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5HT1A Receptors Inhibit Glutamate Inputs to Cardiac Vagal Neurons Post-Hypoxia/Hypercapnia

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Abstract

Synaptic inputs to cardiac vagal neurons (CVNs) regulate parasympathetic activity to the heart. Previous work has shown insults such as hypoxia and hypercapnia (H/H) alter CVN activity by activating post-synaptic serotonergic, purinergic, and glutamatergic receptors in CVNs. This study examines the role of serotonergic 5HT1A receptors in modulating these excitatory neurotransmissions to CVNs during control conditions, H/H and recovery from H/H. Excitatory post-synaptic currents (EPSCs) were recorded from identified CVNs in-vitro before, during and post H/H. The 5HT1A receptor antagonist WAY 100635 had no effect on EPSCs in CVNs before, and during H/H. However during recovery from H/H inspiratory-related excitatory serotonergic and purinergic pathways were recruited to excite CVNs. However when these serotonergic and purinergic pathways are blocked, the 5HT1A receptor antagonist WAY 100635 restores an excitatory glutamatergic neurotransmission to CVNs. This study indicates endogenous activation of serotonergic 5HT1A receptors diminishes glutamatergic neurotransmission to CVNs following H/H, likely via a presynaptic site of action.

Keywords

parasympathetic; serotonin; hypoxia; hypercapnia; ambiguous; cardiac; heart rate; apnea; SIDS

1. Introduction

SIDS remains the leading cause of infant mortality yet, despite many years of research, the underlying basis of SIDS remains unknown. SIDS is postulated to result from failed autoresuscitation and inadequate or inappropriate cardiorespiratory network function and responses to challenges such as hypoxia and/or hypercapnia. Multiple postmortem reports of SIDS victims indicate that hypoxic episodes precipitate respiratory distress which is accompanied and/or preceded by bradycardia before the fatal event (Meny et al., 1994). Genetic and receptor binding studies in postmortem tissues of infants that succumbed to SIDS indicate gross serotonergic and cholinergic abnormalities (Kinney et al., 2001; Nattie and Kinney, 2002; Weese-Mayer et al., 2003; Paterson et al., 2006a; Slotkin et al., 2006a; Slotkin et al., 2006b; Rand et al., 2007).

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Among cardiorespiratory network interactions originating in the brainstem the most ubiquitous is respiratory sinus arrhythmia (RSA). Mechanisms of RSA have been extensively characterized at the level of cardiac vagal neurons (CVNs) located within the nucleus ambiguus (NA) (Neff et al., 2003; Huang et al., 2005). During inspiration CVNs receive tonic non-respiratory related excitatory glutamatergic neurotransmission (Huang et al., 2005), little if any serotonergic or purinergic input, and inspiratory evoked inhibitory GABAergic and glycinergic synaptic inputs (Neff et al., 2003). Glutamatergic neurotransmission to CVNs is diminished while serotonergic and purinergic neurotransmission and post-synaptic 5HT₃ and P2X receptor activation, respectively, sustains excitatory neurotransmission to CVNs during recovery from Hypoxia/Hypercapnia (H/H) (Kamendi et al., 2008). However, the mechanisms responsible for the loss of glutamatergic neurotransmission to CVNs post H/H remain unknown. Given the evidence SIDS is associated with abnormal 5HT receptor function, this study tested whether endogenous activation of serotonergic 5HT_{1A} receptors modulates excitatory neurotransmission to CVNs during normal respiration, H/H or recovery from H/H.

2. Materials and Methods

All animal procedures were performed in compliance with the institutional guidelines at George Washington University and are 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*. All efforts were made to minimize the number of animals used and their suffering.

To identify CVNs *in vitro*, a two-stage procedure was used. In an initial surgery, Sprague-Dawley rats (postnatal days 2–6; Hilltop, Scottsdale, PA) were anesthetized with hypothermia and received a right thoracotomy. Rhodamine (0.05 mL, 1 to 5%) (XRITC, Life Technologies Corporation, Carlsbad, CA) was injected into the pericardial sac to retrogradely label CVNs. The location and identification of these neurons in the NA, was previously described (Bouairi et al., 2006). Specificity of the cardiac vagal labeling was confirmed by the absence of any labeled neurons in the brainstem when rhodamine is injected either outside the pericardial sac or within the pericardial sac after the cardiac branch of the vagus nerve was sectioned ($n = 4$). Recent work demonstrated that this method identifies CVNs localized in the external formation of the NA (Bouairi et al., 2006). In other control experiments ($n = 10$), i.v. injection of up to 10 mg of rhodamine failed to label any neurons in the medulla except for rare labeling of neurons in the area postrema, an area with a deficient blood-brain barrier.

1–4 days later, the animals were anesthetized with isoflurane and killed by rapid cervical dislocation. Electrophysiological experiments were conducted at postnatal age P3–P8. The brain was submerged in cold (4°C) buffer: 140 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 5 mM glucose, and 10 mM HEPES and continually gassed with 100% O₂. A single slice of the medulla (800 µm thickness) that included CVNs, the rostral hypoglossal nucleus and rootlets, and the preBotzinger complex was obtained and submerged in a recording chamber, which allowed perfusion (5–10 mL/min) of ACF at room temperature (24–25°C) containing 125 mM NaCl, 3 mM KCl, 2 mM CaCl₂, 26 mM NaHCO₃, 5 mM glucose, and 5 mM HEPES equilibrated with carbogen (95% O₂ and 5% CO₂, pH 7.4). The thick medullary slice preparation generates rhythmic inspiratory-related motor discharge in hypoglossal cranial nerves. Spontaneous inspiratory-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 (10–300 Hz bandpass; CWE, Ardmore, PA).

Individual CVNs in the NA were identified by the presence of the fluorescent tracer using a Zeiss Axioskop upright microscope (Carl Zeiss Inc., Thornwood, NY) and a 40x water immersion objective. These identified CVNs were then imaged with differential interference contrast optics, infrared illumination, and infrared-sensitive video detection cameras to gain better spatial resolution. Patch pipettes (2.5–3.5 Microns) were filled with a solution consisting of 135 mM K-gluconic acid, 10 mM HEPES, 10 mM EGTA, 1 mM CaCl₂, and 1 mM MgCl₂, pH 7.35 and guided to the surface of individual CVNs. Voltage clamp whole-cell recordings were made at a holding potential of –80 mV with an Axopatch 200B and pClamp 8 software (Axon Instruments, Union City, CA).

All drugs used in these experiments were applied using a pneumatic picopump pressure system (WPI, Sarasota, FL). All drugs were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Drugs were focally released using a picospritzer and pressure ejected from a patch pipette positioned within 30 μ m of the patched CVN. The maximum range of drug application was determined previously to be 100–120 μ m downstream from the drug pipette and was considerably less behind the drug pipette (Wang et al., 2002). Excitatory postsynaptic currents (EPSCs) were isolated by continuous focal application of strychnine (1 μ M) and gabazine (25 μ M) to block glycine and GABAergic receptors, respectively. Other drugs used included ondansetron (100 μ M) to block 5-HT₃ receptors, pyridoxal-phosphate-6-azophenyl-2',4'-disulfonic acid (PPADS, 100 μ M) to block purinergic receptors; and finally d-2-amino-5-phosphonopentanoate (AP-5, 50 μ M) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 50 μ M) were used to block NMDA and AMPA/kainite glutamatergic neurotransmission, respectively.

Rhythmic inspiratory-related activity and EPSCs of CVNs were recorded simultaneously for 10 min in control ACSF, equilibrated with 95% O₂, and 5% CO₂ (normoxia). Slices were then exposed for 10-min to hypoxia/hypercapnia (H/H) by changing control ACSF to ACSF equilibrated with 9% CO₂, 6% O₂, and 85% N₂, and then slices were reoxygenated by returning the perfusate to initial control ACSF equilibrated with 95% O₂, and 5% CO₂ (posthypoxia). Only one experiment was conducted per preparation.

Synaptic events were detected using MiniAnalysis (version 5.6.12; Synaptosoft, Decatur, GA). Threshold was set at root-mean-square noise multiplied by five. The frequency of EPSCs that occurred in CVNs was grouped in 1-s bins and cross-correlated with onset of inspiratory-related hypoglossal activity. Data were analyzed from all bursts during the last 2 min of the condition. Results were presented as means $\pm$ SEM. One-way ANOVA with repeated measures and bonferroni posttest were used to examine the differences between spontaneous and inspiratory related EPSCs within a condition. To examine time-dependent differences in response to various drug application periods the results were analyzed by two-ways ANOVA test with repeated measures, followed by Bonferroni posttest. Significant differences for all data were set at $p < 0.05$.

3. Results

During control conditions CVNs possess tonic glutamatergic EPSCs mediated via NMDA and AMPA/kainate receptor activation. Inspiratory activity did not alter the frequency of EPSCs in CVNs during control conditions, see figure 1. The 5HT_{1A} antagonist WAY100635 had no effect on EPSCs during the control or H/H period, see figure 1.

However during recovery from H/H an inspiratory related excitatory neurotransmission was recruited, see figure 2. The 5HT₃ and P2x receptor antagonists, ondansetron and PPADS, respectively, significantly ($p < 0.05$) inhibited this recruited inspiratory related excitatory neurotransmission to CVNs. Spontaneous excitatory synaptic input to CVNs was also

significantly ($p < 0.05$) inhibited by application of ondansetron and PPADS post-H/H. However application of WAY 100635 restored spontaneous and recruited inspiratory related excitatory neurotransmission to CVNs during the recovery from H/H. Application of CNQX and AP5 completely blocked both the spontaneous and inspiratory-related EPSCs generated by application of the 5HT_{1A} receptor antagonist WAY 100635. The effects of the receptor antagonists were reversible.

4. Discussion

This study demonstrates that under control and H/H conditions, 5HT_{1A} receptors do not alter excitatory neurotransmission to CVNs. However, during recovery from H/H an endogenous serotonergic pathway, acting via 5HT_{1A} receptors and likely via a presynaptic mechanism of action, is recruited to inhibit glutamatergic neurotransmission to CVNs. WAY100635, a 5HT_{1A} receptor antagonist, restored an excitatory glutamatergic pathway to CVNs that persisted in the presence of selective 5HT₃ and P2X receptor antagonists and was subsequently blocked by selective NMDA and AMPA/kainate glutamatergic receptor antagonists. Figure 3 illustrates this complex interaction. During recovery from H/H serotonergic and purinergic pathways, that activate postsynaptic 5HT₃ and P2x receptors in CVNs, respectively, are recruited that provide excitatory input to CVNs, particularly during inspiration. However 5HT release post H/H also inhibits a glutamatergic pathway to CVNs, likely via a 5HT_{1A} receptor mediated presynaptic inhibition of glutamate release, but this glutamatergic neurotransmission is restored upon application of a 5HT_{1A} receptor antagonist.

Studies examining other brain regions also indicate 5-HT receptor activation can inhibit the release of glutamate via presynaptic sites of action. The glutamatergic responses evoked upon stimulation of the pathway from the retina to the suprachiasmatic nucleus is inhibited by serotonin, yet the postsynaptic responses to exogenous glutamate were unaffected, indicating a likely presynaptic site of action for 5HT receptor modulation (Flett and Colwell, 1999). Application of 5HT has also been shown to inhibit the excitation evoked by glutamate in the cerebellar cortex (Kerr and Bishop, 1992).

During normal physiological conditions, the sum of tonic excitatory glutamatergic inputs from the NTS that mediate the baroreflex, and inspiratory-related GABAergic and glycinergic neurotransmission to CVNs that are involved in respiratory sinus arrhythmia are thought to be the major determinants of brainstem parasympathetic outflow to the heart. Although other neurotransmitter containing pathways and receptors have been identified within the nucleus ambiguus, and particularly on CVNs, their physiological role remains unclear. For example, multiple serotonergic terminals and receptor subtypes have been characterized in the nucleus ambiguus region (Izzo et al., 1993; Dergacheva et al., 2007; Audero et al., 2008; Dergacheva et al., 2008; Dergacheva et al., 2009a; Dergacheva et al., 2009b; Duncan et al., 2009; Hodges et al., 2009; Kinney, 2009; Pavone et al., 2009). In particular, 5HT_{1A} receptors are expressed within the nucleus ambiguus and ventrolateral medulla respectively (Chitravanshi and Calaresu, 1992; Thor et al., 1992; Futuro-Neto et al., 1993; Dando et al., 1998; Paterson et al., 2006a; Paterson et al., 2006b). The data from other work supports a critical role for 5HT_{1A} receptors in arousal from sleep during episodes of hypercapnia (Haxhiu et al., 2001; Buchanan and Richerson, 2010). This study demonstrates endogenous 5HT_{1A} receptor mediated neurotransmission does not mediate spontaneous CVN excitation during control or H/H but there is an important endogenous role and release of 5HT and activation of 5HT_{1A} receptors that inhibit glutamatergic neurotransmission to CVNs post H/H.

Alterations in 5HT receptor function are strongly associated with SIDS. Infants that succumb to SIDS have multiple serotonergic abnormalities including hypoplasia of serotonin neurons in the arcuate and raphe nuclei (Filiano and Kinney, 1992; Matturri et al., 2000; Folkerth et al., 2008; Lavezzi et al., 2009), altered numbers of serotonin neurons and receptors (Panigrahy et al., 2000; Kinney et al., 2001; Nattie and Kinney, 2002; Okado et al., 2002; Kinney et al., 2005; Paterson et al., 2006a; Paterson et al., 2006b; Duncan et al., 2009; Paterson et al., 2009; Duncan et al., 2010; Mage and Kollander, 2010) and 5HT transporters polymorphisms (Narita et al., 2001; Weese-Mayer et al., 2003; Rand et al., 2007). This work suggests during recovery from H/H serotonin normally acts via presynaptic 5HT_{1A} receptors to inhibit glutamate neurotransmission to CVNs and prevents excessive excitation of CVNs and an exaggerated bradycardia post H/H. A reduction in 5HT function would reduce this 5HT_{1A} receptor mediated inhibition of glutamatergic excitation and likely result in exaggerated excitation of CVNs and a more pronounced bradycardia and increased frequency and/or duration of bradyarrhythmias. This framework is supported by recent work using mice deficient in the *Pet-1* transcription factor that have a ~70% deficiency of brainstem 5HT neurons (Cummings et al.). These animals exhibit increased spontaneous bradycardias in room air, and delayed gasping, heart rate recovery and mortality in response to anoxia, particularly at 8 days postnatal age (Cummings et al.).

5-HT receptor activation is also likely involved in other cardiorespiratory diseases, including obstructive sleep apnea (OSA). Cardiorespiratory changes induced by chronic intermittent hypoxia likely involve serotonergic synaptic mechanisms. Central respiratory responses to repeated hypoxia are serotonin dependent, and the respiratory plasticity evoked by intermittent hypoxia can be mimicked with intermittent application of serotonin 5HT_{2A} agonists (Feldman et al., 2003; Bocchiaro and Feldman, 2004).

In conclusion, the results in this study indicate an abnormality that decreases 5-HT_{1A} receptor activation or function would exaggerate glutamatergic neurotransmission to CVNs post H/H, and this enhanced excitation of CVNs may be an important mechanism for the pronounced bradycardia as well as increased glutamate excitotoxicity that is known to occur in SIDS victims. Enhanced 5HT activity in patients with OSA may be responsible for the decreased glutamatergic neurotransmission to CVNs, eliciting both a decreased baroreflex control of parasympathetic cardiac activity and a tachycardia.

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Abbreviations

AP-5	d-2-amino-5-phosphonovalerate
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CVNS	cardiac vagal neurons
EPSC	Excitatory Post-Synaptic Current
H/H	Hypoxia/Hypercapnia
NA	Nucleus Ambiguus
PPADS	pyridoxal-phosphate-6-azophenyl-2',4'-disulfonic acid
RSA	Respiratory Sinus Arrhythmia

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Highlights

5HT1A receptors do not alter excitatory neurotransmission to cardiac vagal neurons (CVNs) under control conditions.

However, during recovery from Hypoxia/Hypercapnia (H/H) an endogenous serotonergic pathway, acting via 5HT1A receptors and likely via a presynaptic mechanism of action, is recruited to inhibit glutamatergic neurotransmission to CVNs.

A decrease in 5-HT1A receptor activation or function would exaggerate glutamatergic neurotransmission to CVNs post H/H, and this enhanced excitation of CVNs may be an important mechanism for the pronounced bradycardia as well as increased glutamate excitotoxicity that is known to occur in cardiovascular diseases including Sudden Infant Death Syndrome (SIDS) and Obstructive Sleep Apnea (OSA).

Figure 1.

Inspiratory-related bursting activity was recorded from the hypoglossal rootlet as shown in the middle traces, and integrated hypoglossal activity is shown in the top traces in this figure and figure 2. Fluorescently identified CVNs were patch clamped in the whole cell configuration and a frequency histogram of glutamatergic EPSCs is illustrated in the bottom traces. The 5HT1A receptor antagonist WAY100635 had no effect on glutamatergic EPSCs in CVNs during the control or H/H periods.

Figure 2.

During the recovery period post H/H an excitatory and inspiratory-related neurotransmission was recruited. Application of the 5HT₃ and P2x receptor antagonists ondansetron and PPADS, respectively, blocked the recruited inspiratory related and spontaneous EPSCs in CVNs. Application of WAY100635 during the recovery period restored both the spontaneous and inspiratory related EPSCs in CVNs. Application of CNQX and AP5 completely all EPSCs, and the effects of the receptor antagonists were reversible.

Figure 3.

Serotonergic and purinergic pathways to CVNs are recruited during recovery from H/H and activation of these pathways activate postsynaptic 5HT3 and P2x receptors in CVNs, respectively, particularly during inspiration. However 5HT release post H/H also inhibits a glutamatergic pathway to CVNs via a 5HT1A receptor mediated presynaptic inhibition of glutamate release which can be prevented by 5HT1A receptor antagonists such as WAY100635.