Vanilloid, purinergic and CCK receptors activate glutamate release on single neurons of the nucleus tractus solitarius centralis.

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ABSTRACT

Baroreceptor inputs to nucleus tractus solitarius medialis (mNTS) neurons can be differentiated, amongst other features, by their response to vanilloid- or purinergic- agonists, active only on C- or A-fibers, respectively. A major aim was to examine whether neurons of NTS centralis (cNTS), a subnucleus dominated by esophageal inputs, exhibit a similar dichotomy. Since it has been suggested that cholecystokinin (CCK), exerts its gastrointestinal (GI)-related effects via paracrine activation of vagal afferent C-fibers, we tested whether CCK-sensitive fibers impinging upon cNTS neurons are responsive to vanilloid but not purinergic agonists.

Using whole cell patch clamp recordings from cNTS, we recorded miniature excitatory postsynaptic currents (mEPSCs) to test the effects of the vanilloid agonist capsaicin (CAP), the purinergic agonist α-β-methylene-ATP (α-β-met-ATP) and/or CCK-8s. α-β-met-ATP, CAP and CCK-8s increased EPSC frequency in 37, 71 and 46% of cNTS neurons, respectively. Approximately 30% of cNTS neurons were responsive to both CCK-8s and α-β-met-ATP, to CCK-8s and CAP, or to α-β-met-ATP and CAP while 32% of neurons were responsive to all three agonists. All neurons responding to either α-β-met-ATP or CCK-8s were also responsive to CAP. Perivagal CAP, which is supposed to induce a selective degeneration of C-fibers decreased the number of cNTS neurons responding to CAP or CCK-8s but not those responding to α-β-met-ATP.

In summary, GI inputs to cNTS neurons cannot be distinguished on the basis of their selective responses to α-β-met-ATP or CAP. Our data also indicate that CCK-8s increases glutamate release from purinergic and vanilloid responsive fibers impinging on cNTS neurons.
INTRODUCTION

Vagal afferent (sensory) fibers convey a vast amount of information about the physiological state of the thoracic and abdominal viscera to the central nervous system, specifically, to the nucleus of the tractus solitarius (NTS). Vagal afferent fibers are organized in the NTS in an overlapping topographical manner. Sensory afferents from the stomach and intestine, for example, terminate in the subnuclei commissuralis, and medialis, inputs from the stomach terminate in the subnuclei medialis and gelatinosus, while inputs from the esophagus terminate in the subnucleus centralis (49). Vagal afferent fibers relay this visceral information by a mixture of myelinated (A-type) and unmyelinated (C-type) axons. These two types of afferent fibers display distinct physiological and functional characteristics, including different sensory modalities and conduction velocities and may regulate autonomic homeostatic and regulatory reflexes differentially (4; 35). The differences in responses of C- vs A- fibers have been studied extensively in relation to NTS neurons that comprise the baroreflex (8; 22; 23; 26; 30; 31; 37; 40). Recent studies showed that cardiovascular neurons of the NTS subnucleus medialis (mNTS) receiving C-fibers inputs can be classified based on the presence of the fast-transient Ia current and the response of their synapses to the vanilloid TRPV1 receptor agonist, capsaicin. In contrast, A-fibers impinging on cardiovascular NTS neurons respond to purinergic agonists but not capsaicin (8; 26; 30). Of particular interest are the most recent reports showing that the biophysical and pharmacological characteristics of cardiovascular mNTS neurons are related to their projection target, with clear distinctions between NTS neurons projecting to the hypothalamus (i.e. possibly controlling “slower” homeostatic reflexes) and NTS neurons projecting to the caudal ventrolateral medulla or nucleus ambiguus (i.e. controlling the baroreflex and heart rate) (6; 7). Thus, cardiovascular vagal circuits appear ‘patterned’ and organized into distinct pathways, even at the level of the first central (vagal afferent) synapse. The first aim of our present study was to examine whether this type of organizational dichotomy is a common feature of visceral afferent inputs onto brainstem neurons, specifically, to determine whether the subpopulation of gastrointestinal (GI) inputs onto NTS neurons of the subnucleus centralis (cNTS) could be distinguished on the basis of their responses to vanilloid or purinergic agonists.

The second aim of the present study was to investigate the nature of afferent inputs to cNTS neurons that are responsive to cholecystokinin (CCK). Ingestion of nutrients induces the release of CCK from intestinal I cells resulting in increased pancreatic exocrine secretion, gastric relaxation and short-term satiety (reviewed in (25)). Several studies have suggested that the vagally-mediated effects of CCK are due almost exclusively to a paracrine action of CCK on peripheral, capsaicin-sensitive C-type vagal afferent fibers (12; 21; 39; 42). The main support for this mechanism of action derives from
the observation that capsaicin treatment, either systemic, perivagal or directly to the intestinal mucosa attenuates or abolishes the effects of systemic administration of CCK on functional gastric and pancreatic parameters (33; 34; 42; 46). Behavioral, electrophysiological and pharmacological studies from many groups, including our own, strongly suggest that the effects of CCK may not be limited to a paracrine mechanism of action, however, and effects at other sites, including direct actions on central vagal brainstem circuits, must be considered (5; 9; 10; 16; 27; 43; 56). The second aim of this study was to investigate whether CCK-sensitive afferent inputs to cNTS neurons are, indeed, C-fibers, and are therefore responsive to the vanilloid agonist, capsaicin, but insensitive to purinergic agonists.
METHODS

Research reported in the present manuscript conforms fully to National Institute of Health guidelines and was approved by the Institutional Animal Care and Use Committees.

Electrophysiology

The methods of preparing the brainstem slices and the identification of cNTS neurons have been described previously (9-11). Briefly, 25-35 day old Sprague-Dawley rats of either sex were anesthetized with isoflurane (abolition of the foot pinch withdrawal reflex) before being killed by administration of a bilateral pneumothorax. After removal, the brainstem was glued to the platform of a vibratome, and three-4 coronal slices (300μm-thick) were cut starting from the caudal area postrema and moving rostrally. The slices were incubated at 30±1°C in Krebs’ solution (in mM: 126 NaCl, 25 NaHCO3, 2.5 KCl, 1.2 MgCl2, 2.4 CaCl2, 1.2 NaH2PO4, and 5 dextrose, maintained at pH 7.4 by bubbling with 95% O2-5% CO2) for 60-90 minutes before use. A single slice was then transferred to a perfusion chamber (volume 500μl; Michigan Precision Instruments, Parma, MI), kept in place with a nylon mesh and maintained at 35±1°C by perfusion with warmed Krebs’ solution at a rate of 2.5-3.0ml.min⁻¹. Recordings were made with patch pipettes (6-8MΩ resistance) filled with a potassium gluconate solution (in mM: K gluconate 128, KCl 10, CaCl2 0.3, MgCl2 1, Hepes 10, EGTA 1, ATP 2, GTP 0.25 adjusted to pH 7.35 with KOH) by using either an Axopatch 200B or an Axoclamp 2B amplifier (Axon Instruments, Union City, CA). Liquid junction potential was compensated at the beginning of the experiment. Recordings were accepted only if the series resistance was < 20MΩ. The Krebs’ solution contained 50μM picrotoxin to isolate glutamatergic currents pharmacologically and recordings were conducted at a holding potential = -60mV, i.e. close to ECl. We have shown previously that the currents recorded under these conditions comprise glutamatergic ionotropic currents only (10; 11).

Whole cell recordings of spontaneous and miniature glutamatergic excitatory postsynaptic currents (sEPSC and mEPSC, respectively) were conducted on cNTS neurons located within 100μm medial to the tractus solitarius at levels spanning from the posterior tip of the area postrema to approximately 0.5mm rostral to its anterior portion. The typical experiment consisted of recording sEPSC in control conditions and in the presence of agonists. Cells were classified as responsive if perfusion with capsaicin (100nM), ATP (sodium salt; 100μM), α,β-methylene ATP (α,β-Met-ATP; 10μM) or the sulfated form of cholecystokinin-octapeptide (CCK-8s; 100nM) modified the even frequency by a minimum of 50% from baseline (measured as the average frequency during 1min of recording in control conditions vs 30s of recording centered around peak drug response). If any of the agents affected the event
frequency, the slice was perfused for 10 minutes with tetrodotoxin (1µM) and the drugs were reapplied, i.e. the effects were tested on mEPSC.

Chemical vagal deafferentation (28; 42) was conducted in rats (22 days of age) anesthetized with ketamine/acepromazine/xylazine in saline (80mg/1.6mg/5mg.ml⁻¹, respectively; 0.1ml/100g, i.m.). Once anesthesia was obtained (abolition of the foot pinch withdrawal reflex), a midline neck incision on the ventral side exposed one of the cervical vagus trunks, which was isolated from surrounding tissue by a small piece of Parafilm®. A piece (~1mm²) of gelatin sponge soaked in capsaicin (1% w/v diluted in Ethanol:DMSO:Saline 1:1:8, respectively) was placed unilaterally on the cervical vagus. After 30min, the gelatin sponge was removed, the area was rinsed, dried and the incision was sutured with 5/0 thread. The rat was allowed to recover for five to seven days before experimentation.

Data were sampled at 10 kHz and filtered at 2 kHz, digitized via a Digidata 1322 interface (Axon Instr.), acquired with a PC utilizing pClamp9 software (Axon Instr.) and analyzed with Mini Analysis software (Jaejin Software, Leonia, NJ).

**Drugs and chemicals**

Drugs were applied to the bath via a series of manually operated valves. Tetrodotoxin was purchased from Alomone labs (Jerusalem, Israel). All other drugs and salts were purchased from Sigma (St. Louis, MO). Stock solutions (1000X concentration) were aliquoted, stored at -20°C and diluted to the final concentration in Krebs’ solution just before use.

**Statistical Analysis**

Results are expressed as means ± S.E.M. with significance defined as $P < 0.05$. Results were compared before and after drug administration, with each neuron serving as its own control (Student’s paired t test). Intergroup comparisons were conducted using the Student’s grouped t test or the $\chi^2$ test.
RESULTS

_Capsaicin (CAP) increases glutamatergic currents in a subpopulation of NTS neurons._

In 12 of 23 NTS neurons (i.e. 52%), perfusion with CAP (10nM; 3 minute perfusion) increased the frequency of sEPSCs from $0.9 \pm 0.17$ to $5.7 \pm 1.14$ events s$^{-1}$. Event frequency returned to baseline upon wash-out. The remaining 11 neurons were unresponsive to perfusion with 10nM CAP. In 78 of 109 NTS neurons (i.e. 72%), perfusion with CAP (100nM) induced a larger increase in the frequency of sEPSCs from $1.4 \pm 0.19$ to $12.4 \pm 1.02$ events s$^{-1}$ ($P<0.05$ vs control; $P<0.05$ vs 10nM CAP). Event frequency returned to baseline upon wash-out. The remaining 31 neurons were unresponsive to perfusion with 100nM CAP. Since the proportion of neurons responding to 100nM CAP was larger than the proportion of those responding to 10nM CAP, we used the 100nM concentration for the remaining of the studies.

The response to application with CAP (N=5) did not show tachyphylaxis since repeated applications of CAP to the same neuron, 20-30 minutes apart, gave similar results. In fact, the first perfusion increased the sEPSC frequency from $1.6 \pm 0.96$ to $8.4 \pm 2.53$ events s$^{-1}$ ($P<0.05$ vs control) while the second perfusion increased the frequency of sEPSC to $11.5 \pm 4.58$ events s$^{-1}$ ($P>0.05$ vs first perfusion; Figure 1).

In 6 NTS neurons, perfusion with CAP increased the sEPSC frequency from $1.4 \pm 0.39$ to $14.1 \pm 3.61$ events s$^{-1}$ ($P<0.05$); following wash-out and 15 minutes perfusion with a solution containing the vanilloid TRPV1 antagonist capsazepine (5μM), sEPSC frequency was $1.2 \pm 0.31$ events s$^{-1}$ ($P>0.05$ vs control). In the continued presence of capsazepine, re-application of CAP did not alter sEPSC frequency ($2.6 \pm 0.67$ events s$^{-1}$; $P>0.05$ vs capsazepine alone; $P<0.05$ vs capsaicin alone; data not shown).

To confirm that CAP acted presynaptically to increase event frequency, we conducted further experiments in conditions of synaptic blockade, i.e. we measured mEPSC in the presence of 1μM tetrodotoxin (TTX), with the assumption that the modulation of mEPSC frequency, but not amplitude, in the presence of TTX implies a presynaptic site of action.

In 5 cNTS neurons, following 15 minutes perfusion with TTX, the frequency of mEPSC was $1.8 \pm 0.9$ events s$^{-1}$ and their amplitude was $24.6 \pm 3.2$pA. Re-application of CAP increased mEPSC frequency to $12.2 \pm 3.1$ events s$^{-1}$ ($P<0.05$) but did not affect mEPSC amplitude ($26.9 \pm 4.2$pA; $P>0.05$; Figure 1). Furthermore, the 20-80% rise and 90-37% decay time of the mEPSC were not affected by perfusion with CAP (rise time: $0.91 \pm 0.003$ and $0.95 \pm 0.003$ms in control and CAP, respectively; decay time: $2.83 \pm 0.26$ and $2.43 \pm 0.17$ms in control and CAP, respectively; N=5; $P>0.05$ vs control; Figure 1). These
Purinergic agonists modulate glutamatergic spontaneous currents in a subpopulation of NTS neurons.

In 47 of 128 NTS neurons (i.e. 37%), perfusion with ATP (100 μM) increased the frequency of sEPSC from 2.2±0.32 to 7.3±0.88 events s⁻¹. Event frequency returned to baseline upon wash-out. In these same neurons, perfusion with ATP did not affect the amplitude of the events, i.e. 30.6±1.29 and 34.3±1.85 pA, in control and after ATP respectively (P>0.05). In 74 other neurons (i.e. 58%) perfusion with ATP-Na did not affect the frequency of sEPSC.

In 7 of the 128 neurons tested, perfusion with ATP reduced the frequency of sEPSC from 3.1±0.83 to 0.93±0.22 events s⁻¹ (P<0.05) but did not affect their amplitude. Event frequency returned to baseline upon wash-out. In four of these neurons, 10 minutes perfusion with the adenosine A1 receptor antagonist DPCPX (30nM) prevented the inhibitory effects of ATP, i.e. the frequency of sEPSC was 2.3±0.64 and 2.8±0.74 events s⁻¹ in DPCPX and DPCPX+ATP, respectively (P<0.05 vs ATP alone; not shown). These data indicate that the inhibitory effects of ATP are mediated by activation of adenosine A1 receptors.

In 13 neurons perfusion with ATP (100 μM) increased the frequency of sEPSC from 2.8±0.67 to 7.7±1.44 events s⁻¹ (P<0.05). Event frequency returned to baseline upon wash-out. In these same neurons, subsequent perfusion with the specific but non-selective non-hydrolysable purinergic P2X receptor agonist α-β-Met-ATP (10 μM) increased the frequency of sEPSC from 2.2±0.48 to 6.1±1.29 events s⁻¹ (P<0.05 vs control; P>0.05 vs ATP). In eight neurons in which ATP and α-β-Met-ATP (N=4 each) increased the frequency of sEPSC from 3.2±0.61 to 8.9±1.18 events s⁻¹ (P<0.05), ten minutes perfusion with the specific but non-selective P2X antagonist PPADS (10 μM) antagonized completely the effects of both agonists on sEPSC frequency (i.e. 2.7±0.65 and 2.9±0.79 events s⁻¹ in PPADS and PPADS+agonist; P>0.05; Figure 2).

These data indicate that the excitatory effects of both ATP-Na and α-β-Met-ATP are mediated by activation of P2X receptors, the data obtained with each of these agonists have thus been pooled.

To confirm a presynaptic effect of ATP-Na and α-β-Met-ATP, we conducted the experiments first on sEPSC and then in conditions of action potential blockade, i.e. we measured mEPSC. In 10 cNTS neurons, perfusion with the purinergic agonist increased the sEPSC frequency from 1.7±0.52 to 5.2±0.98 events s⁻¹ and the sEPSC amplitude from 35±3.44 to 41±3.98 pA (P<0.05 for both). Following wash-out and 15 minutes perfusion with TTX, the frequency of mEPSC was 0.9±0.17 events s⁻¹ and
their amplitude was 35±2.92pA; re-application of purine agonist in the continued presence of TTX increased mEPSC frequency to 2.7±0.59 events s\(^{-1}\) (P<0.05) but did not affect mEPSC amplitude (37±3.9pA; P>0.05; Figure 2).

These data indicate that, in our experimental conditions, the excitatory effects of ATP and α-β-Met-ATP on EPSCs frequency are determined by an action at presynaptic P2X receptors located on terminals impinging on cNTS neurons.

**Cholecystokinin-8s (CCK-8s) increases glutamatergic spontaneous currents in a subpopulation of NTS neurons.**

As reported previously (5; 9; 10), perfusion with 100nM CCK-8s increased the frequency of sEPSC from 2.2±0.34 to 11.4±1.46 events s\(^{-1}\) in 44 of 96 cNTS neurons (i.e. 46%) due to actions at presynaptic CCK\(_A\) receptors (data not shown; (9; 10). Event frequency returned to baseline upon wash-out (data not shown). In order to eliminate the possibility that the actions of CCK to increase sEPSC frequency were not due to direct actions on glutamatergic presynaptic terminals but were, instead, due to postsynaptic effects causing release of a retrograde messenger which subsequently acted presynaptically, another series of experiments were conducted in which the recording pipette contained a higher concentration of EGTA (11mM) to counter a postsynaptic rise on calcium level (41). In the presence of 11mM EGTA in the recording pipette, CCK8s (100nM) altered sEPSC frequency in 4 out of 6 neurons (67%), increasing the frequency to 560±133% of control without affecting sEPSC amplitude (104±31% of control; data not shown). These results were not significantly different from those recorded using 1mM EGTA in the patch pipette, indicating that it is unlikely the actions of CCK-8s to modulate glutamatergic synaptic transmission to cNTS neurons are due to release of a retrograde neurotransmitter.

**Responses to CCK-8s, capsaicin and/or purinergic agonists.**

Fifty-one cNTS neurons were tested with CCK-8s followed, after suitable wash-out and recovery, by subsequent perfusion with CAP. In 21 of those neurons, both CCK-8s and CAP increased the frequency of sEPSC. Sixteen of the 51 neurons were unresponsive to CCK-8s but capsaicin increased the frequency of sEPSC, 14 neurons were unresponsive to both agonists. There was no instance in which a neuron responded to CCK-8s but not to capsaicin.

Fifty-four cNTS neurons were tested with CCK-8s followed, after wash-out and suitable recovery, by perfusion with either ATP-Na or α-β-Met-ATP. In 16 of those neurons, both CCK-8s and purinergic agonists increased sEPSC frequency while 8 neurons were unresponsive to CCK-8s but purinergic agonists increased sEPSC frequency. Twenty neurons were unresponsive to both agonists and the remaining 10 neurons responded to CCK-8s but not to purinergic agonists.
These data indicate that all CCK-8s responsive glutamatergic inputs onto cNTS neurons are also excited by capsaicin. There are inputs on cNTS neurons, however, that are excited by capsaicin but not by CCK-8s. Conversely, not all the inputs onto cNTS neurons that respond to CCK-8s also respond to purinergic agonists and, vice versa, not all the purinergic agonist-responsive neurons also respond to CCK-8s.

Forty-five cNTS neurons were tested with purinergic agonists followed, after suitable wash-out and recovery, by subsequent perfusion with capsaicin. In 15 of these neurons, both purinergic agonists and capsaicin increased sEPSC frequency. Twenty of the 45 neurons were responsive to capsaicin but not to purinergic agonists while 10 neurons were unresponsive to both agonists. All purinergic-responsive neurons were also capsaicin-responsive, that is, in every cNTS neuron in which a purinergic agonist increased sEPSC frequency, capsaicin also increased sEPSC frequency.

Twenty eight cNTS neurons were tested, in random order, with α,β-Me-ATP, CAP and CCK-8s, with appropriate wash-out and recovery between perfusions. Nine neurons responded to all 3 agonists with an increase in sEPSC frequency (Figure 3); 5 neurons responded to CCK-8s and CAP but not to purinergic agonists; 2 neurons responded to CAP and purinergic agonists but not CCK-8s and the remaining 4 neurons responded to CAP alone. There were no instances in which a cNTS neuron responded to CCK-8s or purinergic agonists only.

Perivagal capsaicin treatment reduces cNTS responses to CCK-8s and CAP but not purines.

Following perivagal capsaicin treatment, perfusion with CCK-8s was still able to increase the frequency of sEPSC in cNTS neurons from 2.6±1.01 events.s⁻¹ to 10.6±3.14 events.s⁻¹ in 9 out of 46 cells (p<0.05)(9). Treatment with perivagal capsaicin reduced significantly the percentage of cells responsive to CCK-8s (20% compared to the 46% of control cells; P<0.05; Figure 4).

Similarly, while perivagal capsaicin treatment decreased significantly the percentage of neurons responsive to CAP (46% compared to 71% of control cells; P<0.05), perfusion with CAP was still able to increase sEPSC frequency in 12 out of 26 neurons tested, from 1.1±0.31 events.s⁻¹ to 9.9±1.81 events.s⁻¹; Figure 4.

Conversely, treatment with perivagal capsaicin did not alter significantly the percentage of cells responsive to purinergic agonists (25% compared to the 37% of control cells; P<0.05). Specifically, following perivagal capsaicin treatment, perfusion with purinergic agonists increased the frequency of sEPSC in cNTS neurons from 1.1±0.25 events.s⁻¹ to 5±1.38 events.s⁻¹ in 10 out of 40 cells (Figure 4).
These data indicate that capsaicin treatment of neonatal/juvenile rats does not successfully lesion all capsaicin-sensitive neurons and leaves unaffected the terminals impinging on purine-sensitive neurons.
DISCUSSION

The results of the present study suggest that a) putative GI-related cNTS neurons do not form pharmacologically-distinct subgroups based upon the pattern of afferent inputs they receive; b) vanilloid agonists, such as capsaicin, cannot be used to distinguish cNTS neuronal subpopulations; and c) CCK appears to activate a mixture of afferent inputs onto cNTS neurons that are sensitive to both vanilloid- and purinergic-agonists. These results imply that putative GI-related cNTS neurons are organized differently from cardiovascular-related neurons of the mNTS which can be divided into very distinct subgroups based upon their projection targets and the different pharmacological responses of the vagal afferent inputs they receive. Specifically, mNTS neurons that project to the paraventricular nucleus of the hypothalamus, suggesting involvement in longer-term homeostatic regulation, exhibit a fast transient A-type potassium current which is absent in mNTS neurons that project to the caudal ventrolateral medulla or nucleus ambiguus, suggesting involvement in baroreflex and heart rate control (6; 7). Furthermore, mNTS neurons receive inputs that are responsive either to the vanilloid receptor agonist, capsaicin, hence are unmyelinated C-type fibers, or they receive inputs that are responsive to the purinergic receptor agonist, ATP, hence are myelinated A-type fibers (8; 26; 30). That is to say, mNTS neurons appear to receive inputs from either C-type or A-type afferent fibers and, furthermore, that vanilloid or purinergic agonists can be used to distinguish these distinct neuronal subpopulations. Finally, C-type fibers impinging upon mNTS neurons can be distinguished from A-type fibers based upon their neurotransmitter release characteristics. Specifically, NTS neurons receiving capsaicin-sensitive afferents had greater basal frequencies of spontaneous glutamatergic EPSCs as compared to capsaicin-insensitive afferents (41). In contrast, the putative GI-related cNTS neurons had similar sEPSC frequencies irrespective of the pharmacological properties of their afferent inputs.

The results of the present study demonstrate that clear differences exist in the organization of GI- as compared to cardiovascular-related brainstem circuits. Unlike cardiovascular-related mNTS neurons, identification of the physiological and functional organization of gastrointestinal-related cNTS neurons may require detailed investigation at the cellular and genetic level using a combination of electrophysiological, pharmacological and biochemical tools.

These conclusions were reached by the present study demonstrating clearly that cNTS neurons receive a mixture of inputs that are sensitive to both vanilloid- and purinergic-agonists; in fact, all cNTS neurons that received ATP-sensitive synaptic inputs were also sensitive to capsaicin. It is important to remember, however that, due to technical limitations of the coronal brainstem slice preparation, we are not able to distinguish monosynaptic from polysynaptic inputs onto cNTS neurons; neither is it possible,
in the coronal brainstem slice model used herein, to distinguish inputs of vagal origin from non-vagal inputs. A direct consequence of the finding that ATP-sensitive synaptic inputs were also sensitive to capsaicin, is the corollary that capsaicin cannot, therefore, be used as a tool to distinguish different populations of cNTS neurons. In this regard, the present study corroborates results in the guinea-pig and mouse which suggest that the embryonic origin of vagal afferent neurons dictates their phenotype more than their visceral target organ (32; 50). For example, guinea-pig vagal C-fibers innervating the lungs or the esophagus do not respond exclusively to capsaicin, but can be divided into two subpopulations based on whether they also respond to purinergic P2X receptor agonists. Specifically, nodose ganglion vagal C-fibers innervating the lungs or esophagus, which have their embryonic origins in the placode, exhibit a large and sustained response to purinergic P2X receptor agonists. Jugular ganglion vagal C-fibers, which derive embryologically from the neural crest, in contrast have, at best, a small, transient response to P2X receptor agonists (32; 50). The present study, which demonstrates that cNTS neurons that receive purinergic-sensitive inputs also receive vanilloid-sensitive inputs, suggests that, if the rat is similar to the guinea-pig and mouse, GI-related vagal afferents have their embryonic origins in the placode. In contrast, vagal baroreflex inputs to mNTS neurons that are sensitive to either vanilloid or purinergic agonists (but not both), suggesting they may have their embryonic origins in both the placode as well as the neural crest. In the present study, the large majority of inputs onto GI-related cNTS neurons were responsive to capsaicin (72%). This is in agreement with earlier reports, which demonstrated that capsaicin activates gastrointestinal vagal afferents irrespective of the fiber conduction velocity (13). Taken together, all these studies suggest that while vagal afferent fibers can be differentiated based upon their conduction velocity (i.e., into C- versus A-fibers), this does not automatically confer any pharmacological or physiological specificity.

Finally, the present study indicates that CCK does not act exclusively on capsaicin-sensitive vagal C-fibers. Several studies have suggested that the vagally-mediated gastrointestinal actions of CCK are due to paracrine actions on vagal afferent C-fibers (12; 21; 39; 42). This hypothesis arose from the finding that peripheral capsaicin treatment abolishes the gastroinhibitory effects of CCK (33; 34; 42; 46). The results of the present study demonstrates that, while all CCK-sensitive inputs onto GI-related cNTS neurons are capsaicin-sensitive, a significant proportion these neurons received CCK-sensitive inputs that are also responsive to purinergic receptor agonists. This would suggest that, in agreement with earlier studies on GI-related vagal sensory neurons, that some of the vagally-mediated actions of CCK must be mediated via purinergic-sensitive A-type neurons and fibers (44). The failure of perivagal capsaicin to eliminate the ability of capsaicin and CCK8s to increase glutamatergic synaptic transmission to cNTS neurons suggests that either perivagal capsaicin does not induce a selective
degeneration of vagal afferent C fibers, that CCK8s does not act exclusively on vagal afferent C fibers, or that cNTS neurons, in a coronal slice preparation, receive non-vagal synaptic inputs that are also capsaicin sensitive. This provides further evidence to support the idea suggested by several groups, including ours, that the vagally-mediated gastroinhibitory effects of CCK are due to actions at multiple sites, both peripheral and central, that may follow distinct temporal patterns. That is, the CCK released from intestinal I cells in response to ingested nutrients may act initially via a paracrine mechanism to activate vago-vagal inhibitory reflexes, but the resulting gastroinhibition may be prolonged by later, distinct actions of CCK directly on the brainstem or on the stomach itself (9) (38).

Note that, in these experiments, the brainstem slices were cut in the coronal plane since this is the only orientation that allows reliable identification of the centralis subnucleus (2). While the biophysical or morphological properties of NTS or DMV neurons are not altered by the plane of section (36), coronal sectioning does not allow the electrophysiological distinction to be made between monosynaptic vagal afferent inputs and inputs of other origins onto cNTS neurons. In the present study, therefore, it cannot be ruled out that the recorded cNTS neurons receive a mixture of monosynaptic inputs of vagal afferent origin as well as inputs from other brainstem nuclei.

Conclusion

A newly emerging concept hypothesizes that brainstem autonomic circuits are integrative circuits with pathways that are defined uniquely at multiple specific levels, from a neuron’s membrane properties, to its local network connections, its distant network associations as well as to its effector response. This organization represents neuronal specialization, or segregation, into specific functional lines. Each neuron, its input and its target offer a level of potential redundancy, therefore, by allowing adjacent neurons to “recognize” and reinforce each other’s common pathway and goal. This type of cellular organization implies a “task matching” capability where separate subsets of brainstem neurons integrate vital GI, cardiac or respiratory functions.

Increasing evidence from different laboratories, including our own, have shown that even within a relatively restricted group of nuclei such as those comprised in the dorsal vagal complex, fundamental differences exists in the organization of synaptic circuits controlling GI, cardiovascular and respiratory functions (1; 3; 6; 14; 15; 17; 18; 24; 29; 45; 51; 52). This arrangement is quite logical, when considered from a physiological standpoint, since the requirements of each system vary greatly in the type, as well as the timing and duration, of responses. While, generally, one can afford to have a delay in GI-response time of minutes, for example, such an interval would be incompatible with life when applied to
the control of the baroreflex or respiration. In contrast, hypertension induces plasticity within brainstem baroreflex circuits after periods of 1-5 weeks (47; 48; 53-55) while GI brainstem neurocircuits can demonstrate plasticity and neuromodulation after hours (19; 20; 49). Such profound differences further support the concept that vagal pathways within the brainstem are organized into unique neurocircuits that are anatomically and functionally specific.
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Reference List


FIGURE LEGENDS

Figure 1: Capsaicin (CAP) increases EPSC frequency in cNTS neurons
A: Representative traces of sEPSCs recorded from a single cNTS neuron voltage clamped at -60mV. Each figure represents 6 consecutive, overlapping traces. The magnitude of the increase in sEPSC frequency induced by the first application of CAP (100nM; 1st CAP) was similar to that induced by a repeated application of CAP (100nM; 2nd CAP) following a suitable period of wash-out and recovery. These data demonstrate a lack of tachyphylaxis in the response to CAP.
B: Representative traces of mEPSCs recorded in the presence of TTX (1μM) from a single cNTS neuron voltage clamped at -60mV. Each figure represents 6 consecutive, overlapping traces. Compared with control recordings (upper trace), perfusion with CAP (100nM) increased the frequency, but not the amplitude, of mEPSCs. Following washout (lower traces) mEPSC frequency recovered to baseline levels.
C: Graphical representation of the effects of CAP (100nM) on mESPC frequency (Ca), amplitude (Cb), rise-time (Cc) and decay-time (Cd). Note that CAP decreased mEPSC inter-event interval (Ba) i.e., increased event frequency, but had no effect on mEPSC amplitude or event kinetics. These data imply that CAP acts at presynaptic sites to increase mEPSC frequency.

Figure 2: Purinergic agonists increase sEPSC frequency in a subpopulation of cNTS neurons
A: Representative traces from a cNTS neuron voltage clamped at -60mV. Each figure represents 6 consecutive, overlapping traces. Compared with control recordings (upper trace), perfusion with the non-selective agonist, ATP (100μM) as well as the P2X receptor selective agonist, α,β,Me-ATP (10μM) increased sEPSC frequency, but not amplitude. The ability of α,β,Me-ATP to increase sEPSC frequency was prevented by the P2X receptor selective antagonist, PPADS (10μM; lower trace).
B: Graphical representation of the increase in sEPSC frequency, but not amplitude, induced by purinergic agonists. Note that both ATP and α,β,Me-ATP decreased the inter-event interval, i.e., increased sEPSC frequency (left) but had no effect on sEPSC amplitude (right). In the presence of PPADS, however, α,β,Me-ATP no longer induced decreased the sEPSC inter-event interval.
Figure 3:  Differential effects of CCK-8s, CAP and ATP on sEPSC frequency in cNTS neurons
A: Representative traces from a single cNTS neuron voltage clamped at -60mV. Each panel represents 6 consecutive, overlapping traces illustrating the increase in sEPSC frequency in response to \(\alpha,\beta\)-Me-ATP, CCK-8s and CAP as well as the recovery following wash-out between drug treatments.
B: Graphical representation of the response of cNTS neurons to \(\alpha,\beta\)-Me-ATP, CCK-8s and CAP. Eight of the 28 cNTS neurons tested did not respond to any of the agonists and were not included in the graphic. Note that all CCK-8s responsive neurons were also CAP responsive, suggesting that CCK8s may act upon vagal afferent CAP-sensitive C fibers. In contrast, all \(\alpha,\beta\)-Me-ATP-sensitive neurons were also CAP-sensitive and a significant proportion of \(\alpha,\beta\)-Me-ATP-sensitive neurons were also CCK-8s-sensitive. These results suggest that A- and C- afferent inputs onto cNTS neurons cannot be classified pharmacologically on the basis of their distinct responses to purinergic or vanilloid receptor agonists. In particular, all cNTS neurons receiving capsaicin-sensitive inputs (hence, presumably C-fibers) are also sensitive to purinergic agonists.

Figure 4:  Effects of CCK-8s, CAP and ATP on sEPSC frequency in cNTS neurons following perivagal capsaicin
Graphical representation of the proportion of cNTS neurons in which CAP (A), CCK-8s (B) and ATP (C) increase sEPSC frequency in brainstem slices from control rats (white bars) and in brainstem slices from rats following perivagal capsaicin application (black bars). Note that perivagal capsaicin pretreatment decreases, but does not abolish, the ability of CAP and CCK-8s to increase sEPSC frequency in cNTS neurons. In contrast, perivagal capsaicin has no effect upon the proportion of cNTS neurons in which ATP increases sEPSC frequency. These results indicate that perivagal capsaicin a) does not induce a selective degeneration of vagal afferent C fibers and b) that CCK-8s does not act exclusively on capsaicin-sensitive C-fibers.
A

Control
ATP 100 μM
α-β-Met ATP 10 μM
PPADS + α-β-Met ATP

B

\(\alpha-\beta\)-Met ATP
ATP
PPADS + α-β-Met ATP
Control

Cumulative Fraction of sEPSCs

Inter-event Interval (ms)

Cumulative Fraction of sEPSCs

Amplitude (pA)
A

Control                   $\alpha,\beta$-Me-ATP

Wash out                 CCK-8s

Wash out                 Capsaicin

Wash out

100pA

100ms

B

n = 28

$\alpha,\beta$-Me-ATP

0

2

9

CAP

4

5

CCK-8s

0
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- **A**
  - Responsive cells (%) vs. CAP
  - Control: 80%
  - Perivagal Capsaicin: 40%

- **B**
  - Responsive cells (%) vs. CCK-8s
  - Control: 60%
  - Perivagal Capsaicin: 20%

- **C**
  - Responsive cells (%) vs. ATP
  - Control: 40%
  - Perivagal Capsaicin: 20%

Legend:
- Control
- Perivagal Capsaicin

* Denotes statistical significance.