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

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Running title: Synaptic release in the cNTS

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ABSTRACT

The nucleus tractus solitarius (NTS) receives subdiaphragmatic visceral sensory information via vagal A or C-fibers. We have shown recently that, in contrast to cardiovascular NTSmedialis neurons which respond to either purinergic or vanilloid agonists, the majority of esophageal NTScentralis (cNTS) neurons respond to vanilloid agonists while a smaller subset responds to both vanilloid and purinergic agonists.

This study aimed to investigate further 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, 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 when compared to non-responsive neurons. Single-cell RT-PCR revealed that 8/13 αβMeATP responsive neurons expressed mGluR8 mRNA which our previous studies have suggested is a marker of glutamatergic neurons, whereas only 3/13 expressed glutamic acid dehydroxylase (GAD), a marker of GABAergic neurons. A significantly lower proportion of αβMeATP non-responsive neurons expressed mGluR8 (2/30), whereas a greater proportion expressed GAD (12/30). Esophageal distension increased significantly the number of colocalized mGluR8- and c-fos-immunoreactive neurons in 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, 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.

Keywords: nucleus tractus solitarius, vagus, electrophysiology, brainstem
INTRODUCTION

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 CNS. 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, gelatinosus 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 αβMethylene-ATP (αβMeATP), but not to the vanilloid 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 ambiguus (Bailey et al. 2006; Bailey et al. 2007). These findings indicate that, at the level of second-order NTS neurons, baroreceptor inputs to the CNS are organized into distinct functional and anatomical pathways, and that this organization, which is correlated to the afferent fibers inputs and/or the physiological role of the neurons, is revealed by the pharmacological responses to either vanilloid or purinergic agonists.

Similarly, studies in guinea pigs demonstrated that nodose ganglion neurons innervating the lung and the 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 approximately 5-600μm, 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; Baptista et al. 2005b), our recent study has shown that, unlike mNTS which responded either to vanilloid or purinergic agonists, the majority of cNTS neurons were 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 excited 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 diversities 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 (Travagli 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 Travagli 2014; Balfour and Trapp 2007; Browning and Travagli 2007; Browning and Travagli 2009; Browning and Travagli 2010; Gao and Smith 2010; 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 Travagli 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 Travagli 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 this 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 either sex. The protocols were conducted according to the guidelines set forth by the National Institute of Health and were approved by the Penn State University Institutional Animal Care and Use Committee.

Electrophysiological Recording

Rat brainstem slices containing the cNTS were prepared as described previously (Baptista et al. 2005a). Briefly, rats were anesthetized with isoflurane, the brainstem was removed, placed in chilled, oxygenated Krebs’ solution (in mM: 126 NaCl, 25 NaHCO₃, 2.5 KCl, 1.2 MgCl₂, 2.4 CaCl₂, 1.2 NaH₂PO₄, and 11 glucose, maintained at pH7.4 by bubbling with 95% O₂-5% CO₂); 2-3 coronal slices (300μm) at the level of the cNTS (Rogers et al. 1999)(Altschuler et al. 1989) were cut and incubated in Krebs’ solution at 30°C for at least 90min 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 2.5-3.0ml/min.

Whole-cell recordings were made with patch pipettes of resistance 4-6 MΩ when filled with K-gluconate intracellular solution (in mM: 128 K-gluconate, 10 KCl, 0.3 CaCl₂, 1 MgCl₂, 10 HEPES, 1 EGTA, 2 ATP-Na, and 0.25 GTP-Na, adjusted to pH7.35 with KOH).

Synaptic currents (evoked excitatory postsynaptic currents, eEPSCs) were evoked using tungsten bipolar stimulating electrodes (WPI, Sarasota, FL) placed in the tractus solitarius (TS). Stimuli were applied every 10s to evoke submaximal EPSCs. The perfusing Krebs’ solution contained picrotoxin (50μM), to prevent GABAergic currents, and the pipette solution contained the intracellular gNa⁺ blocker QX314 (1μM) to prevent antidromically-activated action potentials.
Equiosmolar Krebs’ with varying concentrations of \([\text{Ca}^{2+}]_e\) and \(\alpha\beta\text{MeATP} (10\mu\text{M})\) were applied via perfusion through a series of manually operated valves. Drugs were applied for periods of time sufficient for the response to reach plateau and neurons were allowed to recover fully between drug additions. Each neuron served as its own control, a minimum variation in eEPSC amplitude of 10% from baseline was considered as responding to \(\alpha\beta\text{MeATP}\).

Variance mean (V-M) analysis of EPSCs from cNTS.

To assess the responsiveness of cNTS synaptic inputs to purinergic agonists, perfusion with \(\alpha\beta\text{MeATP}\) was conducted in 2.4 mM \([\text{Ca}^{2+}]_e\) prior to initiating V-M analysis. V–M analysis of compound inputs obtained upon stimulation of several presynaptic terminals impinging onto cNTS neurons was obtained according to Clements (Clements 2003). This type of analysis permits the calculation of the release probability \((P_R)\), number of functional release sites \((N)\) and quantal size \((q)\). To construct V-M curves, 30-40 eEPSCs were evoked by stimulation of the tractus solitarius (TS; 0.5ms duration) at a frequency of 0.1Hz while perfusing the brainstem slice in Krebs’ with 2.4, 1.5, 0.5 or 0.25 mM \([\text{Ca}^{2+}]_e\). eEPSC amplitudes \((M)\) were measured as the peak current minus the mean baseline current measured in the 15ms preceding the stimulus. Variance \((V)\) was measured as the square of the eEPSC amplitude obtained at each \([\text{Ca}^{2+}]_e\). V-M values were fitted, after constraining the fit to intersect the zero release state using \(V=0\) at \(M=0\), with a least square method using the following equation:

\[
y=Ax+Bx^2
\]

where \(y=\) variance in eEPSC amplitude; \(x=\) mean eEPSC amplitude; \(A=\) quantal size \((q)\); \(1/B=\) number of functional release sites \((N)\). The resulting parabola (fitted with GraphPad Prism®, GraphPad Software, LaJolla, CA) was extended to intersect the X axis to estimate the maximal eEPSC amplitude \((\text{EPSC}_{\text{max}})\) used to normalize data across neuronal recordings. \(P_r\) was calculated as the % ratio of the EPSC amplitude at a given \([\text{Ca}^{2+}]_e\) to the estimated \(\text{EPSC}_{\text{max}}\).

Single-cell RT-PCR

In a group of neurons, after assessing the effects of perfusion with \(\alpha\beta\text{MeATP}\) on the eEPSC, 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 \(\mu\text{l}\); Integrated DNA technologies, Coralville, IA) and RNAse inhibitor (0.25\(\mu\text{l}\) RNAsePLUS; 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 minutes in a thermocycler, followed by 10 minutes on ice. Reverse transcription was
performed using SuperScript II Reverse Transcriptase (Invitrogen, Grand Island, NY) using the following protocol: 45°C for 1 hr, 70°C for 15 min and 37°C for 20 min.

The PCR reaction was performed using the following primers:

**β-actin**
- **GenBank EF157276**
- **outer** Product size 802bp
  - forward ACTGGGACGATATGGAGAAGA
  - reverse ATAGAGCCACCAATCCACACA
- **inner** Product size 138bp
  - forward GCCCCTCTGAACCCTAAG
  - reverse CATCACATGCCAGTGGA

**mGluR8**
- **GenBank U63288**
- **outer** Product size 840bp
  - forward GATCAGAGACCAAACATCAACCG
  - reverse AACTTTGGGCATATAGAGCATTCC
- **inner** Product size 480bp
  - forward ATGATTGCCGCACCTGACA
  - reverse GATGAAAGCTAACCAAATGATGATGCAC

**Gad65**
- **GenBank M72422**
- **outer** Product size 960bp
  - forward TGACATCAACAGCAAACACGAAC
  - reverse GAACTCGCAAACTAGGAGGTACA
- **inner** Product size 360bp
  - forward GCTTTTATCCTCTTCTTGCTGTA
  - reverse CTTTGCTCTCCACACATGAGGCCATA

The reaction was performed in duplicate, adding first the outer then the inner sequence (1μl each), using AmpliTaq DNA polymerase (Invitrogen) according to manufacturer’s instructions. The protocol consisted of a 5 minute-long incubation at 94°C followed by 35 cycles of: 94°C for 30s, 63°C for 1 minute, 72°C for 2 minutes followed by a final extension at 72°C for 7 minutes. 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 (250-450g) were fasted overnight and anesthetized with Inactin® (100-150mg/kg, i.p.); a tracheal cannula was placed to maintain a clear airway. Esophageal distension balloon catheters were made from 1.0mm o.d., 0.5mm i.d. silicone tubing connected to polyethylene tubing (PE-50) attached to a 1ml syringe and a Statham P23 pressure transducer. The balloon was inserted in the esophagus and placed ~5mm from the lower esophageal sphincter. Injection of 0.3ml of saline into the balloon expanded its stressed portion to a final o.d. of approximately 3mm, which we have demonstrated previously to increase esophageal pressure by 14-18mmHg and activate the low-threshold vagal mechanoreceptors (Rogers et al. 1999). Following a minimum recovery period of 60min, the balloon was distended for 1s every 5s for 40min; 90min after the end of the stimulation period, animals were perfused transcardially with saline followed by fixative (4% paraformaldehyde in 0.1M phosphate buffered saline (PBS). The brainstem was removed and stored in fixative with 20%
sucrose at 4°C for at least 48hrs before cutting 40µm-thick coronal slices throughout the length of the DVC.

Two in five brainstem sections were reacted for immunohistochemistry as described previously (Llewellyn-Smith et al. 2012). Sections were rinsed 3x10 min in Tris-PBS (TPBS) containing 0.3% Triton X-100 and 0.05% thimerosal and incubated in a solution of 30% methanol/1% H2O2 to eliminate endogenous peroxidase activity. Following 3x10min rinses in TPBS, sections were blocked in 10% normal horse serum (NHS) in TPBS for at least 30 min. Sections were incubated in the primary rabbit anti c-Fos antibody diluted 1:10000 (Santa Cruz, Dallas, TX) for 3 days at room temperature after which they were rinsed 3x10min in TPBS and placed in biotin-SP-conjugated donkey anti-rabbit for (1:500; Jackson Immunoresearch, West Grove, PA) overnight. Following 3x10min rinses in TPBS, tissue was incubated in ExtrAvidin-HRP (Sigma, St. Louis, MO) for 4-6 hours and the reaction was visualized with Vector SG using glucose oxidase. Sections were rinsed in TPBS and placed in guinea pig anti-mGluR8 (1:1000; Millipore, Temecula, CA). Sections were then rinsed 3x10min in TPBS and placed in either biotinylated donkey anti-guinea pig or biotinylated donkey anti-mouse (both at 1:500 dilution; Jackson Immunoresearch) secondary antibodies overnight. Following 3x10 min rinses in TPBS, sections were incubated in Extravidin-HRP as described above and the reaction product was visualized with DAB using glucose oxidase. Sections were rinsed, mounted on gelatin-coated microscope slides, dehydrated and cover-slipped. Labelling 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 the paired Students’ t-test. scRT-PCR data were analyzed with the χ² 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 mean ± standard error of the mean (SEM) 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 Ca\(^{2+}\).

In 6 of 21 tested neurons, perfusion of the slices with 10\(\mu\)M \(\alpha\beta\text{MeATP}\) increased eEPSC amplitude from 262±53 to 304±56pA (p<0.05). In these neurons, stimulation of the tractus solitarius (TS) in the presence of 2.4mM \([\text{Ca}^{2+}]_e\) evoked EPSCs of 346±64pA amplitude with a variance of 1169±244pA\(^2\). Reducing the \([\text{Ca}^{2+}]_e\) to 1.5mM decreased the eEPSC mean amplitude to 317±54pA, but increased its variance to 2743±1456pA\(^2\). Lowering \([\text{Ca}^{2+}]_e\) to 0.5mM further decreased the mean eEPSC amplitude to 175±37pA and decreased the variance to 1614±312pA\(^2\), whereas in the presence of 0.25 mM [Ca\(^{2+}\)]\(_e\), the mean eEPSC amplitude was 112±39pA and variance was 477±147pA\(^2\).

The characteristics of the parabolic curve illustrating the variance-mean (V-M) analysis and release probabilities of \(\alpha\beta\text{MeATP}\) –sensitive neurons at the various [Ca\(^{2+}\)]\(_e\) are shown in figures 1 and 2 and summarized in Table 1.

In the remaining 15 neurons, \(\alpha\beta\text{MeATP}\) did not affect the eEPSC amplitude. In these neurons, stimulation of the TS in the presence of 2.4mM \([\text{Ca}^{2+}]_e\) evoked EPSCs of 219±34pA with a variance of 1019±354pA\(^2\). Reducing the \([\text{Ca}^{2+}]_e\) to 1.5mM, decreased the mean eEPSC amplitude to 171±30pA and increased the variance to 2533±887pA\(^2\). Perfusion of the slices with 0.5mM \([\text{Ca}^{2+}]_e\) further reduced the mean eEPSC amplitude to 86±17pA and lowered the eEPSC variance to 987±285pA\(^2\). Both eEPSC mean and variance were further reduced in the presence of 0.25mM [Ca\(^{2+}\)]\(_e\) (mean eEPSC amplitude 35±9pA; variance 707±393pA\(^2\)).

The characteristics of parabolic curve illustrating the variance-mean (V-M) analysis and release probabilities of \(\alpha\beta\text{MeATP}\) –insensitive neurons at the various [Ca\(^{2+}\)]\(_e\) showed that these neurons have a significantly lower probability of release and a lower maximal estimated eEPSC amplitude. The data are shown in figures 1 and 2 and are summarized in Table 1.

These data demonstrate that neurons in cNTS can be subdivided into two neuronal populations based on responses to purinergic agonists and that these populations have distinct synaptic properties.

Neurochemical phenotype of cNTS neurons

In another group of cNTS neurons (N=43), the effects of \(\alpha\beta\text{MeATP}\) 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 \(\alpha\beta\text{MeATP}\) with an increase in eEPSC amplitude, while 30 neurons were unresponsive to perfusion with \(\alpha\beta\text{MeATP}\).
The majority (N=8, i.e. 62%) of αβMeATP-responsive neurons expressed mGluR8 (a member of group III mGluR) mRNA, whereas only 3 (i.e. 23%) expressed GAD67 mRNA and 2 (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 mRNA ($\chi^2 <0.05$ compared to αβMeATP-responsive neurons). Fifteen of the non-responsive 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 per section were immunoreactive for c-Fos. The majority of these c-Fos-IR 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 in rostral parts of the NTS (6±1.2). Of these c-Fos-IR 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/section were labelled for c-fos, 5.7±2 % of these neurons were also labelled for mGluR8 (Fig 4).

In seven animals that were subjected to esophageal distension, 17±3.1 NTS neurons per section were immunoreactive for c-Fos. The majority of these c-Fos-IR 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; 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-IR 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 labelled for c-fos, i.e. 27±1.9 % of these neurons were also labelled for mGluR8 (p<0.05 vs control for both; Fig 4).

These data suggest that esophageal distension activates a large subpopulation of cNTS neurons which are also mGluR8-IR.
DISCUSSION

In this 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 the eEPSC compared to neurons that are non-responsive 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) while the majority of neurons with eEPSCs non-responsive 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-IR neurons in cNTS.

It has been demonstrated previously 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; Bailey et al. 2006; Bailey et al. 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 this 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 non-uniform neuronal subpopulations in 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 et al. 2008; Page 1998; 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), 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); these fibers vagal fibers are responsive to capsaicin, respond less intensely to esophageal distension, and their response to distension does not saturate even at high distension pressure. In contrast, non-nociceptive 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 the 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 demonstrated previously that mGluRs display a highly specific organization on synaptic inputs to DMV neurons that innervate the stomach and the 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 current study indicate that group III mGluR do not appear to be present on GABAergic neurons in 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 to non-responsive neurons. Since we have shown recently that in brainstem 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-IR 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 gastro-esophageal 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; Chen et al. 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 et al. 2011; Babic and Travagli 2014; Gao and Smith 2010; Sivarao et al. 1998; Travagli et al. 2006; Travagli and Browning 2011). Furthermore, we have demonstrated that glutamatergic synapses in the DMV are under modulatory control of various neurotransmitters, whereas GABAergic synapses do not appear to be modulated by neurotransmitters negatively coupled to adenylate cyclase under normal physiological conditions (Browning et al. 2004; Browning et al. 2006; Browning and Travagli 2010). In this 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 to 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 the \([Ca^{2+}]_e\) can drop to as low as 0.02mM (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, 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: None
FIGURE LEGENDS

Figure 1: V-M analysis of tractus solitarius-cNTS synapses.
A: Representative traces showing that the response to perfusion with 10μM αβMeATP distinguishes two subgroups of cNTS neurons in which the eEPSC amplitude is either increased (left trace) or unaffected (right trace).
B: Graphical representation showing the relationship between [Ca\(^{2+}\)]\(_e\) and eEPSC amplitude. Note that the eEPSC amplitude was significantly higher in αβMeATP responsive neurons at all [Ca\(^{2+}\)]\(_e\) tested.
C: Group mean values of the V-M relationship of cNTS neurons in which the eEPSC was either sensitive or insensitive to perfusion with αβMeATP. Fits were constrained to pass through the intersection of the X- and Y-axis and had uniformly high r\(^2\) values. Responsive neurons displayed a higher maximal amplitude compared to unresponsive neurons, indicating that synaptic inputs onto αβMeATP responsive neurons were more dependent upon [Ca\(^{2+}\)]\(_e\).
D: V-M relationship of cNTS neurons, normalized to maximal eEPSC mean and maximal variance. Dashed lines represent the probability of glutamate release at 0.5mM [Ca\(^{2+}\)]\(_e\). Note that αβMeATP responsive neurons displayed a higher probability of glutamate release compared to unresponsive neurons.
Data from neurons with eEPSC responsive to αβMeATP in gray; unresponsive to αβMeATP in black.

Figure 2: eEPSC amplitude at varying [Ca\(^{2+}\)]\(_e\) in cNTS neurons.
A: Representative traces showing eEPSC amplitude at 0.25-0.5-1.5 and 2.4mM [Ca\(^{2+}\)]\(_e\). The eEPSC amplitude (M) and variance (V) response to varying concentrations of [Ca\(^{2+}\)]\(_e\) showed a characteristic variation with V maximal at intermediate M. The upper traces are from a neuron in which the eEPSC was responsive to perfusion with αβMeATP, the lower traces are from a neuron in which the eEPSC was unresponsive to perfusion with αβMeATP.
B: Graph showing the variation in eEPSC amplitude at different [Ca\(^{2+}\)]\(_e\). Note that eEPSC amplitude increases at increasing [Ca\(^{2+}\)]\(_e\), whereas variance is low at the low and high extremes of [Ca\(^{2+}\)]\(_e\) and highest at 1.5 mM [Ca\(^{2+}\)]\(_e\). The bar represents the mean of the values for each group.
Data from neurons with eEPSC responsive to αβMeATP in gray; unresponsive to αβMeATP in black.

Figure 3: Single cell RT-PCR showing the expression of mGluR8 and GAD65 in neurons previously tested for eEPSC responsiveness to αβMeATP.
A: Representative 2% agarose gel showing the PCR product for mGluR8 and GAD65 in αβMeATP unresponsive and responsive neurons. PCR product was obtained with the primers specified in the Methods section. cNTS neurons were characterized electrophysiologically before scRT-PCR reactions.

B: Pie charts showing the percentage of cNTS neurons that were unresponsive (left) or responsive (right) to αβMeATP that expressed GAD65 or mGluR8. Note that, compared to αβMeATP responsive cNTS neurons, a significantly greater number of unresponsive cNTS neurons expressed GAD65, whereas a significantly lower number expressed mGluR8.

Figure 4: Esophageal distension activates mGluR-ir neurons in the NTS.

Photomicrographs showing c-fos and mGluR8-ir in the NTS of controls (panels a,b) and animals with esophageal distension (panels c,d). Note that in control animals (panel B), only a small proportion of c-fos-IR neurons (dark blue stain; closed arrows) expressed mGluR8 (brown stain; open arrows). Following esophageal distension (panel D), a greater proportion of c-fos-IR neurons were also immunoreactive for mGluR8 (asterisk).

E: Summary graphic showing the number of c-fos-IR neurons in the NTS according to their localization. Note that the significant increase in c-Fos positive neurons was observed in the intermediate and rostral NTS only, i.e. the areas that comprise the cNTS, whereas esophageal distention did not increase the number of c-Fos –IR neurons in the caudal portion of the NTS.

F: Summary graphic showing the percentage of c-fos-IR neurons in the intermediate and rostral portions of the NTS that also displayed mGluR8-IR. Note the significant increase of c-Fos and mGluR-IR co-localized cNTS neurons following esophageal distension.

Black closed arrows, c-fos-IR neurons; white open arrows, mGluR8-IR neurons; asterisks, double-labeled neurons.

*p<0.05 vs control.
Table 1: Synaptic properties of cNTS neurons that received αβMeATP-sensitive or –insensitive eEPSCs.

<table>
<thead>
<tr>
<th></th>
<th>αβMeATP-sensitive (n=6)</th>
<th>αβMeATP-insensitive (n=15)</th>
</tr>
</thead>
<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>
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<tr>
<td>Quantal size</td>
<td>21±11</td>
<td>20±3</td>
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<tr>
<td>Release probability at 2.4 mM Ca++</td>
<td>70±8</td>
<td>59±7</td>
</tr>
<tr>
<td>Release probability at 1.5 mM Ca++</td>
<td>62±7</td>
<td>46±6*</td>
</tr>
<tr>
<td>Release probability at 0.5 mM Ca++</td>
<td>39±10</td>
<td>23±4*</td>
</tr>
<tr>
<td>Release probability at 0.25 mM Ca++</td>
<td>26±10</td>
<td>7±7*</td>
</tr>
</tbody>
</table>

* P<0.05 vs Responsive neurons
Reference List


Gao, H and Smith, B. N. Tonic GABAA receptor-mediated inhibition in the rat dorsal motor nucleus of the vagus. JNP 103:2, 904-914. 2010.


Figure 1

(a) Graph showing baseline and $\alpha\beta$MeATP responses.
(b) Graph showing $eEPSC$ amplitude against $[Ca^{++}]$.
(c) Graph showing variance against mean $eEPSC$ for responsive and non-responsive categories.
(d) Graph showing variance normalized against mean $eEPSC/eEPSC\ max$.
Figure 2

(a) Graph showing Amplitude (pA) vs. [Ca^{++}]_e (mM) for different concentrations.

(b) Scatter plot showing the amplitude data for non-responsive and responsive categories.

- **Non-responsive**
- **Responsive**

Concentrations tested: 0.25 mM, 0.5 mM, 1.5 mM, 2.4 mM.
### Figure 3

#### a

- **αβMeATP-responsive**
- **αβMeATP-unresponsive**
- **Whole brainstem**

#### b

**Non-responsive**
- mGluR+ 7%
- mGluR- & GAD- 50%
- GAD+ 40%

**Responsive**
- mGluR- & GAD+ 0%
- mGluR & GAD+ 15%
- GAD+ 23%
- mGluR+ 62%

**Legend**
- Primer dimer
- PCR product
- DNA 100bp ladder
- Group III mGluR
- GAD65
- Inner primer
- Single cell, two rounds of nested PCR
- Intracell. solution
Figure 4

Intermediate

Rostral

Total NTS
cNTS

Number of c-fos-ir neurons/section

% Double-labeled neurons

Control

Esophageal distension