RECRUITMENT OF EXCITATORY SEROTONERGIC NEUROTRANSMISSION TO CARDIAC VAGAL NEURONS IN THE NUCLEUS AMBIGUUS POST HYPOXIA AND HYPERCAPNIA.

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Short Title: Activating 5HT3 Receptors Post Hypoxia/Hypercapnia

Word Count: 4641 words

Abstract: 247 words

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ABSTRACT

Inhibitory GABAergic and glycinergic neurotransmission to cardioinhibitory cardiac vagal neurons (CVNs) increase during inspiratory activity and likely mediate respiratory sinus arrhythmia, while the frequency of excitatory postsynaptic currents (EPSCs) in CVNs are unaltered during the different phases of respiration. However, following hypoxia and hypercapnia (H/H) the parasympathetic activity to the heart increases and thus far, identification of the pathways and neurotransmitters that are responsible for exciting CVNs post H/H are unclear. This study identifies different excitatory pathways to CVNs recruited post H/H. Spontaneous and inspiratory related EPSCs were recorded in CVNs before, during and after 10 minutes of H/H, in an in-vitro slice preparation that retains rhythmic respiratory activity. Before and during H/H EPSCs in CVNs were completely blocked by 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX) and D(-)-2-Amino-5-phosphono pentanoic acid (AP5), selective AMPA / kainate and NMDA receptor blockers, respectively. However, after H/H, there was a significant increase in EPSCs during each inspiratory burst. While some of the inspiratory related EPSCs were blocked by the broad purinergic receptor antagonist Pyridoxalphosphate-6-azophenyl-2’, 4’-disulphonic acid (PPADS) and the specific P2X receptor antagonist 2’,3’-O-(2,4,6-Trinitrophenyl) adenosine 5’-triphosphate monolithium trisodium salt (TNP-ATP) a P2X receptor blocker, most of the recruited excitatory neurotransmission to CVNs is serotonergic since odansetron, a selective 5HT3 antagonist, abolished the majority of the spontaneous and inspiratory related EPSCs evoked during recovery from H/H. The results from this study suggest that following episodes of H/H two non-glutamatergic excitatory pathways, purinergic and serotonergic, activating P2X and 5-HT3 receptors, respectively, are recruited to excite CVNs in the post H/H recovery period.
INTRODUCTION

Heart rate is strongly influenced by the changing phases of normal respiration. Heart rate increases with inspiration and decreases with expiration and this occurs in large part due to interactions between respiratory neurons and parasympathetic cardiac vagal neurons (CVNs) in the brainstem. This respiratory sinus arrhythmia (RSA) of CVNs is primarily mediated by inspiratory evoked increases in inhibitory GABAergic and glycinergic synaptic inputs, with no change in excitatory glutamatergic synaptic inputs to CVNs (Evans et al. 2005; Neff et al. 1998). However, whereas RSA occurs under normal conditions, exposure to environmental insults such as hypoxia and/or hypercapnia can dramatically alter cardiorespiratory function, including the inspiratory related modulation of CVN activity (Evans et al. 2005; Griffioen et al. 2007; Huang et al. 2006; Huang et al. 2007; Neff et al. 2003).

Responses to hypoxia and hypercapnia (H/H) include generalized vasodilatation and increased cutaneous temperature that are mediated by the sympathetic nervous system (Simmons et al. 2007). In addition, H/H elicits tachycardia at the onset of the H/H which is followed by bradycardia as the H/H episodes continue (Bonsignore et al. 1994). The biphasic tachycardia followed by bradycardia during H/H is likely partly due to the biphasic increase then decrease in inhibitory GABAergic and glycinergic inputs to CVNs (Neff et al. 2003). Upon recovery from H/H, when normal levels of oxygen and carbon dioxide are restored, increased vagal tone and bradycardia are maintained (Pichot et al. 2000; Roche et al. 2002). The mechanisms responsible for the post hypoxic bradycardia are still not clearly understood.

Neurons in the brainstem responsible for chemosensitivity and initiating cardiorespiratory responses to H/H have not been fully elucidated but likely candidates include ATP (Gourine
2005; Gourine et al. 2005), and glutamate containing neurons of the retrotrapezoid nucleus (Guyenet et al. 2005), as well as glutamate and acetylcholine containing neurons in the arcuate nuclei (Benarroch et al. 2007; Benarroch et al. 2001) and the serotonergic rich medullary raphe neurons (Richerson et al. 2005). Chemoreceptive nuclei could potentially mediate changes in heart rate by activating direct pathways from these neurons to CVNs in the nucleus ambiguus or via polysynaptic pathways. Measurements of neurotransmitters released within the ventral respiratory group (VRG), an area located close to CVNs, in response to hypoxia show transient increases in GABA and glutamate following induction of hypoxia (Richter et al. 1999). As hypoxia progresses, serotonin and ATP levels are released at low levels and the levels of serotonin and ATP peak and remain elevated after termination of the hypoxic episode (Richter et al. 1999). P2X receptors have been also shown to be involved in the responses to hypoxia in CVNs (Griffioen et al. 2007), carotid body (Lahiri et al. 2007), and RVLM neurons (Thomas et al. 1999). Furthermore, P2X2 deficient mice exhibit a diminished response to hypoxia (Rong et al. 2003). The goals of this study were to identify the excitatory pathways recruited and responsible for the excitation of CVNs post H/H. More specifically, the role of glutamate, 5-HT and purinergic pathways to CVNs were examined before, during and in recovery from H/H.

METHODS

Fluorescent Labeling of CVNs and Medullary Slice Preparation.

Neonatal Sprague-Dawley rats (P3-P7; Hilltop, Scottdale, PA) were anesthetized and cooled to ~4°C to slow the heart rate. A right thoracotomy was performed, and the retrograde fluorescent tracer X-rhodamine-5-(and-6)-isothiocyanate (Molecular Probes, Eugene, OR) was injected into the fat pads at the base of the heart. After 24-48 h of recovery, animals were
anesthetized with isoflurane and sacrificed by cervical dislocation, and the brain tissue was placed in a 4°C physiologic saline solution (140 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 5 mM glucose, and 10 mM HEPES) bubbled with 100% O₂ (pH 7.4). All animal procedures were performed with the approval of the Animal Care and Use Committee of The George Washington University in accordance with the recommendations of the panel on euthanasia of the American Veterinary Medical Association and the National Institutes of Health publication, "Guide for the Care and Use of Laboratory Animals."

The medulla was removed with care to preserve the hypoglossal cranial nerve rootlet and was mounted on a cutting block and placed into a vibrating blade microtome (Leica, Nussloch, Germany). Serial transverse sections were sliced in a rostro-caudal progression until the inferior olives and the NA could be visualized on the rostral surface of the tissue. A single thick (770-870 µm) section that included CVNs, the hypoglossal nerve rootlet, the pre-Botzinger complex, and the rostral portion of the hypoglossal nucleus was cut and submerged in a recording chamber that allowed perfusion (4 mL/min) above and below the slice with room temperature artificial cerebrospinal fluid (aCSF; 125 mM NaCl, 3 mM KCl, 2 mM CaCl₂, 26 mM NaHCO₃, 5 mM glucose, and 5 mM HEPES) equilibrated with 95% O₂/5% CO₂ (pH 7.4).

**Recording Respiratory Network Activity.**

The thick medullary slice preparation generates rhythmic inspiratory-related motor discharge in hypoglossal cranial nerves (Smith et al. 1991). Spontaneous respiratory-related activity was recorded by monitoring motor-neuron population activity from hypoglossal nerve rootlets using a suction electrode. Hypoglossal rootlet activity was amplified (50,000 times) and filtered (10- to 300-Hz band pass; CWE Inc., Ardmore, PA) and electronically integrated ([tau] =
50 ms; CWE Inc.).

**Patch-Clamp Techniques.**

CVNs in the NA were identified by the presence of the fluorescent tracer as described previously (Mendelowitz and Kunze 1991). Briefly, slices were viewed with infrared illumination and differential interference optics (Zeiss, Oberkochen, Germany) and under fluorescent illumination with an infrared sensitive cooled charged-coupled device camera (Photometrics, Tucson, AZ). Neurons that contained the fluorescent tracer were identified by superimposing the fluorescent and infrared images on a video monitor (Sony, Tokyo, Japan). Patch pipettes (2.5-3.5 MΩ) were visually guided to the surface of individual CVNs using differential interference optics and infrared illumination (Zeiss, Oberkochen, Germany). CVNs were voltage-clamped at a holding potential of -80 mV. The patch pipettes were filled with a solution that consisted of (in mM) 135 K-gluconic acid, 10 HEPES, 10 EGTA, 1 CaCl₂, and 1 MgCl₂, at a pH of 7.3.

**Hypoxia/Hypercapnia.**

Rhythmic inspiratory-related and spontaneous excitatory post-synaptic currents (EPSCs) in a single CVN were recorded simultaneously for 4 min (control period) in aCSF equilibrated with 95% O₂/5% CO₂. Slices were then perfused with hypoxic/hypercapnic aCSF (equilibrated with 85% N₂/6% O₂/9% CO₂) for 10 min and then returned to the original perfusate for up to 60 minutes during which different drug regimens (see focal drug application below) were applied to isolate the excitatory inputs recruited to CVNs in the post H/H period.

**Focal Drug Application.**
All the drugs used in these experiments were applied using a pneumatic picopump pressure delivery system (WPI, Sarasota, FL). Drugs were ejected from a patch pipette positioned within 30 µm from the patched CVN. The maximum range of drug application has been previously determined to be 100-120 µm downstream from the drug pipette and considerably less behind the drug pipette (Wang et al. 2001). Drugs used included ondansetron (100 µM) to block 5-HT3 receptors, Pyridoxal phosphate-6-azophenyl-2’, 4’-disulphonic acid (PPADS, 100 µM) and 2’,3’-O-(2,4,6-Trinitrophenyl) adenosine 5’-triphosphate (TNP-ATP, 100 µM) to block purinergic and P2X receptors respectively, and finally 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX, 50 µM) and D(--)-2-Amino-5-phosphonopentanoic acid (AP5, 50 µM) were used to block excitatory glutamatergic neurotransmission to CVNs. All drugs were obtained from Sigma Chemical Company (St. Louis, MO).

**Data Analysis.**

Synaptic events were detected using MiniAnalysis version 5.6.12 (Synaptosoft, Decatur, GA). The frequency of EPSCs that occurred in CVNs were grouped into 1-s bins and cross-correlated with the onset of inspiratory-related hypoglossal activity. Data were analyzed from all bursts during the last 2 min of the control period, during the last 2 min of the 10-min period of hypoxia/hypercapnia, and from minutes 6-8 during each 8-min drug regimen application period. In addition at the end of the experiment, the last 2 mins recorded during the drug free period was analyzed. These periods were chosen for analysis because during these periods, hypoxia/hypercapnia-evoked changes and drug application had reached a steady state. Statistical comparisons were performed within a condition using a one-way ANOVA with repeated measures, and a two way ANOVA with repeated measures was utilized to examine the
differences between the spontaneous and respiratory related EPSCs in response to various time-dependent or drug application periods. For the two-way ANOVA with repeated measures comparisons between drug applications or time periods the 5 seconds of spontaneous EPSC frequency before the inspiratory burst was averaged into a single spontaneous frequency value. Significant difference was set at $p < 0.05$.

RESULTS

5-HT3 and purinergic receptors do not Mediate Excitation of CVNs during Normal Respiration

Previous studies have shown that under control conditions CVNs receive spontaneous glutamatergic EPSCs since AP5 and CNQX, NMDA and non-NMDA receptor blockers, respectively, block most, if not all, excitatory inputs to CVNs (Evans et al. 2005; Neff et al. 1998). We tested the hypothesis that CVNs also receive 5-HT3 and purinergic receptor signaling during control conditions. The frequencies and amplitudes of EPSCs in CVNs were not altered by inspiratory bursts, see fig 1. In addition, there was no change in the frequency of EPSCs from spontaneous $4.2 \pm 0.1$ Hz to inspiratory period $4.2 \pm 0.2$ Hz ($n = 6$, $p > 0.05$) see fig 1. Similarly the frequency and amplitude of EPSCs was not altered by ondansetron (100 $\mu$M) or PPADS (100 $\mu$M). However, AP5 and CNQX, NMDA and non-NMDA receptor blockers, abolished all EPSCs, ($4.2 \pm 0.1$ to $0.6 \pm 0.3$ $n = 6$, $p < 0.05$, see figure 1). The block of EPSCs with AP5 and CNQX was reversible, as shown in figure 1.
Inspiratory Related Cardiac Vagal Neuron Responses to Hypoxia and Hypercapnia and During Recovery

Inspiratory bursting frequency and duration significantly (p<0.05) decreased during hypoxia/hypercapnia (from 3.2 ± 0.4 to 2.0 ± 0.2 bursts/min, and 3.3 ± 0.3 to 1.3 ± 0.1 s, respectively), and in recovery both inspiratory frequency and duration significantly (p<0.05) increased (from 2.0 ± 0.2 to 4.2 ± 0.3 bursts/min, and 1.3 ± 0.1 to 12.8 ± 3.2 s, respectively). These changes persisted for the duration of these experiments (up to 60 minutes).

As reported previously CVNs do not receive any inspiratory related excitation during control or 10 minutes of hypoxia and hypercapnia (Huang et al. 2007). However during recovery from H/H there was a significant increase in the frequency of inspiratory related EPSCs in CVNs, see figure 2. The EPSC frequency increased from 5.4 ± 0.4 Hz to 11.0 ± 0.2 Hz (n = 9, p > 0.05) during inspiratory activity. The increase in excitatory inputs to CVNs during recovery persisted for up to 1 hour after termination of the hypoxic hypercapnic period, see figure 2 (10.1 ± 0.7 Hz at 6-8 mins and 11.1 ± 0.3 Hz at 46-48 mins).

NMDA and AMPA Receptor Antagonists do not Diminish the Inspiratory Related Excitation of CVNs

To test the hypothesis that the increase in inspiratory related excitation post H/H was glutamatergic, CNQX and AP5 were applied. Surprisingly application of AP5 and CNQX did not significantly alter (11.1 ± 0.3 Hz to 10.8 ± 0.5 Hz n= 10, P> 0.05) the excitatory pathway to CVNs recruited post H/H, see figure 3 and figure 4.
**5HT3 Receptors Mediate the Spontaneous and Inspiratory Related Excitation of CVNs**

Since both purinergic and 5-HT neurons can mediate chemosensitivity and pH signaling within the brainstem (Ling et al. 2001), and 5-HT fibers are known to make axo-somatic connections with CVNs (Izzo et al. 1993; Takeuchi et al. 1983), we hypothesized that either purinergic or 5-HT pathways acting via P2 or 5-HT3 receptors, respectively, could mediate inspiratory related excitation of CVNs post H/H. To test these hypotheses PPADS and ondansetron were added sequentially by focal application. PPADS significantly inhibited, but did not eliminate, the inspiratory related EPSCs from 11.6 ± 0.2 Hz to 8.4 ± 0.3 Hz n=8 p< 0.05), see figure 3A. Ondansetron subsequently completely blocked the increase in inspiratory related excitation of CVNs (inspiratory; from 8.4 ± 0.3 Hz to 2.7 ± 0.5 Hz n=8 p< 0.05), see figure 3A, and in addition, decreased the spontaneous EPSCs (5.4 ± 0.4 Hz to 2.69 ± 0.52 Hz n=8 p< 0.05) see figure 3B. The inhibition by PPADS and ondansetron were reversible.

**TNP-ATP a P2X Receptor Antagonist Significantly Inhibits Inspiratory Related Excitation During Recovery from Hypoxia and Hypercapnia**

In another set of experiments, PPADS was substituted with TNP-ATP, a more specific P2X antagonist (Gever et al. 2006) and ondansetron was applied before TNP-ATP. Similar to the previous series of experiments, CNQX and AP5 did not significantly diminish the inspiratory related excitation of CVNs (10.1 ± 0.5 Hz to 9.9 ± 0.6 Hz n= 9, P> 0.05) post H/H, see figure 4A. Ondansetron inhibited both the inspiratory related (9.9 ± 0.6 Hz to 5.8 ± 0.5 Hz n= 10, P< 0.05) see figure 4A, and spontaneous EPSCs (5.9 ± 0.6 Hz to 1.9 ± 0.7 Hz n= 10, P< 0.05), in CVNs post H/H, see figure 4B. Addition of TNP-ATP abolished the inspiratory related EPSCs from (5.8 ± 0.5 Hz to 1.9 ± 0.5 Hz n= 10, P< 0.05), figure 4A, with no further significant change.
to spontaneous EPSCs, see figure 4B. The inhibition by TNP-ATP and ondansetron were
reversible, (inspiratory 1.9 ± 0.5 Hz to 10.87 ± 0.77 Hz, n = 8 p< 0.05 and spontaneous 1.9 ± 0.7
Hz to 5.3 ± 0.7 Hz n = 9 p< 0.05), see figure 4.

Discussion

In this study we sought to determine the synaptic mechanisms that are involved in
exciting CVNs following hypoxia and hypercapnia. The findings of this study are; 1) Purinergic
P2X and serotonergic 5-HT3 receptors do not mediate excitation of CVNs during normal
inspiratory activity. 2) A long-lasting inspiratory related excitation to CVNs is recruited during
recovery from H/H which is not mediated by glutamatergic neurotransmission. 3) Following
hypoxia and hypercapnia, both spontaneous EPSCs and the inspiratory related increase in EPSCs
are mediated by the activation of 5-HT pathway, and in addition, during inspiration a purinergic
pathway is also recruited which activates P2X receptors in CVNs.

Previous studies from this lab have demonstrated that under control conditions EPSCs in
CVNs are primarily mediated by glutamate via NMDA and non NMDA receptors (Neff et al.
1998). For example, a likely link in the baroreflex arc is the glutamate pathway evoked upon
stimulation of the NTS (Neff et al. 1998) as well as vagal afferent fibers (Evans et al. 2003).
Similar to previously reported results CVNs do not receive inspiratory related excitation during
normal respiration (Evans et al. 2005; Huang et al. 2007). This study confirms that excitatory 5-
HT3 and P2 receptors are not spontaneously active in CVNs under control conditions.

However H/H significantly alters cardiorespiratory function. The results in this study
demonstrate a long lasting (up to 1 hour) increase in inspiratory related excitation to CVNs
following an acute 10 minute period of hypoxia and hypercapnia. It is possible this post
hypoxic/hypercapnic augmentation of inspiratory related EPSCs is elicited by long term facilitation (LTF) of the respiratory network resulting from exposure to hypoxia and/or hypercapnia. Respiratory LTF is mediated by modulation of the respiratory network by both serotonin and ATP in both awake and anesthetized rats subjected to hypoxia and hypercapnia (Fuller et al. 2000; Kinkead et al. 2001; Kinkead and Mitchell 1999).

The results in this study that show there is little or no glutamatergic signaling involved in the control of CVNs post H/H is surprising. NMDA and non NMDA receptor antagonists did not significantly inhibit the spontaneous and inspiratory related EPSCs in CVNs post H/H. However, this study reveals the first endogenously active role of serotonergic neurotransmission to CVNs. Within the nucleus ambiguus CVNs receive the most dense axo-somatic 5-HT contacts in the brainstem (Takeuchi et al. 1983). However the physiological role of the 5-HT contacts has not been previously elucidated. This study establishes a critical role for 5-HT3 receptors as a 5-HT pathway to CVNs that is selectively recruited post H/H. The 5-HT3 receptor antagonist ondansetron inhibited both the spontaneous and inspiratory related excitation of CVNs during recovery from hypoxia and hypercapnia indicating both spontaneous and inspiratory evoked 5-HT activity to CVNs are increased post H/H. The present work also demonstrates P2X receptors are involved and partly mediate an additional inspiratory evoked selective purinergic excitation of CVNs post H/H.

In conclusion, normal brainstem cardiorespiratory interactions and the central cardiorespiratory responses to H/H is complex. Under control conditions RSA is mediated by increases in GABA and glycine activity during inspiratory activity with no changes in excitatory glutamatergic, serotonergic, or purinergic neurotransmission to CVNs. The initial responses to H/H include an increase in inhibitory GABA and glycine neurotransmission to CVNs which is
likely responsible for the initial tachycardia that occurs with H/H. This first phase is replaced by reduced GABA and glycine neurotransmission and this likely is responsible for the bradycardia that follows the tachycardia in the biphasic responses to H/H. Finally there is a phase of augmented parasympathetic activity in the recovery period post H/H (Pichot et al. 2000; Roche et al. 2002). This maintained increase in parasympathetic cardiac vagal activity post H/H is not mediated by glutamatergic neurotransmission but is rather mediated by the recruitment of an excitatory purinergic pathway to CVNs evoked during inspiratory activity, as well as the recruitment of a serotonergic pathway that elicits both spontaneous and inspiratory related 5-HT3 mediated EPSCs in CVNs.
Acknowledgments:

Sources of Funding: This work was supported by NIH grants HL49965 and HL59895 to DM.
Figure Legends

Figure 1. 5-HT3 and P2X receptor antagonists do not mediate excitation of CVNs during control respiratory activity. Inspiratory-related bursting activity was recorded from the hypoglossal rootlet (XII) and electronically integrated (top). Fluorescently identified CVNs were patch clamped in the whole-cell configuration, and glutamatergic neurotransmission was isolated by focal application of GABAergic (Gabazine; 25 M) and glycinergic (strychnine; 1 M) receptor antagonists in this and all subsequent figures. 100µM ondansetron, 100 µM PPADS, and 25µM CNQX /1µM AP5 were applied sequentially to CVNs with a 5 minute period between applications. A typical experiment is shown in A, and the average results from 6 CVNs are illustrated in panel B. There was no significant difference in mEPSC frequency or amplitude between the control, ondansetron and PPADS applications. In the presence of CNQX and AP5 EPSC events were blocked, * p < 0.001.

Figure 2. Hypoxia/hypercapnia evokes a long lasting inspiratory related excitation in CVNs. Changing the perfusate from aCSF equilibrated with 95% O2/5% CO2 to aCSF equilibrated with 9% CO2, 6% O2, 85% N2, H/H, did not alter the frequency of EPSCs to CVNs. However H/H induced an increase in the frequency of EPSCs to CVNs during recovery from H/H which lasted for up to 48 minutes. * denotes a statistically significant difference of P<0.0001 between spontaneous and respiratory evoked EPSC frequency using a one-way ANOVA with repeated measures in this and all subsequent figures.

Figure 3. Activation of 5-HT3 receptors mediates respiratory related EPSC neurotransmission to CVNs during recovery from hypoxia/hypercapnia. CNQX, AP5, PPADS and ondansetron were
applied sequentially to characterize the excitatory synaptic inputs to CVNs. A typical experiment from an unexposed animal is shown at the top, A, and the average data from 10 cells is shown in the histograms, figure 3A, bottom panel. AP5 and CNQX did not significantly alter respiratory related excitatory neurotransmission to CVNs during recovery from hypoxia/hypercapnia. Rather, PPADS, a broad purinergic antagonist, inhibited the inspiratory related excitation while ondansetron, the 5-HT3 receptor blocker inhibited both the spontaneous and inspiratory related EPSCs. The inhibition by ondansetron and PPADS was reversible. # and an unfilled bar denotes a statistically significant difference of $P< 0.0001$ between respiratory related EPSC frequency in a period compared to control values using a 2-way ANOVA with repeated measures in this and all subsequent figures. + denotes a statistically significant difference of $P<0.001$ using a two-way ANOVA with repeated measures comparing the spontaneous EPSC frequency in the control and during each drug application in this and all subsequent figures.

Figure 4. Activation of P2X purinergic receptors mediates respiratory related EPSC neurotransmission to CVNs during recovery from hypoxia/hypercapnia. In this series of experiments, TNP-ATP was used instead of PPADS and ondansetron was applied before TNP-ATP. As in the previous series of experiments, AP5 and CNQX did not significantly decrease the spontaneous or respiratory related excitatory neurotransmission to CVNs during recovery from hypoxia/hypercapnia. The 5-HT3 receptor antagonist ondansetron significantly diminished both the spontaneous and respiratory related EPSCs. TNP-ATP significantly ($p<0.01$) decreased the remaining respiratory related EPSCs but did not significantly change the spontaneous EPSCs.
REFERENCES


