Characterization of synapses in the rat subnucleus centralis of the nucleus tractus solitarius

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Babic T, Ambler J, Browning KN, Travagli RA. Characterization of synapses in the rat subnucleus centralis of the nucleus tractus solitarius. J Neurophysiol 113: 466–474, 2015. First published October 29, 2014; doi:10.1152/jn.00598.2014.—The nucleus tractus solitarius (NTS) receives subdiaphragmatic visceral sensory information via vagal A- or C-fibers. We have recently shown that, in contrast to cardiovascular NTS medialis neurons, which respond to either purinergic or vanilloid agonists, the majority of esophageal NTS centralis (cNTS) neurons respond to vanilloid agonists, whereas a smaller subset responds to both vanilloid and purinergic agonists. The present study aimed to further investigate the neurochemical and synaptic characteristics of cNTS neurons using whole cell patch-clamp, single cell RT-PCR and immunohistochemistry. Excitatory postsynaptic currents (EPSCs) were evoked in cNTS by tractus solitarius stimulation, and in 19 of 64 neurons perfusion with the purinergic agonist αβ-methylene ATP (αβMeATP) increased the evoked EPSC amplitude significantly. Furthermore, neurons with αβMeATP-responsive synaptic inputs had different probabilities of release compared with nonresponsive neurons. Single cell RT-PCR revealed that 8 of 13 αβMeATP-responsive neurons expressed metabotropic glutamate receptor 8 (mGluR8) mRNA, which our previous studies have suggested is a marker of glutamatergic neurons, whereas only 3 of 13 expressed glutamic acid dehydroxylase, a marker of GABAergic neurons. A significantly lower proportion of αβMeATP-nonresponsive neurons expressed mGluR8 (2 of 30 neurons), whereas a greater proportion expressed glutamic acid dehydroxylase (12 of 30 neurons). Esophageal distension significantly increased the number of colocализed mGluR8- and c-Fos-immunoreactive neurons in the cNTS from 8.0 ± 4% to 20 ± 2.5%. These data indicate that cNTS comprises distinct neuronal subpopulations that can be distinguished based on their responses to purinergic agonists and that these subpopulations have distinct neurochemical and synaptic characteristics, suggesting that integration of sensory inputs from the esophagus relies on a discrete organization of synapses between vagal afferent fibers and cNTS neurons.

nucleus tractus solitarius; vagus; electrophysiology; brain stem

THE NUCLEUS TRACTUS SOLITARIUS (NTS) comprises second-order neurons that receive and integrate visceral afferent fibers from the gustatory, gastrointestinal (GI), pulmonary, and cardiovascular systems with reciprocal inputs connecting the NTS with higher centers in the central nervous system. Within the NTS, vagal afferent fibers are organized in an overlapping topographical manner. For example, cardiovascular afferents from the aortic depressor nerve terminate in the interstitial, lateral, and medial (mNTS) subnuclei of the NTS (Barraco et al. 1992; Ciriello and Calaresu 1981), afferents from the stomach and intestine terminate in the commissural, gelatinous, and medial subnuclei, whereas inputs from the esophagus terminate exclusively in the subnucleus centralis (cNTS) (Altschuler et al. 1989; Rogers et al. 1999). It is not surprising, therefore, that NTS neurons are not homogenous but rather comprise different neuronal subgroups that display distinct physiological and functional characteristics, including responses to different sensory modalities, conduction velocities, and neurochemical phenotypes (Brookes et al. 2013; Mendelowitz 1999).

The different properties of NTS neuronal subgroups have been characterized thoroughly in relation to their inputs from baroreceptors (Andresen and Kunze 1994; Mifflin 2007). Recent studies have demonstrated that mNTS neurons receiving inputs from myelinated (A-type) baroreceptor fibers respond to purinergic agonists such as ATP and αβ-methylene-ATP (αβMeATP) but not to the vanilloid transient receptor potential (TRPV1) receptor agonist capsaicin, whereas unmyelinated (C-type) fibers respond to capsaicin but not to purinergic agonists (Andresen et al. 2004; Bailey et al. 2002; Jin et al. 2004). Furthermore, it has also been demonstrated that electrophysiological and pharmacological properties of mNTS neurons projecting to the hypothalamus differ from those of mNTS neurons projecting to the caudal ventrolateral medulla or nucleus ambiguous (Bailey et al. 2006 and 2007). These findings indicate that, at the level of second-order NTS neurons, baroreceptor inputs to the central nervous system are organized into distinct functional and anatomic pathways and that this organization, which is correlated to the afferent fibers inputs and/or the physiological role of the neurons, is revealed by pharmacological responses to either vanilloid or purinergic agonists.

Similarly, studies in guinea pigs have demonstrated that nodose ganglion neurons innervating the lung and esophagus can be differentiated from jugular and dorsal root ganglion neurons based on their responses to purinergic and TRPV1 agonists (Brozmanova et al. 2011; Kwong et al. 2008); however, these studies did not investigate whether these agonists can be used to further differentiate subtypes of vagal afferents originating in the esophagus.

Electrophysiological properties of NTS neurons that receive sensory inputs from the GI tract, however, have not been characterized as thoroughly. Afferents from the GI tract are not confined to a discrete subnucleus of the NTS but are intermingled with afferents from other viscera, presenting difficulties in the investigation of GI-related NTS neurons. The exception is represented by cNTS neurons, which receive exclusively afferent fibers originating from the subdiaphragmatic esophagus and are located adjacent to the fibers of the tractus solitarius starting from the middle level of the area postrema and extending rostrally for ~5–600 μm and are activated by esophageal distention (Altschuler et al. 1989; Rogers et al. 1999).
Following the initial characterization of cNTS neurons (Baptista et al. 2005a and 2005b), our recent study has shown that, unlike mNTS neurons, which responded either to vanilloid or purinergic agonists, the majority of cNTS neurons are responsive to the vanilloid agonist capsaicin, whereas only a small subpopulation responded to both purinergic and vanilloid agonists (Browning et al. 2011). These data suggested that inputs from esophageal vagal afferent fibers can be separated into at least two cNTS neuronal subpopulations based on whether or not they respond to purinergic agonists such as αβMeATP (Browning et al. 2011). The release properties of the fibers impinging on these neurons and their neurochemical characteristics, however, are not known. Furthermore, unlike capsaicin, which excites the vast majority of cNTS neurons, αβMeATP excites only a small subpopulation of cNTS neurons and thus is a more suitable agent to distinguish potentially diverse sensory inputs in afferent fiber properties.

GI and cardiorespiratory sensory information is relayed by the afferent vagus to NTS neurons via a glutamatergic synapse (Andresen and Kunze 1994; Baptista et al. 2005b; Barraco et al. 1992; Ciriello and Calaresu 1981; Jean 2001; Mifflin and Felder 1990). NTS neurons integrate this sensory information, and GI-related signals are transmitted to parasympathetic preganglionic neurons of the dorsal motor nucleus of the vagus (DMV) via glutamatergic, GABAergic, and catecholaminergic synapses (Travagl et al. 2006). Several laboratories, including ours, have shown that synaptic inputs from the NTS to the DMV are under modulatory control by a plethora of neurotransmitters and neuromodulators, which fine tune synaptic transmission, adapting it to constantly changing physiological conditions (Babic and Travagl 2014; Balfour and Trapp 2007; Browning and Travagl 2007; Holmes et al. 2013). Among other neuromodulators, we have demonstrated that metabotropic glutamate receptors (mGlurS) display a highly specific organization on synaptic terminals that impinge on gastric- and pancreas-projecting neurons (Babic et al. 2012; Browning and Travagl 2007). In particular, we have shown that the glutamatergic synapse between NTS and DMV neurons is modulated by group III mGlurS; conversely, a tonic glutamatergic input activates group II mGlurS selectively and modulates GABAergic synaptic transmission (Browning and Travagl 2007).

Taken together, these data suggest that esophageal sensory information may rely on a yet-uncharacterized, extremely specialized pattern of synaptic transmission and neurotransmitter release from vagal afferent fibers to cNTS neurons.

The aim of the present study was to use a combination of patch-clamp electrophysiology, single cell RT-PCR, and immunohistochemistry to investigate whether subgroups of cNTS neurons display distinct pharmacological, synaptic, and neurochemical characteristics.

MATERIALS AND METHODS

All experiments were conducted on Sprague-Dawley rats of both sexes. Protocols were conducted according to the guidelines set forth by the National Institutes of Health and were approved by the Penn State University Institutional Animal Care and Use Committee.

Electrophysiological recording. Rat brain stem slices containing the cNTS were prepared as previously described (Baptista et al. 2005a). Briefly, rats were anesthetized with isoflurane, and the brain stem was removed, placed in chilled, oxygenated Krebs solution [containing (in mM) 126 NaCl, 25 NaHCO3, 2.5 KCl, 1.2 MgCl2, 2.4 CaCl2, 1.2 NaH2PO4, and 11 glucose, maintained at pH 7.4 by bubbling with 95% O2-5% CO2. Two to three coronal slices (300 μm) from the level of the cNTS (Altshuler et al. 1989; Rogers et al. 1999) were cut and incubated in Krebs solution at 30°C for at least 90 min before recording. A single slice was transferred to a custom-made perfusion chamber, kept in place with a nylon mesh, and maintained at 30°C by perfusion with warm Krebs solution at a rate of 2.5–3.0 ml/min.

Whole cell recordings were made with patch pipettes of resistance 4–6 MΩ when filled with K-glucuronate intracellular solution [containing (in mM) 128 K-glucuronate, 10 KCl, 0.3 CaCl2, 1 MgCl2, 10 HEPES, 1 EGTA, 2 ATP-Na, and 0.25 GTP-Na, adjusted to pH 7.35 with KOH].

Synaptic currents [evoked excitatory postsynaptic currents (eEPSCs)] were evoked using tungsten bipolar stimulating electrodes (WPI, Sarasota, FL) placed in the tractus solitarius. Stimuli were applied every 10 s to evoke submaximal EPSCs. The perfusing Krebs solution contained picrotoxin (50 μM) to prevent GABAergic currents, and the pipette solution contained the intracellular γNa blocker QX314 (1 μM) to prevent antidromically activated action potentials.

Equisynomolar Krebs solution with varying concentrations of extracellular Ca2+ concentration ([Ca2+]o) before variance-mean (V-M) analysis was initiated. V-M analysis of compound inputs obtained upon stimulation of several presynaptic terminals impinging onto cNTS neurons was obtained according to Clements (2003). This type of analysis permits the calculation of the release probability, number of functional release sites, and quantal size. To construct V-M curves, 30–40 eEPSCs were evoked by stimulation of the tractus solitarius (0.5-ms duration) at a frequency of 0.1 Hz while the brain stem slice was perfused in Krebs solution with 2.4, 1.5, 0.5, or 0.25 mM [Ca2+]o. eEPSC amplitudes (mean) were measured as the peak current minus the mean baseline current measured in the 15 ms preceding the stimulus. Variance was measured as the square of the eEPSC amplitude obtained at each [Ca2+]o. V-M values were fitted after the fit was constrained to intersect the zero release state using variance = 0 at mean = 0, with a least-square method using the following equation: y = Ax + Bx2, where y is the variance in eEPSC amplitude, x is the mean eEPSC amplitude, A is the quantal size, and 1/B is the number of functional release sites. The resulting parabola (fitted with GraphPad Prism, GraphPad Software, LaJolla, CA) was extended to intersect the x-axis to estimate the maximal eEPSC amplitude (EPPSCmax) used to normalize data across conditions (Babic and Travagl 2014; Balfour and Trapp 2007; Holmes et al. 2013). Among other neuromodulators, we have shown that synaptic inputs to purinergic agonists, perfusion with αβMeATP was conducted in 2.4 mM [Ca2+]o before variance-mean (V-M) analysis was initiated. V-M analysis of compound inputs obtained upon stimulation of several presynaptic terminals impinging onto cNTS neurons was obtained according to Clements (2003). This type of analysis permits the calculation of the release probability, number of functional release sites, and quantal size. To construct V-M curves, 30–40 eEPSCs were evoked by stimulation of the tractus solitarius (0.5-ms duration) at a frequency of 0.1 Hz while the brain stem slice was perfused in Krebs solution with 2.4, 1.5, 0.5, or 0.25 mM [Ca2+]o. eEPSC amplitudes (mean) were measured as the peak current minus the mean baseline current measured in the 15 ms preceding the stimulus. Variance was measured as the square of the eEPSC amplitude obtained at each [Ca2+]o. V-M values were fitted after the fit was constrained to intersect the zero release state using variance = 0 at mean = 0, with a least-square method using the following equation: y = Ax + Bx2, where y is the variance in eEPSC amplitude, x is the mean eEPSC amplitude, A is the quantal size, and 1/B is the number of functional release sites. The resulting parabola (fitted with GraphPad Prism, GraphPad Software, LaJolla, CA) was extended to intersect the x-axis to estimate the maximal eEPSC amplitude (EPPSCmax) used to normalize data across neuronal recordings. The release probability was calculated as the percent ratio of the EPSC amplitude at a given [Ca2+]o to the estimated EPPSCmax.

Single cell RT-PCR. In a group of neurons, after the effects of perfusion with αβMeATP on eEPSC had been assessed, the cytoplasm of the cell was pulled into a patch pipette using negative pressure. The pipette tip was then broken in a PCR tube containing resuspension buffer (1 μl, Integrated DNA Technologies, Coralville, IA) and RNAase inhibitor (0.25 μl RNAasePLUS, Promega, Madison, WI). Samples of the intracellular solution and water used for the extracellular solution were collected for control. Samples where then heated at 70°C for 2 min in a thermocycler followed by 10 min on ice. Reverse transcription was performed using SuperScript II reverse
transcriptase (Invitrogen, Grand Island, NY) using the following protocol: 45°C for 1 h, 70°C for 15 min, and 37°C for 20 min.

PCR was performed using the following primers: β-actin (GenBank Accession No. EF157276), outer product size 802 bp, forward 5'-ACTGGAGCAGATGACAGAAGA-3' and reverse 5'-ATAGAGCCACCATCTCCACACA-3', and inner product size 138 bp, forward 5'-GCCCTCTGACACAGTTAGAAG-3' and reverse 5'-ATCCTGAATGAGCTACATCC-3', and inner product size 480 bp, forward 5'-ATGATTGCCGACCAAGCTGACA-3' and reverse 5'-GATGAAAAGTACCAAATGATGATGCAC-3'; and glutamic acid dehydrogenase (Gad)65 (GenBank Accession No. M72242), outer product size 960 bp, forward 5'-TGACATCACAAGCAAAACAGAAGC-3' and reverse 5'-GAACTCGCAAACTAGGAGGTACA-3', and inner product size 360 bp, forward 5'-GCTTTGATCCTCTCCTGTTGTA-3' and reverse 5'-CTTGTCTCTCCACACATAAGGCACATAA-3'.

The reaction was performed in duplicate, with the outer sequence added first and then the inner sequence (1 µl each), using AmpliTaq DNA polymerase (Invitrogen) according to the manufacturer's instructions. The protocol consisted of a 5-min-long incubation at 94°C followed by 35 cycles of 94°C for 30 s, 63°C for 1 min, and 72°C for 2 min followed by a final extension at 72°C for 7 min. Products were then visualized in ethidium bromide-stained 2% agarose gels and photographed. PCR products were verified by direct sequencing (Eurofins Genomics, Huntsville, AL).

**Esophageal distension and immunohistochemistry.** Male Sprague-Dawley rats (250–450 g) were fasted overnight and anesthetized with Inactin (100–150 mg/kg ip), and a tracheal cannula was placed to maintain a clear airway. Esophageal distension balloon catheters were made from 1.0-mm outer diameter, 0.5-mm inner diameter silicone tubing connected to polyethylene tubing (PE-50) attached to a 1-ml syringe and a Statham P23 pressure transducer. The balloon was inserted in the esophagus and placed ~5 mm from the lower esophageal sphincter. Injection of 0.3 ml saline into the balloon expanded its stressed portion to a final outer diameter of ~3 mm, which we have previously demonstrated to increase esophageal pressure by 14–18 mmHg and activate low-threshold vagal mechanoreceptors (Rogers et al. 1999). After a minimum recovery period of 60 min, the balloon was distended for 1 s every 5 s for 40 min; 90 min after the end of the stimulation period, animals were perfused transcardially with saline followed by fixative (4% paraformaldehyde in 0.1 M PBS). The brain stem was removed and stored in fixative with 20% sucrose at 4°C for at least 48 h before being cut into 40-µm-thick coronal slices throughout the length of the dorsal vagal complex.

Two in five brain stem sections were reacted for immunohistochemistry as previously described (Llewellyn-Smith et al. 2012). Sections were rinsed three times for 10 min in Tris-PBS (TPBS) containing 0.3% Triton X-100 and 0.05% thimerosal and incubated in a solution of 30% methanol and 1% H2O2 to eliminate endogenous peroxidase activity. After three 10-min rinses in TPBS, sections were blocked in 10% normal horse serum in TPBS for at least 30 min. Sections were incubated in primary rabbit anti c-Fos antibody diluted a 1:500, Jackson Immunoresearch, West Grove, PA) overnight. After three 10-min rinses in TPBS, sections were incubated in ExtrAvidin-horseradish peroxidase as described above, and the reaction product was visualized with diaminobenzidine using glucose oxidase. Sections were rinsed, mounted on gelatin-coated microscope slides, dehydrated, and coverslipped. Labeling was examined using a light-field microscope (Nikon E400) and photographed using CellSens software (Olympus, Tokyo, Japan).

**Statistical analysis.** For electrophysiological recordings, each neuron served as its own control, i.e., eEPSC magnitude was assessed before and after drug application using a paired Student’s t-test. Single cell RT-PCR data were analyzed with the $\chi^2$-test. For immunohistochemistry, the total number of c-Fos neurons double labeled for mGluR8 was counted for caudal, intermediate, and rostral sections of the NTS and expressed as the average number of neurons per section. The number of neurons was compared between control and esophageal distension groups using an unpaired Student’s t-test.

All data are expressed as means ± SE with significance defined as P < 0.05.

**RESULTS**

**Synaptic characteristics of cNTS neurons.** To identify synaptic characteristics of cNTS neurons, eEPSCs were evoked by tractus solitarius stimulation under conditions that alter the probability of glutamate release, i.e., varying concentrations of extracellular Ca2+.

In 6 of 21 tested neurons, perfusion of the slices with 10 µM αβMeATP increased eEPSC amplitude from 262 ± 53 to 304 ± 56 pA (P < 0.05). In these neurons, stimulation of the tractus solitarius in the presence of 2.4 mM [Ca2+]e evoked eEPSCs of 346 ± 64 pA amplitude with a variance of 1,169 ± 244 pA2. Reducing [Ca2+]e to 1.5 mM decreased the eEPSC mean amplitude to 317 ± 54 pA but increased its variance to 2,743 ± 1,456 pA2. Lowering [Ca2+]e to 0.5 mM further decreased mean eEPSC amplitude to 175 ± 37 pA and decreased the variance to 1,614 ± 312 pA2, whereas in the presence of 0.25 mM [Ca2+]e, the mean eEPSC amplitude was 112 ± 39 pA and variance was 477 ± 147 pA2.

The characteristics of the parabolic curve illustrating the V-M analysis and release probabilities of αβMeATP-sensitive neurons at the various [Ca2+]e are shown in Figs. 1 and 2 and in Table 1.

In the remaining 15 neurons, αβMeATP did not affect the eEPSC amplitude. In these neurons, stimulation of the tractus solitarius in the presence of 2.4 mM [Ca2+]e evoked eEPSCs of 219 ± 34 pA with a variance of 1,019 ± 354 pA2. Reducing [Ca2+]e to 1.5 mM decreased the mean eEPSC amplitude to 171 ± 30 pA and increased the variance to 2,533 ± 887 pA2. Perfusion of the slices with 0.5 mM [Ca2+]e further reduced the mean eEPSC amplitude to 86 ± 17 pA and lowered the eEPSC variance to 987 ± 285 pA2. Both eEPSC mean and variance were further reduced in the presence of 0.25 mM [Ca2+]e (mean eEPSC amplitude: 35 ± 9 pA; variance: 707 ± 393 pA2).

The characteristics of the parabolic curve illustrating the V-M analysis and release probabilities of αβMeATP-insensitive neurons at various [Ca2+]e showed that these neurons have a significantly lower probability of release and a lower maximal estimated eEPSC amplitude. The data are shown in Figs. 1 and 2 and in Table 1.

These data demonstrate that neurons in the cNTS can be subdivided into two neuronal populations based on responses to purinergic agonists and that these populations have distinct synaptic properties.
expressed both genes (Fig. 3). 

pressed neither mGluR8 nor GAD67, whereas one neuron (i.e., 7%) that did not respond to αβMeATP expressed mGluR8 mRNA, whereas 12 neurons (i.e., 40%) expressed GAD67 mRNA, whereas only three αβMeATP-responsive neurons were more dependent on [Ca^{2+}]_{e} tested. 

Note that αβMeATP-responsive neurons displayed a higher probability of glutamate release compared with unresponsive neurons. Data from neurons with eEPSC responsive to αβMeATP are in gray; those unresponsive to αβMeATP are in black.

Neurochemical phenotype of cNTS neurons. In another group of cNTS neurons (N = 43), the effects of αβMeATP was tested on eEPSC, and, immediately after, the neuronal content was collected to perform single cell RT-PCR analysis. Thirteen of these cNTS neurons responded to perfusion with αβMeATP with an increase in eEPSC amplitude, whereas thirty neurons were unresponsive to perfusion with αβMeATP.

The majority (N = 8, i.e., 62%) of αβMeATP-responsive neurons expressed mGluR8 (a member of group III mGluR) mRNA, whereas only three αβMeATP-responsive neurons (i.e., 23%) expressed GAD67 mRNA and two αβMeATP-responsive neurons (i.e., 15%) expressed neither mGluR8 nor GAD67 mRNA.

In contrast to αβMeATP-responsive neurons, only 2 neurons (i.e., 7%) that did not respond to αβMeATP expressed mGluR8 mRNA, whereas 12 neurons (i.e., 40%) expressed GAD67 (\chi^2 < 0.05 compared with αβMeATP-responsive neurons). Fifteen of the nonresponsive neurons (i.e., 50%) expressed neither mGluR8 nor GAD67, whereas one neuron expressed both genes (Fig. 3).

These data demonstrate that cNTS neurons that respond to purinergic agonists are more likely to express group III mGluR, but not GAD, than αβMeATP-non responsive neurons.

Immunohistochemistry. To determine the neurochemical phenotype of cNTS neurons that are activated by esophageal distension, we performed double-labeling immunohistochemistry for c-Fos and mGluR8 in animals that received esophageal distension. In five control rats that received a sham esophageal distension (i.e., the distention balloon was placed in the esophagus but not inflated), 8 ± 1.8 NTS neurons/section were immunoreactive for c-Fos. The majority of these c-Fos-immunoreactive neurons (12 ± 3.7 neurons/section) were located in the intermediate NTS, with fewer neurons located in the caudal (7 ± 1.5 neurons/section) and rostral (6 ± 1.2 neurons/section) parts of the NTS. Of these c-Fos-immunoreactive neurons in the NTS, 9 ± 2.3% were also immunoreactive for mGluR8, with the highest number of c-Fos and mGluR double-labeled neurons (8.3 ± 3.4%) in the intermediate NTS. When the analysis was confined to the cNTS of control rats, 0.2 ± 0.05.
neurons. Neurons showing eEPSC amplitude at varying \([Ca^{2+}]_e\) concentrations are shown in Fig. 2. The eEPSC amplitude (mean) and variance response to varying concentrations of \([Ca^{2+}]_e\) showed a characteristic variation with variance maximal at intermediate mean. The top traces are from a neuron in which eEPSC was responsive to perfusion with \(\alpha\beta\text{MeATP}\); the bottom traces are from a neuron in which eEPSC was unresponsive to perfusion with \(\alpha\beta\text{MeATP}\). The bar represents the mean of the values for each group. Data from neurons with eEPSC responsive to \(\alpha\beta\text{MeATP}\) are in gray; those unresponsive to \(\alpha\beta\text{MeATP}\) are in black.

In seven animals that were subjected to esophageal dissection, 17 ± 3% of cNTS neurons that received eEPSC were labeled for c-Fos, and 5.7 ± 2% of these neurons were also labeled for mGluR8 (Fig. 4).

In seven animals that were subjected to esophageal dissection, 17 ± 3% of cNTS neurons that received eEPSC were labeled for c-Fos, and 5.7 ± 2% of these neurons were also labeled for mGluR8 (Fig. 4).

Table 1. Synaptic properties of nucleus tractus solitarius centralis neurons that received \(\alpha\beta\text{MeATP}-\text{sensitive or insensitive evoked excitatory postsynaptic currents}

<table>
<thead>
<tr>
<th></th>
<th>(\alpha\beta\text{MeATP}-\text{Sensitive Neurons})</th>
<th>(\alpha\beta\text{MeATP}-\text{Insensitive Neurons})</th>
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<tbody>
<tr>
<td>Maximal estimated amplitude, pA</td>
<td>474 ± 76</td>
<td>277 ± 47*</td>
</tr>
<tr>
<td>Number of release sites</td>
<td>26 ± 7</td>
<td>17 ± 4</td>
</tr>
<tr>
<td>Quantal size</td>
<td>21 ± 11</td>
<td>20 ± 3</td>
</tr>
<tr>
<td>Release probability at 2.4 mM ([Ca^{2+}]_e)</td>
<td>70 ± 8</td>
<td>59 ± 7</td>
</tr>
<tr>
<td>Release probability at 1.5 mM ([Ca^{2+}]_e)</td>
<td>62 ± 7</td>
<td>46 ± 6*</td>
</tr>
<tr>
<td>Release probability at 0.5 mM ([Ca^{2+}]_e)</td>
<td>39 ± 10</td>
<td>23 ± 4*</td>
</tr>
<tr>
<td>Release probability at 0.25 mM ([Ca^{2+}]_e)</td>
<td>26 ± 10</td>
<td>7 ± 7</td>
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Values are means ± SE; \(n = 6\) \(\alpha\beta\)-methylene ATP (\(\alpha\beta\text{MeATP}\))-sensitive neurons and 15 \(\alpha\beta\text{MeATP}-\text{insensitive neurons}. \(*P < 0.05\) vs. \(\alpha\beta\text{MeATP}-\text{responsive neurons.}

Fig. 2. cEPSC amplitude at varying \([Ca^{2+}]_e\) in cNTS neurons. A: representative traces showing eEPSC amplitude at 0.25–0.5–1.5 and 2.4 mM \([Ca^{2+}]_e\). The eEPSC amplitude (mean) and variance response to varying concentrations of \([Ca^{2+}]_e\) showed a characteristic variation with variance maximal at intermediate mean. The top traces are from a neuron in which eEPSC was responsive to perfusion with \(\alpha\beta\text{MeATP}\); the bottom traces are from a neuron in which eEPSC was unresponsive to perfusion with \(\alpha\beta\text{MeATP}\). B: pie charts showing the percentage of cNTS neurons that were unresponsive (left) or responsive (right) to \(\alpha\beta\text{MeATP}\) that expressed GAD65 or mGluR8. Note that compared with \(\alpha\beta\text{MeATP}-\text{responsive cNTS neurons, a significantly greater number of unresponsive cNTS neurons expressed GAD65, whereas a significantly lower number expressed mGluR8.}

Fig. 3. Single cell RT-PCR showing the expression of metabotropic glutamate receptor 8 (mGluR8) and glutamic acid dehydroxylase (GAD65) in neurons previously tested for eEPSC responsiveness to \(\alpha\beta\text{MeATP}\). A: representative 2% agarose gel showing PCR products for mGluR8 and GAD65 in \(\alpha\beta\text{MeATP}-\text{unresponsive and -responsive neurons. The PCR products were obtained with the primers specified in MATERIALS AND METHODS. cNTS neurons were characterized electrophysiologically before single-cell RT-PCR. B: pie charts showing the percentage of cNTS neurons that were unresponsive (left) or responsive (right) to \(\alpha\beta\text{MeATP}\) that expressed GAD65 or mGluR8. Note that compared with \(\alpha\beta\text{MeATP}-\text{responsive cNTS neurons, a significantly greater number of unresponsive cNTS neurons expressed GAD65, whereas a significantly lower number expressed mGluR8.}

The majority of these c-Fos-immunoreactive neurons (25 ± 7.6 neurons/section) were located in the intermediate NTS, with fewer neurons located in the rostral parts of the NTS (17 ± 2.6 neurons/section, \(P < 0.05\) vs. control for both areas). The number of neurons in the caudal
portion of the NTS (8 ± 3.0 neurons/section) was similar to that of control animals, indicating that esophageal distention did not increase c-Fos in areas that do not receive esophageal inputs. Of these c-Fos-immunoreactive neurons in the NTS, 22 ± 1.5% were also immunoreactive for mGluR8, with the highest number of c-Fos and mGluR double-labeled neurons (24 ± 2.6%, \( P < 0.05 \) vs. control) in the intermediate NTS. When the analysis was confined to the cNTS, 2.9 ± 0.5 neurons/section were labeled for c-Fos, i.e., 27 ± 1.9% of these neurons were also labeled for mGluR8 (\( P < 0.05 \) vs. control for both; Fig. 4).

These data suggest that esophageal distension activates a large subpopulation of cNTS neurons that are also mGluR8 immunoreactive.

**DISCUSSION**

In the present study, we provide evidence that cNTS neurons can be separated into two subpopulations based on the effects of purinergic agonists on the glutamatergic synapse impinging upon them and that these subpopulations have unique synaptic properties and neurochemical phenotypes. Specifically, we show that 1) cNTS neurons that receive glutamatergic inputs that are modulated by purinergic agonists have a higher probability of glutamate release and higher maximal amplitude of eEPSC compared with neurons that are nonresponsive to purinergic agonists; 2) the majority of neurons with eEPSCs responsive to purinergic agonists express mGluR8 and only a small proportion of these neurons express GAD65; 3) whereas the majority of neurons with eEPSCs nonresponsive to purinergic agonists express neither mGluR8 nor GAD65, a large proportion of these neurons express GAD65; and 4) esophageal distension activates a subpopulation of mGluR8-immunoreactive neurons in the cNTS.

It has been previously demonstrated that vagal afferent fibers impinging on NTS neurons are not uniform but rather display distinct physiological and functional characteristics (Andresen and Peters 2008; Bailey et al. 2002, 2006, and 2007; Browning et al. 2011; Jin et al. 2004). While the properties of different...
populations of cardiovascular-related mNTS neurons have been well characterized, the properties of vagal afferent fibers that carry sensory information from the GI tract have yet to be elucidated completely. In the present study, we provide evidence that cNTS neurons that receive vagal afferent inputs from the esophagus comprise two separate neuronal subpopulations, which can be differentiated based on the responsiveness of the glutamatergic synapses impinging upon them to purinergic agonists, and that these subpopulations have distinct synaptic and neurochemical characteristics. This suggests that transmission of sensory information from esophageal vagal afferent fibers to second-order cNTS neurons relies on specific patterns of neurotransmitter release onto neurons with specific neurochemical phenotypes.

Identification of nonuniform neuronal subpopulations in the cNTS is consistent with previous studies, which have demonstrated that different types of esophageal vagal afferent fibers can be characterized based on their responses to mechanical or chemical stimulation (Brookes et al. 2013; Goyal et al. 2001; Page 1998; Page et al. 2008; Sengupta et al. 1989; Yu et al. 2005). An in vitro study in ferrets has shown that esophageal vagal afferent fibers can be divided into those that respond to mucosal stroking (mucosal receptors) and to circular tension (tension receptors) and those that respond to both modalities (Page 1998). Moreover, a study in the guinea pig esophagus has also identified vagal afferent fibers with nociceptive properties (Yu et al. 2005); in these fibers, vagal fibers are responsive to capsaicin and respond less intensely to esophageal distension, and their response to distension does not saturate even at high distension pressure. In contrast, nonnociceptive fibers do not respond to capsaicin and their response to esophageal distension saturates at non-noxious pressures (Yu et al. 2005). Nociceptive vagal fibers can be further subdivided into those that are sensitive to purinergic agonists and those that do not respond to purinergic agonists but contain substance P (Yu et al. 2005). Taken together, these data indicate that esophageal vagal afferents consist of distinct populations of fibers that differ based on their responses to mechanical and noxious stimuli as well as their chemical characteristics. Our study is the first to show how this diverse information is relayed from the sensory fibers to second-order neurons in the cNTS. We show that neurons responsive to the purinergic agonist αβMeATP have a higher probability of glutamate release and maximal eEPSC amplitude. Although this study did not investigate whether neurons sensitive to αβMeATP receive afferent inputs carrying a specific sensory modality, our results may suggest that differences in the sensitivity of vagal afferents to specific sensory stimuli such as noxious stimulation or tension may be integrated in the cNTS by different patterns of neurotransmitter release. Further studies are required, however, to determine whether a correlation exists between different sensory stimuli and specific patterns of synaptic transmission in the cNTS.

Our study demonstrated that cNTS neurons that respond to αβMeATP also contained mGluR8, a member of group III mGluR family, whereas only a small proportion of these neurons expressed GAD65. We have previously demonstrated that mGluRs display a highly specific organization on synaptic inputs to DMV neurons that innervate the stomach and pancreas (Babic et al. 2012; Browning and Travagli 2007). Group III mGluRs are present on excitatory, but not inhibitory, synaptic inputs impinging on gastric-projecting DMV neurons, whereas both inhibitory and excitatory synaptic terminals impinging on pancreas-projecting DMV neurons express group III mGluRs (Babic et al. 2012; Browning and Travagli 2007). The results of our present study indicate that group III mGluRs do not appear to be present on GABAergic neurons in the cNTS, suggesting a similar organization of these receptors on vagal neurocircuits regulating gastric and esophageal functions. Furthermore, neurons that expressed mGluR8 displayed a higher probability of glutamate release and maximal eEPSC amplitude compared with nonresponsive neurons. Since we have recently shown that in brain stem vagal neurocircuits group III mGluRs inhibit glutamatergic synaptic transmission (Babic et al. 2012; Browning and Travagli 2007), their presence on cNTS neurons that received more robust glutamatergic input may serve to dampen a potentially deleterious increased excitatory synaptic transmission between cNTS and DMV neurons. It is highly likely, however, that not all glutamatergic cNTS neurons express mGluR8 immunoreactivity, and the possibility that these neurons also express other receptors and neuromodulators cannot be excluded.

In the present work, we did not investigate the in vivo effects of mGluR8 and αβMeATP; however, previous studies have shown that both purinergic and group III mGluRs agonists modulate GI functions. ATP and its metabolites are released by mechanical and chemical stimuli and activate primary sensory neurons throughout the GI tract (Burnstock 2006; Finger et al. 2005; Wynn et al. 2003). ATP has been shown to be involved in sensory signaling in the colorectum (Wynn et al. 2003), stomach, esophagus (Page et al. 2000), intestine (Bertrand and Bornstein 2002), and gustatory nerves (Finger et al. 2005), suggesting that purines modulate sensory information at all levels of the GI tract. Our data suggest that ATP may also modulate GI functions via effects on synaptic transmission between sensory vagal afferents and second-order neurons in the cNTS.

Group III mGluRs have been shown to modulate GI functions via both peripheral and central sites of action. For example, group III mGluR agonists inhibit the mechanosensitivity of gastroesophageal vagal afferent fibers (Page et al. 2005) and inhibit transient lower esophageal sphincter relaxation in response to gastric load (Frisby et al. 2005), whereas intracerebroventricular administration of mGluR8 antagonists reduces the gastric distension-induced activation of NTS neurons (Chen et al. 2007a and 2007b; Young et al. 2008). In our study, esophageal distension increased c-Fos activity in cNTS neurons that express mGluR8, further supporting the suggestion that these receptors also modulate esophageal functions at a central site of action.

The present study showed that only a small proportion of cNTS neurons that receive glutamatergic inputs, respond to αβMeATP, and express mGluR8 are GABAergic. Data from this and other laboratories have shown that tonic GABAergic inputs provide the predominant NTS-mediated influence over the activity of vagal preganglionic neurons in the DMV, whereas tonic glutamatergic inputs do not play a prominent role in the regulation of GI functions (Babic and Travagli 2014; Babic et al. 2011; Gao and Smith 2010; Sivarao et al. 1998; Travagli and Browning 2011; Travagli et al. 2006). Furthermore, we have demonstrated that glutamatergic synapses in the DMV are under modulatory control of various neurotransmit-
letters, whereas GABAergic synapses do not appear to be modulated by neurotransmitters negatively coupled to adenylate cyclase under normal physiological conditions (Browning et al. 2004 and 2006; Browning and Travagli 2010). In the present study, we show that the parabola describing the V-M relationship of GABAergic cNTS neurons (i.e., those that received a glutamatergic input that was not responsive to αβMeATP) had a tighter curvature compared with that of neurons that received an αβMeATP-sensitive glutamatergic input. The narrow fit of the parabola indicates that the activity of these neurons is not as dependent on [Ca\(^{2+}\)]\(_e\) that is, the activity of these neurons would be maintained even under conditions of low [Ca\(^{2+}\)]\(_e\). Since the tonic GABAergic input onto DMV neurons is essential in the regulation of GI functions, it is crucial to preserve the activity of these neurons under conditions, such as in low [Ca\(^{2+}\)]\(_e\), which have been shown to occur in several pathophysiological states, such as ischemia and hypoglycemic coma, where [Ca\(^{2+}\)]\(_e\) can drop to as low as 0.02 mM (Kristian et al. 1993; Kristian et al. 1994) as well as during sustained neuronal activity (Su et al. 2001). Our data also demonstrate that GABAergic neurons displayed lower maximal eEPSC amplitude and a lower probability of glutamate release. Taken together with our previous data showing that unlike GABAergic synapses, glutamatergic synapses impinging onto DMV neurons can be modulated by various neurotransmitters and physiological conditions, these data indicate that due to the lower glutamatergic input from the vagal afferents, the synaptic inputs onto GABAergic neurons in the cNTS are not as open to modulation, further ensuring that the tonic activity of these critical neurons remains stable.

In summary, we have shown that cNTS neurons that receive inputs from esophageal vagal afferents comprise two neuronal subpopulations with different synaptic and neurochemical characteristics and that these neuronal subpopulations can be distinguished based on their responses to purinergic agonists. These data indicate that integration of sensory inputs from the esophagus relies on discrete organization of synaptic inputs onto second-order cNTS neurons.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS


REFERENCES


