NTS-DMV and regulation of digestion, feeding, and satiety (Berthoud 2008; Faipoux et al. 2008; Wan et al. 2008), especially during meals, when signals arising from mechanoreceptors activated in the stomach wall are transmitted to the NTS via vagal afferents. Some of these effects incorporate activation of NTS-DMV nAChRs (Ferreira et al. 2002; Jo et al. 2002). Specifically, activation of nAChRs in the NTS-DMV alters gastric motility and secretion (Ferreira et al. 2000, 2002; Nagata and Osumi 1990, 1991), which may be associated with the nAChR-mediated modulation of glutamatergic release in the NTS-DMV (Jo et al. 2002). The relevance of nAChRs to digestion is emphasized by the fact that nicotine is emetic (Beleslin and Krstic 1987; Laffan and Borison 1957) and exerts profound anorexigenic effects in humans and animals by suppressing appetite and decreasing food intake per meal size without significant changes in meal frequency (Blaha et al. 1998; Grunberg et al. 1986; Jo et al. 2002; Miyata et al. 2001). In humans, smoking cessation leads to significant gains in body weight, affecting the effectiveness of smoking cessation programs (Nordstrom et al. 1999; Pomerleau et al. 2000). However, as DMV-projecting caudal NTS neurons can be both excitatory (e.g., glutamatergic) and inhibitory (e.g., GABAergic) (Davis et al. 2004; Glatzer et al. 2003; Herman et al. 2009; Travagli et al. 2006), the integrated net effect of nAChR activation on the excitability of DMV neurons remains uncertain. Moreover, the sources of nicotine-sensitive glutamatergic terminals within the NTS are not known and may include high-fidelity inputs from the solitary tract and/or low-fidelity inputs from within the NTS or other brain regions including those that receive NTS projections. However, it seems unlikely that the primary high-fidelity afferents could benefit from expression of nAChRs. It is more likely that the efficacy of low-fidelity glutamatergic synapses can be enhanced by activation of functional presynaptic nAChRs by nicotine and other nicotinic agents including endogenous ACh.

In addition to local NTS cholinergic interneurons, the potential cholinergic sources may include the key brain stem cholinergic nuclei: the DMV and the pedunculopontine nucleus. It is intriguing to speculate that the NTS and the DMV establish reciprocal functional connections. Because of the close proximity of these nuclei (Fig. 2A), cholinergic DMV neurons may not need to establish synaptic connections with the NTS but may transmit signaling to the NTS via volume transmission of ACh (Lendvai and Vizi 2008) and/or dendritic release of ACh (Bergquist and Ludwig 2008). If DMV-projecting nicotine-responsive caudal NTS neurons are GABAergic, then the cholinergic volume transmission inputs to the NTS from the DMV may act as a negative feedback inhibiting overly excited DMV neurons via GABAergic neurotransmission. In this scheme, a moderate activity of DMV neurons may not cause a robust release of ACh and its volume transmission to the NTS, and thus may not be sufficient for activation of nAChRs in the NTS and NTS-mediated inhibition of DMV activity. However, an increased DMV activity might in turn increase ACh volume transmission, consequently enhancing excitatory influences on GABA neurons, resulting in inhibition of the originating DMV activity. This volume transmission feedback mechanism may be less effective for presynaptic and somatodendritic NTS nAChRs with a higher potency for desensitization (e.g., α7) (Uteshev et al. 1996, 2003). Moreover, although only nAChRs were investigated in this study, muscarinic AChRs (mAChRs; e.g., m2 subtype) have also been reported in the NTS (Endoh 2007; Shihara et al. 1999; Uteshev and Smith 2006). The m2 mAChRs are often expressed presynaptically on glutamatergic and cholinergic terminals, and their activation inhibits release of the corresponding neurotransmitters (Allen 1999; Li et al. 2002). However, mAChRs and nAChRs are usually expressed by different neurons (Shihara et al. 1999; Uteshev and Smith 2006), and thus the spatiotemporal profile of nAChR and mAChR activation would be expected to be the key determinant of the integrated net NTS response to cholinergic inputs.

Using the experimental approach established in this study, future studies may confirm and expand the target-specific profile of nAChR expression within the caudal NTS. Therefore, nicotine-sensitive caudal NTS pathways and their relevance to the effects of endogenous and exogenous nicotinic agents on the autonomic nervous system may be elucidated in greater detail. These results may support development of new therapeutic strategies for selective targeting of specific autonomic pathways and impaired autonomic homeostasis.

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DISCLOSURES

No conflicts of interest (financial or otherwise) are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: L.F., E.A.S., A.G.G., and V.V.U. performed experiments; V.V.U. drafted manuscript. L.F., E.A.S., A.G.G., and V.V.U. interpreted results of experiments; L.F. and V.V.U. edited and contributed to the design of research; V.V.U. drafted manuscript.

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