Mechanisms of facilitation of synaptic glutamate release by nicotinic agonists in the nucleus of the solitary tract.

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The nucleus of the solitary tract (NTS) is the principal integrating relay in the processing of visceral sensory information. Functional nicotinic acetylcholine receptors (nAChRs) have been found on presynaptic glutamatergic terminals in subsets of caudal NTS neurons. Activation of these receptors has been shown to enhance synaptic release of glutamate and thus, may modulate autonomic sensory-motor integration and visceral reflexes. However, the mechanisms of nAChR-mediated facilitation of synaptic glutamate release in the caudal NTS remain elusive. This study uses rat horizontal brainstem slices, patch-clamp electrophysiology and fluorescent Ca$^{2+}$ imaging to test the hypothesis that a direct Ca$^{2+}$ entrance into glutamatergic terminals through active presynaptic non-$\alpha$7 or $\alpha$7 nAChR-mediated ion channels is sufficient to trigger synaptic glutamate release in subsets of caudal NTS neurons. The results of this study demonstrate that in the continuous presence of 0.3 µM tetrodotoxin, a selective blocker of voltage-activated Na$^+$ ion channels, facilitation of synaptic glutamate release by activation of presynaptic nAChRs (detected as an increase in the frequency of miniature excitatory postsynaptic currents) requires external Ca$^{2+}$, but does not require activation of presynaptic Ca$^{2+}$ stores and presynaptic high- and low-threshold voltage-activated Ca$^{2+}$ ion channels. Expanding the knowledge of mechanisms and pharmacology of nAChRs in the caudal NTS should benefit therapeutic approaches aimed at restoring impaired autonomic homeostasis.
INTRODUCTION

The nucleus of the solitary tract (NTS) is the site of the first synapse for primary afferents to the central nervous system (CNS) from autonomic sensory receptors located in the gastrointestinal tract, heart, blood vessels and other visceral tissues. The NTS plays a key role in the maintenance of autonomic homeostasis and projects within the brainstem (e.g., to the dorsal motor nucleus of the vagus (DMV) and the ventrolateral medulla) supporting parasympathetic and sympathetic visceral reflexes (e.g., gastrointestinal and baroreflex), as well as to higher brain regions (e.g., the thalamus and the hypothalamus) supporting behavioral and endocrine functions (2, 46). Therefore, modulation of neuronal activity and synaptic transmission in the NTS by biologically active compounds such as nicotinic agonists can have potent effects on basic autonomic functions and visceral reflexes.

The NTS contains a highly heterogeneous population of neurons characterized by a broad spectrum of morphological, electrophysiological and cytochemical properties and multiple projection targets (3, 6, 12, 17, 20, 23, 44, 55). Expression of nicotinic (nAChRs) and muscarinic (mAChRs) acetylcholine receptors also varies across the NTS region (44, 55). Electrophysiological recordings from the NTS in brainstem slices revealed that nicotinic and muscarinic AChRs are likely expressed by different NTS neurons (43, 55). Moreover, subsets of small (somal area, ~137 μm²) elongated (form factor, ~0.62) caudal NTS neurons have been found to express functional presynaptic α3β4-containing nAChRs (i.e., α3β4*) (44) whose activation by nicotine, a broad spectrum agonist of nAChRs, or cytisine, a potent agonist of β4* nAChRs, was found to enhance synaptic release of glutamate via a presynaptic mechanism (44).
The presence of presynaptic and/or pre-terminal nAChRs in the CNS has been previously documented (1, 19, 28, 31, 34, 44, 57). Several studies reported presynaptic and/or pre-terminal β2-containing (likely α4β2*) nAChRs corresponding to high affinity binding sites for nicotine (1, 13, 24, 28, 32-34, 42). These nAChRs can be selectively blocked by low μM concentrations of Dihydro-beta-erythroidine (DHβE). In the caudal NTS, DHβE failed to block the facilitating effects of nicotine on glutamatergic miniature excitatory postsynaptic currents (mEPSCs, (44)) indicating that α4β2* nAChRs are unlikely contributors to the observed presynaptic effects of nicotine on synaptic glutamate release. The second most common presynaptic or pre-terminal nAChR in the CNS is composed of α7 subunits (1, 14, 16, 19, 35, 39, 42). Activation of presynaptic nAChRs is able to facilitate neurotransmitter release either directly, via increasing Ca^{2+} influx through nAChR-mediated ion channels (15, 19, 24, 27, 39, 45); or indirectly, via transient depolarization and activation of voltage-activated Ca^{2+} channels (VACCs) (27, 47, 56). The mechanisms of nAChR-mediated facilitation of synaptic glutamate release in the caudal NTS remain unknown and are investigated in the present study using patch-clamp electrophysiology, fluorescent Ca^{2+} imaging and rat horizontal brainstem slices. Specifically, the roles of external Ca^{2+} ions, presynaptic voltage-gated Ca^{2+} ion channels, presynaptic Ca^{2+} stores, presynaptic non-α7 and α7 nAChRs are elucidated. Understanding the mechanisms and pharmacology of nAChRs and nicotinic effects in the NTS may benefit development of therapeutic approaches for selective targeting of specific autonomic pathways (e.g., gastrointestinal, cardiorespiratory).
MATERIALS AND METHODS

Animals. Young Sprague-Dawley rats (P21-30) of both genders were used in experiments. Animal care was in accordance with the Guide for the Care and Use of Laboratory Animals (NIH 865-23, Bethesda, MD) and was approved by the Animal Care and Use Committee of Southern Illinois University School of Medicine.

Slice Preparation. Upon euthanizing, brains were rapidly removed and placed in ice-cold oxygenated sucrose-based solution of the following composition (in mM): sucrose 250, KCl 2.5, NaH$_2$PO$_4$ 1.23, MgCl$_2$ 5, CaCl$_2$ 0.5, NaHCO$_3$ 26, glucose 10 (pH 7.4), when bubbled with carbogen (95% O$_2$ and 5% CO$_2$). The brainstem was then separated from the rest of the brain and transferred to the brain slicer chamber (Vibratom-1000plus, Vibratome, St. Louis, MO) and two-three horizontal brainstem slices (~260 μm thick) were cut. Slices were transferred to a temporary storage chamber, where they were perfused at ~30°C for 30 min in an oxygenated artificial cerebrospinal fluid (ACSF) solution of the following composition (in mM): NaCl 125, KCl 2.5, NaH$_2$PO$_4$ 1.23, MgCl$_2$ 1, CaCl$_2$ 2, NaHCO$_3$ 26, glucose 10 (pH 7.4). Slices were then transferred to a long-term storage chamber and perfused with an identical oxygenated ACSF at ~24°C for up to 10 h. For patch-clamp experiments, slices were transferred to the recording chamber and perfused with an oxygenated ACSF at a rate of 1 ml/min using a 2232 Microperpex S peristaltic pump (LKB, Upsalla, Sweden). Whole-cell recordings were conducted at room temperature (~24°C) unless otherwise specified.
**Electrophysiology.** Neurons from the caudal NTS were visually selected for electrophysiological patch-clamp recordings using an infrared Olympus BX-51WI microscope (Olympus America Inc, Center Valley, PA). Current recordings were made in voltage-clamp configuration using a MultiClamp-700B amplifier equipped with Digidata-1440A A/D converter (Molecular Devices, Sunnyvale, CA). Data were filtered at 5-10 kHz, sampled at 20-50 kHz and stored on a personal computer for an offline analysis. For recordings, patch pipettes were pulled using a Sutter P-97 horizontal puller (Sutter Instruments, Novato, CA). The pipette resistance was ~4-6 MΩ when filled with the internal solution (see below). After formation of a stable gigaseal (>2 GΩ), the whole-cell configuration was established. The access resistance was between 15 and 30 MΩ, and typically, was not compensated. Patches with access resistances higher than 30 MΩ were corrected by applying an additional negative pressure or discarded. Nicotinic agonists were applied via picospritzer pipettes (application pressure 5-8 psi, Parker Hannifin Instrumentation, Cleveland, OH, USA) identical to those used for patch-clamp recordings. The tip of the application pipette was positioned within 15 μm from the recorded neuron. The extracellular solution was identical to ACSF which was used for the brain tissue preparation. The intracellular electrode solution contained (in mM): K-gluconate 140, NaCl 1, MgCl₂ 2, Mg-ATP 2, Na-GTP 0.3, HEPES 10, KOH 0.42 (pH 7.38). Membrane voltages were not corrected for the liquid junction potentials $V_{LJ}(\text{K-gluconate})=16.2$ mV. The membrane voltage was maintained at -60 mV unless otherwise stated. In experiments utilizing 0.2 mM Cd²⁺ and 0.2-1 mM Ni²⁺, phosphate salts were removed from ACSF to avoid potential precipitation of Cd²⁺ and Ni²⁺ ions. When necessary, a GENIE Plus syringe pump (Kent Scientific,
Torrington, CT) was used to add 0.3 µM tetrodotoxin (TTX, a selective Na⁺ ion channel antagonist) or 20 nM methyllycaconitine (MLA, a selective α7 nAChR antagonist) to the ACSF just before it entered the recording chamber. The final drug concentrations in the chamber were then calculated based on the known concentrations of drug stock solutions and adjustable rates of all pumps. A detailed description of this application method can be found in the Methods sections in (10, 11, 44, 50, 52). In brief, if the flow rate of ACSF is X ml/min, an addition of Y ml/min of a stock antagonist solution results in an (X/Y)-fold dilution. The important advantages of this drug application method include: 1) drugs can be added to and withdrawn from ACSF rapidly (<1 min) and repetitively without contamination of connective tubing because drugs are added to the mixing chamber located near the recording chamber; and 2) only one stock solution needs to be prepared for each drug while multiple ACSF drug concentrations can be easily generated during an experiment by adjusting the flow rates of the pumps.

To record electrophysiological data at various temperatures between 24 C and 32 C, ACSF was pre-heated using a heating penal (WPI, ) before ACSF entered the recording chamber. The ACSF temperature was measured using a temperature sensor (WPI, ) positioned on the wall of the recording chamber next to the slice. During the experiment, ACSF temperature was recorded and monitored continuously and synchronously with the electrophysiological data using a designated input channel in MultiClamp-700B.

Fluorescent Ca²⁺ imaging. A stock solution (500 µM) of Ca-Green-1 fluorescent dye (K_d=190 nM, Invitrogen Inc., Carlsbad, CA USA) was prepared in 30 µl aliquots and
stored at -80 °C. Before each experiment, the dye was mixed with the filtered intracellular solution for the final concentration of 50 µM. Fluorescent signals were recorded using a cooled Retiga-EXi digital camera and analyzed on- and off-line using InVivo software package (Media Cybernetics Inc., Bethesda, MD). Ca-Green-1 emits fluorescent signals monotonically related to [Ca^{2+}], within the 10–1000 nM range (Invitrogen Inc., Carlsbad, CA USA). NTS neurons were filled with fluorescent dye via patch-clamp recording pipettes. The dye binding/unbinding of free Ca^{2+} ions is very rapid and relatively insensitive to pH. Changes in [Ca^{2+}], were estimated in terms of the ratio F/F0, where F is the peak amplitude of cytosolic Ca^{2+} signal and F0 is the background [Ca^{2+}].

Drugs. Tetrodotoxin citrate was purchased from Ascent Scientific (Bristol, UK). Cyclopiazonic acid was purchased from Tocris Bioscience (Ellisville, MO). Other chemicals were purchased from Sigma-Aldrich Inc. (St. Louis, MO). 4OH-GTS-21 (i.e., 3-(4-hydroxy-2-methoxy-benzylidene)anabaseine) was synthesized in the Department of Pharmacology and Therapeutics at the University of Florida, Gainesville, FL, USA.

Data analysis. To quantify presynaptic effects of nicotine in the caudal NTS, the mean frequency (Hz) and amplitude (pA) of mEPSCs before and after brief focal nicotine puffs were estimated and analyzed off-line using MiniAnalysis 6.0.3 (Synaptosoft Inc., Fort Lee, NJ; http://www.synaptosoft.com). Caudal NTS neurons were defined as presynaptically-responsive only if the mean frequency of mEPSCs estimated over 5 s immediately after nicotine puffs was at least twice as high as the mean mEPSC
frequency estimated over 5 s immediately before nicotine puffs. Experimental tests were only conducted on neurons defined as presynaptically-responsive. Therefore, a simple but robust online criterion (such as the 2-fold increase in the mEPSC frequency) was necessary during data acquisition to reliably define neurons as presynaptically-responsive or –unresponsive in the beginning of each experiment. By contrast, nicotine-elicited changes in mEPSC amplitudes were monitored, but not used to classify neurons.

All data are presented as means ±SD. The level of statistical significance was defined by the p-value <0.05. To determine the statistical significance of nicotine-mediated effects related to the same pool of NTS cells investigated under various experimental conditions (e.g., in the absence and presence of various drugs), standard Student’s paired t-tests were conducted using an online version of GraphPad (GraphPad Software, Inc., LaJolla, CA; http://graphpad.com). To evaluate the effects of nicotine puffs on mEPSC amplitudes in a single presynaptically-responsive neuron, cumulative amplitude distributions of mEPSCs recorded before and after nicotine puffs were compared using the Kolmogorov-Smirnov two-sample test and the p-values were then determined.

To analyze the temperature dependence of presynaptic facilitation, the coefficient of determination (i.e., $R^2$) was evaluated from the linear regression of shifts in the mean mEPSC frequency and amplitude as a function of ACSF temperature (Figures 8D and 8F, respectively). The t-value was then determined from the $R^2$-value using the following equation Eq. 1 (40):
\[ t = R \sqrt{\frac{n - 2}{1 - R^2}} \quad \text{Eq. 1} \]

where \( n \) is the number of experiments and \( R \) is the correlation coefficient. The p-value was then calculated using its definition (Eq. 2, (40)):

\[
p = 1 - \frac{ \Gamma \left( \frac{v + 1}{2} \right) }{ \Gamma \left( \frac{v}{2} \right) \sqrt{\pi v} } \int_{-t}^{t} \left( 1 + \frac{x^2}{v} \right)^{-\frac{v+1}{2}} \, dx \quad \text{Eq. 2}
\]

where \( v = n - 2 \) is the number of degrees of freedom. If \( p < 0.05 \) (\( p > 0.05 \)), the linear regression was defined as significantly (insignificantly) different from the horizontal line.

To solve mathematical equations and to build mEPSC frequency and amplitude histograms, Mathematica 2.2.3 software package (Wolfram Research, Inc. Champaign, IL) was used. Gray traces and histograms always illustrate control data (i.e., before nicotine puffs), while black traces and histograms illustrate data recorded after a nicotine puff.

**Methodological Advantages and Limitations.** Nicotinic agonists often exhibit greater potencies for desensitization than activation of nAChRs (52). Therefore, while bath administration of agonists is an appropriate model of systemic drug administration allowing for a full control of the final ACSF concentration of nicotinic agonists, it may cause asynchronous activation and desensitization of nAChRs and thus, hinder the
detection and quantification of nicotine-mediated mEPSCs. More importantly, in this study, the knowledge of the final ACSF concentration of nicotinic agonists is not critical because nAChRs would likely desensitize before the application of nicotinic agonists to the bath is completed and the agonist concentration is equilibrated in the slice. This challenge is eased by using focal rapid pressure administration of nicotinic agonists (i.e., “puffs”) which produces synchronous activation and minimal desensitization of nAChRs during each response allowing rapid washout of applied drugs and multiple applications of nicotinic agonists to the same cell as required by this study. Addressing the probabilistic nature and the transient character of the nicotine puff-mediated release of glutamatergic vesicles, this approach (analogous to electrical stimulation) allows repeatable stimulation, detection and quantification of multi-fold transient elevations in the frequency of nicotine-mediated mEPSCs. The responsiveness of glutamatergic synapses to nicotine is then used to define and characterize the pharmacology and Ca$^{2+}$ dependence of nicotine-mediated mechanisms of glutamatergic synaptic release.

Similar experiments could be conducted using acutely dissociated NTS neurons with attached presynaptic glutamatergic terminals, however the feasibility of such a preparation in the caudal NTS is uncertain as acute dissociation of NTS neurons may not preserve nicotine-responsive glutamatergic synapses. Moreover, in such a preparation, the proportion of presynaptically-responsive NTS neurons would most likely reflect the success rate of acute dissociation rather than the prevalence of nicotine-responsive glutamatergic presynaptic terminals. One advantage of such a preparation would be a capability to control the concentration of applied nicotinic agonists. Although advantageous in some studies, this capability brings limited value to this investigation.
as it is the stability of stimulation (i.e., analogous to electrical stimulation) and not the stimulus intensity per se, that is crucial for comparison of synaptic responses under various experimental conditions (e.g., in the presence and absence of Cd\textsuperscript{2+}/Ni\textsuperscript{2+}) required for this study.

Finally, this study did not investigate the nature and the distribution of presynaptically-responsive cells within the caudal NTS. However, in this and previous studies (44), presynaptic responsiveness to nicotinic agonists was observed in a substantial proportion of randomly chosen caudal NTS cells (see Results) suggesting the presence and broad distribution of functional nAChRs in presynaptic glutamatergic terminals within the caudal NTS. These and other aspects of nicotinic effects in the caudal NTS will be investigated in future studies.

RESULTS

Whole-cell voltage-clamp experiments were conducted in rat horizontal brainstem slices to investigate the mechanisms of facilitation of synaptic glutamate release by activation of presynaptic nAChRs. The mean capacitance, input resistance and access resistance of the recorded NTS cells were estimated to be 33.6±17.8 pF, 510±258 MΩ and 13.4±9.4 MΩ, respectively (n=169). When present, presynaptic nAChRs could be activated by brief focal pressure administration of 0.1-0.5 mM nicotine via a picospritzer pipette positioned within 15 µm from the recorded caudal NTS neuron (44). The observed facilitation of glutamatergic release by puffs of nicotinic agonists was detected as an increase in the frequency of miniature excitatory postsynaptic currents (mEPSCs) that lasted for less than a minute, but could be repeated as often as
once a minute (44). Experiments in this study were conducted in the continuous presence of 0.3 µM tetrodotoxin (TTX) to inhibit voltage-gated Na⁺ channels and action potentials. Therefore, the observed presynaptic effects of nicotine were attributed to activation of presynaptic (i.e., expressed directly on presynaptic terminals) and not pre-terminal nAChRs (i.e., expressed antidromically to presynaptic terminals) (28, 57). Together the current and previous (44) studies have demonstrated that ~40% (i.e., 86 out of 217) of caudal NTS neurons are presynaptically-responsive to nicotine (see Methods). Therefore, in subsets of caudal NTS neurons synaptic release of glutamate can be potentiated by activation of presynaptic nAChRs, most likely α3β4-containing, as discussed previously (44).

Nicotine-mediated facilitation of excitatory postsynaptic currents. In the presence of 0.3 µM TTX, brief (~100 ms) focal pressure administration of 0.1-0.5 mM nicotine transiently increased the frequency of mEPSCs (Figure 1A). mEPSCs were reversibly blocked by 15 µM 6,7-dinitroquinoxaline-2,3-dione (DNQX, n=6, Figure 1B-C) supporting the involvement of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors. Examples of mEPSCs recorded from a presynaptically-responsive caudal NTS neuron and the corresponding analysis of mEPSCs before (gray traces, histograms and curves) and after (black traces, histograms and curves) nicotine puffs are illustrated in Figure 1D-F. In many presynaptically-responsive cells a nicotine-elicited increase in mEPSC frequency (Figure 1E) was accompanied by an increase in mEPSC amplitude (Figure 1F). The Kolmogorov-Smirnov two-sample test applied to cumulative mEPSC amplitude distributions (Figure 1F, right graph) demonstrated that in
75% of tested presynaptically-responsive cells (i.e., 27 out of 36), nicotine significantly potentiated the amplitudes of mEPSCs. In the remaining 25% (i.e., 9 out of 36) of tested presynaptically-responsive cells, nicotine puffs did not significantly change mEPSC amplitudes (not shown). Therefore, these results indicate the existence of two sub-groups of presynaptically-responsive caudal NTS neurons. In the first sub-group of neurons, activation of presynaptic nAChRs by nicotine significantly increases the mean frequency, but not the mean amplitude of mEPSCs. In the second sub-group of neurons, activation of presynaptic nAChRs by nicotine significantly increases both the mean frequency and amplitude of mEPSCs.

**Effects of 10 μM mecamylamine.** The nicotine-mediated potentiation of mEPSC frequency was completely blocked by 10 μM mecamylamine, a non-selective nAChR antagonist with a higher potency for α3β4 nAChRs (37). Examples of current traces recorded from a presynaptically-responsive caudal NTS neuron before and after nicotine puffs in the absence and presence of mecamylamine in ACSF are shown in Figure 2A-B. In nine experiments with presynaptically-responsive caudal NTS neurons nicotine significantly enhanced the mean mEPSC frequency (n=9, Student’s paired t-test, Figure 2C, two left columns) and this enhancement was eliminated by adding 10 μM mecamylamine to ACSF (n=9, Student’s paired t-test, Figure 2C, two right columns). In these nine experiments, the mean amplitude of mEPSCs was not significantly altered by nicotine puffs in the absence (two left columns) and presence (two right columns) of 10 μM mecamylamine (n=9, Student’s paired t-test, Figure 2D). The mean frequency of mEPSCs after nicotine puffs (Figure 2C, filled columns) were significantly reduced by
mecamylamine (n=9). By contrast, the mean frequency of spontaneous mEPSCs (i.e.,
before nicotine puffs, Figure 2C, open columns) was not significantly altered by
mecamylamine (n=9). The mean mEPSC amplitudes before and after nicotine puffs
were not significantly different in the absence and presence of mecamylamine (n=9,
Figure 2D). These results demonstrate that in presynaptically-responsive caudal NTS
cells, nicotine facilitates synaptic glutamate release via activation of functional
presynaptic nAChRs.

Ca²⁺ dependence. To test for the Ca²⁺-dependence of nicotine effects on
glutamatergic release in presynaptically-responsive caudal NTS neurons, zero-Ca²⁺
ACSF (i.e., ACSF-0) was made by an addition of 5 mM EGTA (i.e., ethylene glycol-
bis(2-aminoethylether)-N,N',N,N'-tetraacetic acid) to nominally Ca²⁺-free ACSF. To test
the effectiveness of ACSF-0, Ca²⁺ influx into NTS neuronal somata was induced in
voltage-clamp experiments by brief (2.5 s) depolarizing voltage steps to 0 mV from -80
mV (n=2, Figure 3A-B). Intracellular Ca²⁺ concentration ([Ca²⁺]) was monitored using a
fluorescent Ca²⁺ imaging system (see Methods). Voltage-steps generated strong
elevations in [Ca²⁺], (Figure 3A, control, black trace) which were completely (Figure 3A,
gray trace), but reversibly (Figure 3B) blocked by a 10 min perfusion of the slice in
ACSF-0. These experiments confirmed that ACSF-0 is not a substantial source of Ca²⁺
influx into NTS neurons and thus, can be used for testing the Ca²⁺-dependence of
nicotine-mediated facilitation of mEPSCs.

In four experiments, ACSF was replaced with ACSF-0 and at least 5 min were
allowed for equilibration of ACSF-0 within the slice before patch-clamp recordings
Examples of current traces recorded from a presynaptically-responsive caudal NTS neuron before and after nicotine puffs in ACSF and ACSF-0 are shown in Figure 3C-D. In ACSF-0, nicotine-mediated facilitation of mEPSCs vanished rendering the same NTS neuron presynaptically-unresponsive (Figure 3D). A summary of results from four experiments is shown in Figure 3E-F. In ACSF, nicotine significantly enhanced the mean mEPSC frequency (n=4, Student’s paired t-test, Figure 3E, two left columns) and this enhancement was eliminated by ACSF-0 (n=4, Student’s paired t-test, Figure 3E, two right columns). In these four experiments, the mean amplitude of mEPSCs was not significantly altered by nicotine in either ACSF or ACSF-0 (n=4, Student’s paired t-test, Figure 3F). Moreover, ACSF-0 did not significantly alter the mean frequency of spontaneous mEPSCs (i.e., before nicotine puffs, Student’s paired t-test, Figure 3E, open columns), but significantly reduced the mean amplitude of spontaneous mEPSCs (paired t-test, Figure 3F, open columns). The mean frequency (Student’s paired t-test, Figure 3E, filled columns) and the mean amplitude of mEPSCs (Student’s paired t-test, Figure 3F, filled columns) after nicotine puffs were also significantly reduced in ACSF-0. These results demonstrate that external Ca$^{2+}$ is essential for nicotine-mediated facilitation of synaptic glutamate release in presynaptically-responsive caudal NTS neurons.

**Insensitivity to 0.2 mM Cd$^{2+}$ plus 0.2 mM Ni$^{2+}$ ions.** Activation of presynaptic nAChRs by nicotine may not enhance synaptic release of glutamate via a direct Ca$^{2+}$ influx through presynaptic nAChR-mediated ion channels. Instead, it may potentiate glutamate release indirectly, via depolarizing presynaptic glutamatergic terminals and
activating presynaptic high-threshold (i.e., HVACCs (27, 47, 48, 56)) or low-threshold (i.e., LVACCs (4, 36, 38)) voltage-activated Ca^{2+} channels (VACCs). This possible involvement of presynaptic VACCs as amplifying agents of presynaptic nAChRs was investigated in voltage-clamp experiments using potent blockers of HVACCs and LVACCs. Specifically, in eight experiments, a mixture of 0.2 mM Cd^{2+} (a potent blocker of HVACCs (48)) plus 0.2 mM Ni^{2+} (a potent blocker of Ca_{V3.2} LVACCs (22)) was added to ACSF for at least 5 min and focal 0.5 mM nicotine puffs were applied every 3 min in the continuous presence of 0.3 μM TTX and 20 nM MLA in ACSF.

The facilitation of mEPSCs by nicotine remained in the presence of 0.2 mM Cd^{2+} plus 0.2 mM Ni^{2+} in ACSF. Examples of current traces recorded from a presynaptically-responsive caudal NTS neuron before and after nicotine puffs in the absence and presence of 0.2 mM Cd^{2+} plus 0.2 mM Ni^{2+} in ACSF are shown in Figure 4A-B. A summary of results from eight experiments with presynaptically-responsive caudal NTS neurons is shown in Figure 4C-D. In these experiments, in the absence of 0.2 mM Cd^{2+} plus 0.2 mM Ni^{2+}, nicotine significantly enhanced both the mean mEPSC frequency (n=8, Student’s paired t-test, Figure 4C, two left columns) and the mean amplitude of mEPSCs (n=8, Student’s paired t-test, Figure 4D, two left columns). In the presence of 0.2 mM Cd^{2+} plus 0.2 mM Ni^{2+}, nicotine still significantly increased both the mean frequency (n=8, Student’s paired t-test, Figure 4C, two right columns) and the mean amplitude of mEPSCs (n=8, Student’s paired t-test, Figure 4D, two right columns). The mean frequency of mEPSCs after nicotine puffs (Figure 4C, filled columns) as well as the mean frequency of spontaneous mEPSCs (i.e., before nicotine puffs, Figure 4C, open columns) were not significantly altered by an addition of 0.2 mM Cd^{2+} plus 0.2 mM
Ni$^{2+}$ to ACSF (n=8, Student’s paired t-test, Figure 4C). Similarly, the mean mEPSC amplitudes before and after nicotine puffs were not significantly different in the absence and presence of 0.2 mM Cd$^{2+}$ plus 0.2 mM Ni$^{2+}$ (n=8, Student’s paired t-test, Figure 4D). These results demonstrate that presynaptic HVCCs (which are sensitive to 0.2 mM Cd$^{2+}$) and Ca$_{v}$3.2 LVACCs (which are sensitive to 0.2 mM Ni$^{2+}$) are not responsible for the observed facilitation of mEPSCs by nicotine.

In these experiments TTX, MLA, Cd$^{2+}$ and Ni$^{2+}$ were added to ACSF at least 5 min prior to nicotine application. This pre-incubation time was sufficient for a complete block of $\alpha$7$^*$ nAChRs and presynaptic VACCs because somatodendritic $\alpha$7$^*$ nAChR-mediated responses were completely blocked after only 3 min of perfusion with 20 nM MLA (not shown, but see Figure 2 in (44)). Moreover, in a similar horizontal brainstem slice preparation and under similar experimental settings, perfusion of slices with 0.2 mM Cd$^{2+}$ plus 0.2 mM Ni$^{2+}$ for 5 min was sufficient for a complete but reversible block of: 1) evoked EPSCs recorded in voltage-clamp in the absence of TTX (Figure 4E); and 2) putative VACC-dependent spontaneous oscillations of the NTS membrane voltage recorded in current-clamp in the continuous presence of 0.5 $\mu$M TTX (Figure 4F). In experiments utilizing Cd$^{2+}$ and Ni$^{2+}$, phosphate salts were removed from ACSF to avoid potential precipitation of Cd$^{2+}$ and Ni$^{2+}$ ions.

Effects of 1 mM Ni$^{2+}$ on mEPSCs. Activation of presynaptic nAChRs in subsets of caudal NTS neurons may recruit presynaptic Ca$_{v}$3.1 and Ca$_{v}$3.3 LVACCs which are relatively insensitive to 0.2 mM Ni$^{2+}$ (8, 22, 26, 38). To test this possibility, in twelve experiments the concentration of Ni$^{2+}$ in ACSF was elevated to 1 mM and 0.2 mM
nicotine was pressure-applied to presynaptically-responsive caudal NTS neurons in the absence (control) and presence (>8 min exposure) of 1 mM Ni\textsuperscript{2+} in ACSF. TTX (0.3 μM), MLA (20 nM) and Cd\textsuperscript{2+} (0.2 mM) were continuously present in ACSF to inhibit voltage-gated Na\textsuperscript{+} ion channels, α7\textsuperscript{*} nAChRs and HVACCs, respectively.

The nicotine-mediated increase in the frequency of mEPSCs remained in the presence of 0.2 mM Cd\textsuperscript{2+} plus 1 mM Ni\textsuperscript{2+} in ACSF. A summary of results from twelve experiments is shown in Figure 4G-H. In these experiments, in the absence of 0.2 mM Cd\textsuperscript{2+} plus 1 mM Ni\textsuperscript{2+}, nicotine significantly enhanced both the mean mEPSC frequency (n=12, Student’s paired t-test, Figure 4G, two left columns) and the mean amplitude of mEPSCs (n=12, Student’s paired t-test, Figure 4H, two left columns). In the presence of 0.2 mM Cd\textsuperscript{2+} plus 1 mM Ni\textsuperscript{2+}, nicotine still significantly increased the mean mEPSC frequency (n=12, Student’s paired t-test, Figure 4G, two right columns), but not the mean amplitude of mEPSCs (n=12, Student’s paired t-test, Figure 4H, two right columns). The mean frequency of mEPSCs after nicotine puffs (Figure 4G, filled columns) as well as the mean frequency of spontaneous mEPSCs (i.e., before nicotine puffs, Figure 4G, open columns) were not significantly altered by an addition of 0.2 mM Cd\textsuperscript{2+} plus 1 mM Ni\textsuperscript{2+} to ACSF (Student’s paired t-test, Figure 4G). Similarly, in these experiments, the mean mEPSC amplitudes before and after nicotine puffs were not significantly different in the absence and presence of 0.2 mM Cd\textsuperscript{2+} plus 1 mM Ni\textsuperscript{2+} (Student’s paired t-test, Figure 4H).

These results indicate that presynaptic HVACCs and LVACCs do not contribute to the observed facilitation of mEPSCs by nicotine and activation of presynaptic nAChRs provides the required direct influx of Ca\textsuperscript{2+} ions into presynaptic glutamatergic
terminals sufficient to trigger synaptic release of glutamate. However, 1 mM Ni^{2+} blocks nicotine-mediated potentiation of mEPSC amplitudes (Figure 4H, two right columns) and this effect may reflect inhibition of presynaptic non-α7 nAChRs by 1 mM Ni^{2+}.

**Effects of 1 mM Ni^{2+} on NTS somatodendritic nAChRs.** To determine whether 1 mM Ni^{2+} indeed inhibits activation of non-α7 nAChRs by nicotine, caudal NTS neurons expressing functional somatodendritic non-α7 nAChRs were identified by a random search and responses of somatodendritic non-α7 nAChRs to pressure-applied 0.2 mM nicotine were investigated in the continuous presence of 0.3 μM TTX and 20 nM MLA (Figure 5). In some experiments, 0.2 mM Cd^{2+} was also present in ACSF. Nicotine (0.2 mM, ~100 ms) was pressure-applied every 2-4 min via a picospritzer pipette in the absence of 1 mM Ni^{2+} (Figure 5A) and during the wash-in phase of 1 mM Ni^{2+} (Figure 5B-C). Whole-cell responses were recorded, averaged over several experiments, normalized to the control responses (i.e., responses recorded prior to application of 1 mM Ni^{2+}) and plotted as a diagram (Figure 5C). Each point represents data obtained from two to six experiments. These results demonstrate that 1 mM Ni^{2+} considerably inhibits nicotine-evoked responses of somatodendritic non-α7 nAChRs in caudal NTS neurons leaving on average only 53.6±7.5% (dashed arrow, Figure 5C) of those receptors available for activation by 0.2 mM nicotine upon 8-30 min (i.e., averaged over 12 amplitudes) of 1 mM Ni^{2+} wash-in. Nevertheless, activation of the remaining available presynaptic nAChRs by 0.2 mM nicotine appears to be sufficient for triggering synaptic release of glutamate in experiments with presynaptically-responsive caudal NTS neurons (Figure 4). However, this block may contribute to the inhibition of
nicotine-mediated potentiation of mEPSC amplitudes by 1 mM Ni^{2+} (Figure 4H). These conclusions were made on the basis of an assumption that somatodendritic and presynaptic non-α7 nAChRs in the caudal NTS are functionally and pharmacologically similar.

**Contribution of presynaptic α7* nAChRs.** Although the nicotine-mediated facilitation of mEPSCs occurs in the presence of 20 nM MLA and thus, in the absence of activation of highly Ca^{2+}-permeable α7* nAChRs (Figures 4 and (44)), in some cases presynaptic glutamatergic terminals may co-express functional α3β4* and α7* nAChRs and activation of either of those nAChRs may be sufficient for facilitation of synaptic glutamate release. To test this hypothesis, the responsiveness of presynaptically-responsive caudal NTS neurons to 10 μM 4OH-GTS-21, a selective α7 nAChR agonist, was investigated. This concentration of 4OH-GTS-21 has been shown to produce a nearly maximum α7* nAChR-mediated current net charge (51) and thus, would be expected to produce a nearly maximum Ca^{2+} influx into presynaptic glutamatergic terminals if these terminals indeed express functional α7* nAChRs. In these experiments, pressure-applied 0.2 mM nicotine was used as a positive control to identify presynaptically-responsive NTS cells. Examples of current traces recorded in these experiments under different experimental conditions are shown in Figure 6A-D.

A summary of results obtained from nineteen presynaptically-responsive caudal NTS neurons is shown in Figure 6E. To evaluate and compare the effects of nicotine and 4OH-GTS-21 pertaining to the same caudal NTS neuron, the mean mEPSC frequencies measured within 5 s before and 5 s after nicotine and 4OH-GTS-21 puffs
were estimated and their ratios \( f = \frac{\text{frequency after puff}}{\text{frequency before puff}} \) were calculated (Figure 6E). Fourteen out of nineteen (i.e., ~74%) presynaptically-responsive (to nicotine) caudal NTS neurons (Figure 6A and Figure 6E, left filled column) did not respond to 10 µM 4OH-GTS-21 \( (f=1.14+0.37; \) Figure 6B and Figure 6E, left open column) and therefore, the effects of 0.2 mM nicotine and 10 µM 4OH-GTS-21 on the mEPSC frequency were significantly different \( (n=14, \text{Student's paired t-test, Figure 6E, two left columns}) \). In the remaining five cases (i.e., ~26%), 0.2 mM nicotine (Figure 6C, middle trace) and 10 µM 4OH-GTS-21 (Figure 6D, middle trace) produced similar increases in the mEPSC frequency \( (n=5, \text{Student's paired t-test, Figure 6E, two right columns}) \) suggesting the expression and activation of presynaptic functional \( \alpha 7 \) nAChRs. In two of those five experiments, mEPSC facilitation elicited by nicotine (Figure 6C) and 4OH-GTS-21 (Figure 6D) was completely blocked by 20 nM MLA added to ACSF (Figure 6C-D, bottom traces) indicating that glutamatergic terminals synapsing onto these two cells expressed functional \( \alpha 7^* \) nAChRs, while presynaptic non-\( \alpha 7 \) nAChRs were either not expressed or not effective in triggering synaptic release of glutamate. In the remaining three presynaptically-responsive (to nicotine) cells, facilitation of mEPSCs by 10 µM 4OH-GTS-21 was not apparent during data acquisition and thus, MLA was not used during recordings. Those three cells were later identified as presynaptically-responsive (to both nicotine and 4OH-GTS-21) as a result of post-experimental analysis.

These results suggest that the majority of glutamatergic terminals in the caudal NTS do not express functional \( \alpha 7^* \) nAChRs at densities sufficient for triggering nicotine-mediated glutamate release. However, some glutamatergic terminals in the caudal NTS
express functional α7* nAChRs and do not express functional non-α7 nAChRs, as evidenced by the sensitivity of nicotine- and 4OH-GTS-21-mediated facilitation of mEPSCs to 20 nM MLA (n=2, Figure 6C-D, bottom traces) and by the similarity of effects elicited by 0.2 mM nicotine and 10 µM 4OH-GTS-21 (Figure 6E, two right columns). Pressure-applied 0.2 mM nicotine resulted in a similar increase in mEPSC frequency in cells innervated by non-α7 and α7* nAChR-expressing presynaptic glutamatergic terminals (Student’s unpaired t-test, Figure 6E, filled columns). As expected, pressure-applied 10 µM 4OH-GTS-21 resulted in a significantly greater potentiation of mEPSC frequency mediated by presynaptic α7* than non-α7 nAChRs (Student’s unpaired t-test, Figure 6E, open columns).

A lack of contribution of presynaptic Ca\(^{2+}\) stores. Facilitation of synaptic glutamate release by nicotine may require a rapid recruitment of Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) from presynaptic Ca\(^{2+}\) stores. To test for the presence of functional and rapidly available presynaptic Ca\(^{2+}\) stores in presynaptically-responsive caudal NTS neurons, 10 mM caffeine, a potent and rapid mediator of CICR was used in fourteen experiments. Focal pressure application of 0.2 mM nicotine (Figure 7A) or 0.2 mM cytisine (not shown) was used to identify presynaptically-responsive caudal NTS neurons. Focal pressure administration of 10 mM caffeine onto presynaptically-responsive cells (n=14, Figure 7B) did not reproduce the effects of 0.2 mM nicotine (n=7, Figure 7B) or 0.2 mM cytisine (n=7, not shown) in any of the fourteen cells tested. A summary of results is shown in Figure 7C. To compare the effects of 0.2 mM nicotine and 10 mM caffeine on the same presynaptically-responsive caudal NTS neuron, the mean mEPSC
frequencies measured over 5 s before and 5 s after nicotine and caffeine puffs were estimated and their ratios \[ f = \frac{\text{frequency after puff}}{\text{frequency before puff}} \] were calculated (Figure 7C). Pressure-applied 0.2 mM nicotine produced significant increases in the frequency of mEPSCs (14.17±11.26, n=7, Figure 7C, filled column, Student’s paired t-test) compared to puffs of 10 mM caffeine (0.90±0.14, n=7, Figure 7C, open column). Caffeine and nicotine were applied to the recorded NTS neurons alternately via two picospritzer pipettes positioned within 15 µm from each other and the recorded cell. These results demonstrate that presynaptic Ca\(^{2+}\) stores are unlikely to play a role in facilitation of synaptic glutamate release in presynaptically-responsive caudal NTS neurons.

As a positive control for the effects of caffeine on internal Ca\(^{2+}\) stores, in two experiments caudal NTS neurons were filled with 50 µM Ca-Green-1 via a recording patch-clamp pipette. Caffeine (10 mM) was then pressure-applied via a picospritzer pipette positioned within 15 µm from the recorded neuron and changes in the level of somatic Ca\(^{2+}\) (i.e., \([Ca^{2+}]_i\)) were recorded using a fluorescent Ca\(^{2+}\) imaging system (see Methods). Pressure-applied 10 mM caffeine produced robust transient elevations in \([Ca^{2+}]_i\). A typical example of somatic \([Ca^{2+}]_i\), responses in caudal NTS neurons is shown in Figure 7D. These responses were completely blocked by 50 µM cyclopiazonic acid (CPA, a potent blocker of Ca\(^{2+}\)-ATPase in the intracellular Ca\(^{2+}\) stores) added to ACSF (Figure 7E). These tests revealed that focal pressure-applied 10 mM caffeine is effective in triggering somatic CICR in caudal NTS neurons and therefore, the lack of caffeine-mediated facilitation of mEPSCs in presynaptically-responsive caudal NTS
neurons (Figure 7B) is likely due to the absence of presynaptic Ca\textsuperscript{2+} stores or their ineffectiveness in facilitating glutamate release in these neurons.

**Temperature dependence of mEPSC facilitation.** In this study, all results were obtained from patch-clamp experiments conducted at room temperature (24°C). To extrapolate these results to physiological temperatures, six experiments with presynaptically-responsive caudal NTS neurons were conducted at higher temperatures (up to 32°C, Figure 8A) and the effects of nicotine on mEPSCs were investigated as a function of ACSF temperature (Figure 8B-F). In these experiments, presynaptically-responsive NTS cells were identified by the presence of nicotine-evoked facilitation in mEPSC frequency at room temperature (Figure 8B, top trace) as in other experiments of this study. Then, nicotine puffs were delivered every 3 minutes and mEPSCs were recorded (Figure 8B, bottom trace) while ACSF temperature was gradually raised to ~32°C and then, reduced back to ~24°C (n=6, Figure 8A). To compare the effects of nicotine puffs on the same presynaptically-responsive caudal NTS neuron at different ACSF temperatures, the mean mEPSC frequencies and amplitudes measured over 5 s before and 5 s after nicotine puffs were estimated at 24°C and 32°C and their ratios \( f = \frac{\text{value after puff}}{\text{value before puff}} \) were calculated. These ratios were then plotted as a function of time (Figures 8C and 8E) or the corresponding ACSF temperature (Figures 8D and 8F). The correlations between nicotine-mediated increases in the frequency and amplitude of mEPSCs and ACSF temperature were evaluated by a linear regression fit and the coefficients of determination were calculated: \( R^2 = 0.17 \) (Figure 8D) and \( R^2 = 0.15 \) (Figure 8F), respectively. The statistical significance of differences
between the linear regression and the horizontal line was estimated in each case: 

\( p > 0.18 \) and \( p > 0.21 \), respectively (Methods). The estimated p-values indicated that in both cases, linear regressions were insignificantly different from the horizontal line (Figures 8D and 8F). The large variability in the increase in mEPSC frequency at \( \sim 31^\circ C \) (Figure 8D) was a result of two strong consecutive responses to nicotine puffs. When those two responses were ignored (not shown), the p-values (\( p > 0.38 \) and \( p > 0.39 \) for mEPSC frequency and amplitudes, respectively) still revealed insignificant differences between regression fits and the horizontal line. Therefore, these data indicate that in presynaptically-responsive caudal NTS neurons, the effects of nicotine on mEPSC frequency and amplitudes do not significantly depend on ACSF temperature within the range between 24°C and 32°C. These results suggest that the key conclusions of this study are directly applicable to nicotine-responsive caudal NTS synapses operating at higher ACSF temperatures and possibly, physiological temperatures as well.

**DISCUSSION**

In subsets of caudal NTS neurons, synaptic release of glutamate can be enhanced by activation of presynaptic nAChRs (Figure 1 and (44)). In this study, facilitation of synaptic release of glutamate was found to: 1) require activation of functional presynaptic non-\( \alpha 7 \) (~74%) and \( \alpha 7^* \) (~26%) nAChRs (Figure 6), as evidenced by a complete block of nicotine effects by 10 \( \mu M \) mecamylamine (Figure 2), a non-selective antagonist of nAChRs with a greater potency for \( \alpha 3\beta 4^* \) nAChRs (37); 2) require external \( Ca^{2+} \) ions because facilitation of mEPSCs by nicotine completely
vanished in zero-Ca\(^{2+}\) ACSF (i.e., in the presence of nominal 0 mM Ca\(^{2+}\) plus 5 mM EGTA in ACSF; Figure 3); 3) not require activation of presynaptic HVACCs or LVACCs (Figure 4) because facilitation of mEPSCs persisted in the presence of 0.2 mM Cd\(^{2+}\) (a potent blocker of HVACCs (21, 48)) plus 1 mM Ni\(^{2+}\) (a potent blocker of LVACCs (38)); and 4) not require involvement of presynaptic internal Ca\(^{2+}\) stores because 10 mM caffeine did not reproduce facilitation of mEPSCs by nicotine (Figure 7). Moreover, the effects of nicotine on glutamatergic mEPSCs were insensitive to ACSF temperatures between 24°C and 32°C (Figure 8).

We have previously reported (44) that in ~69% of presynaptically-responsive caudal NTS neurons, the nicotine-elicited increase in the mean mEPSC frequency is accompanied by an increase in the mean mEPSC amplitude. In the present study, a slightly higher proportion (i.e., 75%; or 27 out of 36) of caudal NTS neurons exhibited this phenomenon. Although the exact reason for this variability is presently unknown, several explanations can be offered. It is possible that some presynaptically-responsive NTS neurons express subgroups of high-amplitude-low-probability (i.e., HALP) glutamatergic synapses (41, 53, 54). These HALP synapses generate larger mEPSCs, but their probability of release is low. Therefore, in the absence of stimulation, HALP synapses would be expected to remain relatively inactive and thus, inaudible (53), but they can be transiently activated by nicotine puffs to generate mEPSCs with higher amplitudes. Not all caudal NTS neurons may express HALP synapses giving rise to the observed variability of results. Another possibility is that some caudal NTS glutamatergic synapses may exhibit a nicotine-mediated synchronous release of multiple glutamatergic vesicles increasing the amplitudes of corresponding mEPSCs.
However, multivesicular release may require activation of presynaptic intracellular Ca\textsuperscript{2+}
stores (18, 30) which have not been detected in this study (Figure 7). Other potential
causes of variability in mEPSC amplitudes may include variability in the size of
 glutamatergic vesicles (5), random summation of mEPSCs, and potentiation of
postsynaptic AMPARs by nicotine. These possibilities have not been investigated in
this study and are the subject of future research.

The majority of experiments in this study were conducted in the continuous
presence of 0.3 \(\mu\)M TTX in ACSF to inhibit Na\textsuperscript{+} voltage-gated ion channels and action
potentials. Therefore, facilitation of mEPSCs was attributed to activation of presynaptic
(i.e., expressed directly on presynaptic terminals) and not pre-terminal nAChRs (i.e.,
expressed antidromically to presynaptic terminals) (27, 57). Activation of presynaptic
nAChRs is able to facilitate neurotransmitter release either directly, via increasing Ca\textsuperscript{2+}
influx through nAChR-mediated ion channels or indirectly, via causing transient
depolarization of presynaptic terminals and activation of presynaptic VACCs (15, 19, 24,
27, 39, 45, 47, 56). To distinguish between these two possibilities, nicotine-mediated
facilitation of mEPSCs in the caudal NTS was investigated using potent antagonists of
HVACCs and LVACCs (Figure 4). This focus was especially important because the
majority of experiments were conducted in the presence of 20 nM MLA in ACSF and
thus, in the absence of activation of highly Ca\textsuperscript{2+}-permeable \(\alpha 7^*\) nAChRs. Facilitation of
glutamatergic mEPSCs in presynaptically-responsive caudal NTS neurons has been
previously reported to be mediated by presynaptic \(\alpha 3\beta 4^*\) nAChRs (44). The
permeability of \(\alpha 3\beta 4^*\) nAChR-mediated ion channels to Ca\textsuperscript{2+} ions is relatively low
compared to \(\alpha 7^*\) nAChRs (7, 25, 49) and therefore, activation of presynaptic \(\alpha 3\beta 4^*\)
nAChRs may not be sufficient for triggering glutamate release unless it is amplified by HVACCs and/or LVACCs. The presented experimental results argue against this latter hypothesis demonstrating that activation of presynaptic HVACCs and/or LVACCs is not required for triggering nicotine-mediated facilitation of synaptic glutamate release in the caudal NTS (Figure 4). However, activation of presynaptic VACCs by nAChR-mediated depolarization of presynaptic glutamatergic terminals may amplify these direct facilitating effects of nAChRs in the absence of TTX, when presynaptic voltage-gated Na⁺ ion channels are active and thus, can amplify activation of presynaptic VACCs.

The use of high concentrations of metals (e.g., 0.2 mM Cd²⁺ plus 1 mM Ni²⁺, Figure 4) as a pharmacological tool poses the danger of misinterpreting data because of a possible direct inhibition of nAChR-mediated ion channels. For example, 0.2 mM Cd²⁺ has been shown to partially inhibit α7* nAChRs in hypothalamic tuberomammillary neurons (50). To avoid a potential misinterpretation of data, the effects of 1 mM Ni²⁺ on non-α7 nAChRs were investigated in greater detail using functional somatodendritic nAChRs expressed in some caudal NTS neurons (Figure 5). In the presence of 1 mM Ni²⁺ added to ACSF for at least 8 min, somatodendritic responses of caudal NTS neurons to pressure-applied 0.2 mM nicotine were inhibited by ~46% (Figure 5). These experiments were conducted in the continuous presence of 0.3 µM TTX and 20 nM MLA in ACSF. In some experiments, 0.2 mM Cd²⁺ was also present in ACSF. If similar types of functional non-α7 nAChRs are expressed on glutamatergic terminals of presynaptically-responsive caudal NTS neurons, it would be expected that only ~54% of those receptors would remain available for activation by 0.2 mM nicotine after >8 min of 1 mM Ni²⁺ wash-in (horizontal dashed arrow, Figure 5C). Nevertheless, these
remaining presynaptic nAChRs appear sufficient for facilitation of synaptic release of glutamate (Figure 4) via a direct Ca\(^{2+}\) influx into presynaptic glutamatergic terminals. One potential pitfall includes a possibility that presynaptic and somatodendritic nAChRs in caudal NTS neurons contain different nAChR subunits and exhibit different sensitivity to Ni\(^{2+}\). However, regardless of the exact subunit compositions of presynaptic and somatodendritic nAChRs in the caudal NTS, the results of this study demonstrate that 0.2 mM Cd\(^{2+}\) plus 1 mM Ni\(^{2+}\) (an inhibitory mixture sufficient to completely block all VACCs) did not eliminate nicotine-mediated facilitation of synaptic glutamate release and thus, presynaptic VACCs are not required for such facilitation.

Experiments with 10 μM 4OH-GTS-21 demonstrated that in a sub-population of caudal NTS neurons, presynaptic α7* nAChRs contribute to and play the key role in facilitation of mEPSCs by nicotine because in five out of nineteen experiments (i.e., ~26%) the effects of both 4OH-GTS-21 and nicotine were completely blocked by 20 nM MLA (Figure 6C-D). In the remaining fourteen presynaptically-responsive NTS cells (i.e., ~74%), 4OH-GTS-21 did not facilitate mEPSCs (Figure 6A-B). These results suggest that a co-expression of functionally-relevant densities of non-α7 and α7* nAChRs in the same presynaptically-responsive glutamatergic terminals of caudal NTS neurons is unlikely. Instead, glutamatergic presynaptic terminals appear to either not express nAChRs at functionally-relevant densities (i.e., in presynaptically-unresponsive neurons, Types III-IV, (44)); or express predominantly functional non-α7 (Figure 6A-B) or predominantly functional α7* nAChRs (Figure 6C-D) (i.e., in presynaptically-responsive neurons). Therefore, the results of this study define two functionally distinct sub-groups of presynaptically-responsive glutamatergic terminals in the caudal NTS: 1)
terminals (~74%) that express predominantly or exclusively functional non-α7 nAChRs and thus, do not respond to 10 μM 4OH-GTS-21, while responding to 0.2 mM nicotine by generating mEPSCs (Figure 6A-B); and 2) terminals (~26%) that express predominantly or exclusively functional α7* nAChRs and thus, generate mEPSCs as a response to both 0.2 mM nicotine and 10 μM 4OH-GTS-21 (Figure 6C-D, middle traces); this responsiveness is completely blocked by 20 nM MLA (Figure 6C-D, bottom traces). To date, in the caudal NTS, presynaptic glutamatergic terminals that co-express functionally-relevant densities of non-α7 and α7* nAChRs have not been detected (n=19, Figure 6).

The exact sources of cholinergic projections to the NTS are unknown and may include the key brainstem cholinergic nuclei (e.g., the pedunculopontine nucleus or the dorsal motor nucleus of the vagus (DMV)) as well as local NTS cholinergic interneurons. The DMV acts as one of the prime projection targets of NTS neurons (6, 9, 17, 20, 46). Both glutamatergic and GABAergic neurotransmissions have been reported for NTS-DMV projections (6, 9, 17, 20, 46). Therefore, an intriguing possibility points to cholinergic inputs arriving to the NTS neurons from the neighboring DMV. Because of its close vicinity, cholinergic DMV neurons may not need to directly synapse on NTS neurons, but instead, may be able to activate NTS nAChRs (including presynaptic nAChRs expressed on glutamatergic terminals) via volume transmission of ACh (29). This possibility may be particular attractive if nicotine-responsive NTS neurons are inhibitory GABAergic cells projecting to the DMV. In this case, cholinergic inputs to the NTS from the DMV may act as negative feedback inhibiting overly-excited DMV neurons. On the other hand, a moderate activity of DMV neurons may not cause a
robust release of ACh and its volume transmission, and thus, may not be sufficiently strong for activation of nAChRs in the NTS and NTS-mediated inhibition of its own (i.e., DMV) activity.

The results of this study further support heterogeneity of neuronal properties and nAChR expression in the NTS (43, 44, 55) and suggest that activation of functional nAChRs expressed on presynaptic glutamatergic terminals in the caudal NTS may enhance the efficacy of glutamatergic neurotransmission in this brain region and thus, enhance the integration of viscerosensory information with inputs arriving from other brain regions and modulate autonomic visceral functions and reflexes.

Although the presence of functional presynaptic nAChRs and their ability to modulate synaptic release of glutamate are clearly demonstrated in this study (see also (44)), the sources of nicotine-responsive glutamatergic terminals remain unknown. Presynaptically-responsive caudal NTS neurons may receive high-fidelity glutamatergic inputs from the solitary tract (ST) and/or low-fidelity glutamatergic inputs from within the NTS (both ipsilateral and contralateral) or other brain regions including some of the NTS primary targets (e.g., the paraventricular nucleus, the parabrachial nucleus) with which the NTS establishes reciprocal connections. It seems unlikely (but not impossible) that the primary high-fidelity ST afferents could draw physiological benefits from expressing presynaptic nAChRs and presynaptic facilitation by nicotinic agonists. It is more likely that some of the higher order low-fidelity glutamatergic synapses can be strengthened by activation of presynaptic nAChRs by nicotine or endogenous ACh. This enhanced fidelity of nicotine-responsive glutamatergic terminals may increase the impact of centrally-originating information versus vagal afferent-originating information. Moreover,
the expression of specific subtypes of presynaptic and somatodendritic nAChRs in the
caudal NTS may be function-specific and correlate with cytochemical identity, neuronal
morphology, projection targets and other cellular properties. These and other aspects
of nicotinic function in the caudal NTS are the subject of future investigation.
Understanding the mechanisms and pharmacology of nicotinic effects in the caudal
NTS may benefit development of new therapies for selective targeting of specific
autonomic pathways (e.g., gastrointestinal, cardiorespiratory) and impaired autonomic
homeostasis.

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**Figure 1.** Nicotine-mediated facilitation of mEPSCs in subsets of caudal NTS neurons. Brief (~100 ms) focal pressure administration of 0.2 mM nicotine onto subsets of caudal NTS neurons facilitated miniature excitatory postsynaptic currents (mEPSCs) (A and D-E). B-C) mEPSCs were completely and reversibly blocked by 15 µM DNQX, a selective antagonist of AMPA receptors. D) Current traces recorded from a presynaptically-responsive caudal NTS neuron within 5 s before (gray trace, left) and after (black trace, right) nicotine puffs in the continuous presence of 0.3 µM TTX. Nicotine application artifacts are not shown. E-F) Analysis of mEPSC frequencies (E) and amplitudes (F) from traces shown in (D) before (gray histograms and curves) and after (black histograms and curves) nicotine puffs. Nicotine puffs more than doubled the frequency of mEPSCs (E) defining this neuron as presynaptically-responsive (see Methods) and significantly increased mEPSC amplitudes as determined from cumulative amplitude distribution (F, right graph) by the Kolmogorov-Smirnov two-sample test (see Methods). Not all presynaptically-responsive caudal NTS neurons exhibited nicotine-mediated increase in mEPSC amplitudes (see text).

**Figure 2.** Nicotine-mediated facilitation of synaptic release of glutamate requires functional presynaptic nAChRs. Examples of current traces recorded from a presynaptically-responsive caudal NTS neuron within 5 s before (gray traces, left) and after (black traces, right) nicotine puffs in the absence (A) and presence (B) of 10 µM mecamylamine. Nicotine application artifacts are not shown. Mecamylamine (10 µM)
added to ACSF, completely eliminated facilitation of mEPSCs by nicotine. C-D) A summary of results obtained from nine presynaptically-responsive caudal NTS neurons. The mean mEPSC frequencies and amplitudes were calculated over 19-37 s of recordings before (open columns, C-D) and after (filled columns, C-D) nicotine puffs. A Student’s paired t-test was used for statistical analysis. In these experiments, nicotine significantly increased the mean mEPSC frequency (n=9, C, two left columns), but not the mean amplitude of mEPSCs (n=9, D, two left columns). Mecamylamine (10 µM) completely eliminated nicotine-mediated increases in the mean mEPSC frequency (n=9, C, two right columns). The mean frequency of mEPSCs after nicotine puffs (C, filled columns) were significantly altered by mecamylamine (n=9). By contrast, the mean frequency of spontaneous mEPSCs (i.e., before nicotine puffs, C, open columns) were not significantly altered by mecamylamine (n=9). The mean mEPSC amplitudes before and after nicotine puffs were not significantly different in the absence and presence of mecamylamine (n=9, D). All experiments were conducted in the presence of 0.3 µM TTX.

**Figure 3. Ca$^{2+}$-dependence of nicotine-mediated facilitation of synaptic release of glutamate.** Voltage-steps (2.5 s duration) to 0 mV from -80 mV were applied in voltage-clamp experiments using caudal NTS neurons. The influx of Ca$^{2+}$ ions into NTS somata was monitored using a fluorescent Ca$^{2+}$ imaging system (A-B). Removing external Ca$^{2+}$ resulted in a complete (gray trace, A), but reversible (B) elimination of Ca$^{2+}$ influx (black trace, A) demonstrating that ACSF-0 is not a substantial source of Ca$^{2+}$. C-F) Current traces and data analysis obtained from presynaptically-responsive
caudal NTS neurons. Examples of current traces recorded from a presynaptically-responsive caudal NTS neuron within 5 s before (gray traces, left) and after (black traces, right) nicotine puffs in ACSF (C) and ACSF-0 (D). In ACSF-0, facilitation of mEPSCs by nicotine completely vanished (D). Nicotine application artifacts are not shown. E-F) A summary of results obtained from four presynaptically-responsive caudal NTS neurons under similar experimental conditions. The mean mEPSC frequencies and amplitudes were calculated over 19-37 s of recordings before (open columns, E-F) and after (filled columns, E-F) nicotine puffs. A Student's paired t-test was used for statistical analysis. In these experiments, nicotine significantly increased the mean mEPSC frequency (n=4, E, two left columns), but not the mean amplitude of mEPSCs (n=4, F, two left columns). In ACSF-0, nicotine-mediated increases in the mean mEPSC frequency completely vanished (n=4, E, two right columns). Nicotine-mediated changes in the mean mEPSC amplitude remained insignificant in ACSF-0 (n=4, F, two right columns). ACSF-0 did not significantly alter the mean frequency of spontaneous mEPSCs (i.e., before nicotine puffs, n=4, E, open columns), but significantly reduced the mean amplitude of spontaneous mEPSCs (n=4, F, open columns). Both the mean frequency (n=4, E, filled columns) and the mean amplitude of mEPSCs (n=4, F, filled columns) after nicotine puffs were significantly reduced in ACSF-0. All experiments were conducted in the presence of 0.3 µM TTX.

Figure 4. Insensitivity of nicotine-mediated facilitation of mEPSCs to 0.2 mM Cd^{2+} plus 0.2-1 mM Ni^{2+} ions. A-F) Current traces and data analysis obtained from presynaptically-responsive caudal NTS neurons. Examples of current traces recorded
from a presynaptically-responsive caudal NTS neuron within 5 s before (gray traces, left) and after (black traces, right) nicotine puffs. Nicotine application artifacts are not shown. mEPSCs were recorded under control conditions (i.e., 0.3 µM TTX + 20 nM MLA), (A) and after an addition of 0.2 Cd²⁺ plus 0.2 mM Ni²⁺ (B). C-D) A summary of results obtained from eight presynaptically-responsive caudal NTS neurons under similar experimental conditions. The mean mEPSC frequencies and amplitudes were calculated over 19-37 s of recordings before (open columns, C-D) and after (filled columns, C-D) nicotine puffs. A Student’s paired t-test was used for statistical analysis.

In control experiments, pressure-applied nicotine significantly increased the mean mEPSC frequency (n=8, C, two left columns) and the mean amplitude of mEPSCs (n=8, D, two left columns). In the presence of 0.2 mM Cd²⁺ plus 0.2 mM Ni²⁺, nicotine still significantly increased both the mean frequency (n=8, C, two right columns) and the mean amplitude of mEPSCs (n=8, D, two right columns). The mean frequency of mEPSCs after nicotine puffs (C, filled columns) as well as the mean frequency of spontaneous mEPSCs (i.e., before nicotine puffs, C, open columns) were not significantly altered by 0.2 mM Cd²⁺ plus 0.2 mM Ni²⁺. Similarly, the mean mEPSC amplitudes before and after nicotine puffs were not significantly different in the absence and presence of 0.2 mM Cd²⁺ plus 0.2 mM Ni²⁺ (n=8, D). E-F) In a similar horizontal brainstem slice preparation and under similar experimental conditions, perfusion of slices with 0.2 mM Cd²⁺ plus 0.2 mM Ni²⁺ for 5 min completely, but reversibly blocked: 1) evoked EPSCs recorded in voltage-clamp in the absence of TTX (E); and 2) putative VACC-dependent spontaneous oscillations of the NTS membrane voltage recorded in current-clamp in the continuous presence of 0.5 µM TTX (F). Horizontal bars in F)
indicate the membrane voltage of -50 mV. In experiments involving electrical
stimulation (E), the stimulation electrode was positioned near the solitary tract and short
(0.1-0.4 ms) single pulses (0.5-0.8 mA) were applied every 30 s to evoke EPSCs (E).
G-H) A summary of results obtained from twelve presynaptically-responsive caudal
NTS neurons. mEPSCs were recorded before and after nicotine puffs under control
ACSF conditions (i.e., 0.3 μM TTX + 20 nM MLA + 0.2 mM Cd^{2+}) and after an addition
of 1 mM Ni^{2+} to ACSF. The mean mEPSC frequencies and amplitudes were calculated
over 19-37 s of recordings before (open columns, G-H) and after (filled columns, G-H)
nicotine puffs. A Student’s paired t-test was used for statistical analysis. In control
experiments, pressure-applied nicotine significantly increased the mean mEPSC
frequency (n=12, G, two left columns) and the mean amplitude of mEPSCs (n=12, H,
two left columns). In the presence of 1 mM Ni^{2+}, nicotine still significantly increased the
mean mEPSC frequency (n=12, G, two right columns), but not the mean amplitude of
mEPSCs (n=12, H, two right columns). The mean frequency of mEPSCs after nicotine
puffs (G, filled columns) and the mean frequency of spontaneous mEPSCs (i.e., before
nicotine puffs, G, open columns) were not significantly altered by 1 mM Ni^{2+} (n=12, G).
Similarly, the mean mEPSC amplitudes before and after nicotine puffs were not
significantly different in the absence and presence of 1 mM Ni^{2+} (n=12, H).

Figure 5. Effects of 1 mM Ni^{2+} on somatodendritic non-α7 nAChRs in the caudal
NTS. The effects of 1 mM Ni^{2+} on non-α7 nAChRs were investigated using caudal NTS
neurons expressing functional somatodendritic non-α7 nAChRs. ACSF contained 0.3
μM TTX and 20 nM MLA. In some experiments, 0.2 mM Cd^{2+} was also present in
Nicotine (0.2 mM, 50-100 ms) was pressure-applied via a picospritzer pipette every 2-4 min in the absence of 1 mM Ni\(^{2+}\) (0 min, A) and during the wash-in phase of 1 mM Ni\(^{2+}\) (30 min, B). Whole-cell responses were recorded, averaged over several cells, normalized to the control responses (i.e., before 1 mM Ni\(^{2+}\)) and plotted as a diagram (C). Each point represents data obtained from two to six cells (C). A horizontal dashed arrow marks the position of the mean peak amplitude of whole-cell currents (53.6±7.5%, n=12) elicited by focal pressure application of 0.2 mM nicotine and recorded between 8 min and 30 min (n=12) of the wash-in phase of 1 mM Ni\(^{2+}\) added to ACSF.

**Figure 6. Contribution of presynaptic \(\alpha^{7*}\) nAChRs.** Fourteen out of nineteen (i.e., ~74%) presynaptically-responsive (to nicotine) caudal NTS neurons (A) did not respond to 10 µM 4OH-GTS-21 (B). In the remaining five cells (C-D), both 0.2 mM nicotine and 10 µM 4OH-GTS-21 facilitated mEPSCs (C-D, middle traces). Traces shown in C-D were obtained from the same cell ~5 min apart. E) A summary of results obtained from nineteen presynaptically-responsive (to nicotine) caudal NTS neurons. The majority (~74%) of these cells did not respond to 4OH-GTS-21 (n=14, Student’s paired t-test, E, two left columns) indicating that the majority of glutamatergic terminals do not express functionally-relevant densities of \(\alpha^{7*}\) nAChRs. The remaining ~26% of cells responded equally to both nicotine and 4OH-GTS-21 (n=5, Student’s paired t-test, E, two right columns). Pressure-applied 0.2 mM nicotine resulted in similar mEPSC frequencies in cells innervated by non-\(\alpha 7\) and \(\alpha^{7*}\) nAChR-expressing glutamatergic terminals (Student’s unpaired t-test, E, filled columns). By contrast, pressure-applied 10 µM 4OH-GTS-21 resulted in a significantly greater mEPSC frequency in cells innervated by only
α7⁺ nAChR-expressing glutamatergic terminals (Student’s unpaired t-test, E, open columns). ACSF contained 0.3 µM TTX.

**Figure 7. Lack of contribution of presynaptic Ca²⁺ stored.** Representative traces of responses to focal pressure application of 0.2 mM nicotine (A) and 10 mM caffeine (B) to the same presynaptically-responsive (to nicotine) caudal NTS neuron. A lack of responsiveness to 10 mM caffeine (B) suggests a lack of expression of presynaptic Ca²⁺ stores or their ineffectiveness at triggering synaptic release of glutamate. C) A summary of results obtained from seven presynaptically-responsive (to nicotine) cells. The mean mEPSC frequencies measured over 5 s before and 5 s after nicotine and caffeine puffs were estimated and their ratios \( f = (\text{frequency after puff}) / (\text{frequency before puff}) \) were plotted (C). Pressure-applied 0.2 mM nicotine produced significant increases in the frequency of mEPSCs \( (14.17±11.26, n=7, C, \text{filled column, Student’s paired t-test}) \) compared to puffs of 10 mM caffeine \( (0.90±0.14, n=7, C, \text{open column, Student’s paired t-test}) \). Caffeine and nicotine were applied to the recorded NTS neurons alternately (at least 3 min apart) via two picospritzer pipettes positioned within 15 µm from each other and the recorded cell. D) As a positive control, in two experiments, caudal NTS neurons were filled with 50 µM Ca-Green-1 via a recording patch-clamp pipette and caffeine (10 mM) was then briefly (400 ms) applied on the recorded cell via a picospritzer pipette resulting in robust elevations in \([\text{Ca}^{2+}]\). These elevations were completely blocked by 50 µM CPA added to ACSF (E) confirming the effectiveness of pressure-applied 10 mM caffeine for Ca²⁺ release from internal stores.
Figure 8. Temperature dependence of nicotine-elicited facilitation of mEPSCs. In six presynaptically-responsive caudal NTS neurons, nicotine-elicited facilitation of mEPSCs was tested at different ACSF temperatures between 24°C and 32°C. The same temperature profile (A) was repeated in each of the six experiments: ACSF temperature was increased transiently from 24°C to 32°C during a 3 min ramp and then it was allowed to recover to the baseline level (A). Nicotine puffs were applied every 1.5 min and the ratios of the mean mEPSC frequencies (C) and amplitudes (E) recorded over 5 s before and 5 s after nicotine puffs were determined for each nicotine application (i.e., \( f = \frac{\text{value after puff}}{\text{value before puff}} \)). These ratios were then plotted as a function of time (C and E) and ACSF temperature (D and F). Data were fitted with a linear regression and the coefficients of determinant were determined (\( R^2 = 0.17 \) (D) and \( R^2 = 0.15 \) (F)). These values translated into the corresponding p-values of \( p > 0.18 \) (D) and \( p > 0.21 \) (F) indicating that the linear regressions are only insignificantly different from the horizontal line and thus, pointing to a lack of dependence on ACSF temperature.


Washout DNQX
+ 15 μM DNQX, 5 min

F

0.2 mM nic

0.2 mM nic

Washout DNQX

0.2 mM nic

Before nicotine

After nicotine

mEPSC frequency

mEPSC amplitude

Before 0.5 mM nicotine puff

After 0.5 mM nicotine puff

0.3 mM TTX

0.3 mM TTX

50 pA 800 ms

50 pA 800 ms

* p<0.05
Mean mEPSC frequency

Before 0.5 mM nicotine puff

After 0.5 mM nicotine puff

Before 0.5 mM nicotine puff

After 0.5 mM nicotine puff

Mean mEPSC amplitude

Before 0.5 mM nicotine puff

After 0.5 mM nicotine puff

Before 0.5 mM nicotine puff

After 0.5 mM nicotine puff

0.3 mM TTX

0.3 mM TTX + 10 mM Mecamylamine

*
**Control Nicotine**

**mPSC amplitude, pA**

$-80 \text{ mV}$

$2+ 0 \text{ mM Ca}^{2+} + 5 \text{ mM EGTA}, 10 \text{ min}$

**Washout, 10 min**

$-80 \text{ mV}$

Before 0.5 mM nicotine puff

After 0.5 mM nicotine puff

$0 \text{ mM Ca}^{2+} + 5 \text{ mM EGTA}$

Mean mEPSC frequency

Mean mEPSC amplitude
Figure A: 0.2 mM nicotine, 0 mM Ni^{2+} (0 min)

Figure B: 0.2 mM nicotine, +1 mM Ni^{2+} (30 min)

Figure C: Relative peak amplitude vs. Duration of 1 mM Ni^{2+} application, min

The graph shows the relative peak amplitude over time for different concentrations of Ni^{2+} in the presence of 0.2 mM nicotine. The data points are plotted with error bars indicating variability.
Presynaptic non-\(\alpha7\) nAChRs

A. Before nicotine
B. Before 4OH-GTS-21

C. Before nicotine
D. Before 4OH-GTS-21

E. Bar graph showing the number of mEPSCs (5 s after puff) for 0.2 mM nicotine, 10 \(\mu\)M 4OH-GTS-21, 0.2 mM nicotine + 20 nM MLA, and 10 \(\mu\)M 4OH-GTS-21 + 20 nM MLA for both presynaptic non-\(\alpha7\) nAChRs and presynaptic \(\alpha7\) nAChRs.