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Review Article

Role of Purinergic and Nicotinic Receptors in the Hypoxia/Hypercapnia Evoked Excitation of Parasympathetic Cardiac Vagal Neurons in the Brainstem

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Abstract

Hypoxia and hypercapnia are among the strongest challenges to the cardiorespiratory system, and these responses are altered by prenatal nicotine exposure. However the mechanism(s) responsible for these cardiorespiratory responses, and their alteration by prenatal nicotine exposure are unknown. We used an *in vitro* medullary slice that allows simultaneous examination of rhythmic respiratory-related activity and synaptic neurotransmission to cardiac vagal neurons (CVNs) that control heart rate. Respiratory-related increases in excitatory neurotransmission only occurred upon recovery from hypoxia/hypercapnia in unexposed animals. These responses were mediated in part by purinergic receptors. Prenatal nicotine exposure transformed central cardiorespiratory responses; CVNs received a respiratory-related neurotransmission not during recovery but during hypoxia/hypercapnia which was wholly dependent upon nicotinic receptor activation. In the presence of nicotinic antagonists, the responses in prenatal nicotine animals reverted to the pattern of responses in unexposed animals. These data identify a new functional role for purinergic receptors in the cardiorespiratory responses to hypoxia/hypercapnia and their role in occluding nicotinic receptor activation with prenatal nicotine exposure. (*Tzu Chi Med J* 2008;20(1):1–10)

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1. Introduction

The respiratory and cardiovascular systems are highly intertwined, both anatomically and physiologically. The most ubiquitous cardiorespiratory interaction is respiratory sinus arrhythmia. During each respiratory cycle, the heart beat slows during expiration and heart rate increases during inspiration. Respiratory sinus arrhythmia helps match pulmonary blood flow to lung inflation and maintain the appropriate diffusion gradient for oxygen in the lungs (1). Recent work, both from *in vivo* and *in vitro* preparations, has shown that respiratory sinus arrhythmia does not involve excitatory pathways but rather is mediated

mainly by an increase in both inhibitory GABAergic and glycinergic neurotransmission to cardiac vagal neurons (CVNs) during inspiration [2–4].

Among the strongest interactions between the cardiovascular and respiratory systems are the responses to hypoxia and hypercapnia. Hypoxia and hypercapnia initially elicit an increase, followed by a dramatic decrease in respiratory frequency [5]. Prolonged hypoxia and hypercapnia eventually produces a terminal apnea. Likewise, hypoxia and hypercapnia evoke an initial transient increase in heart rate followed by a parasympathetically mediated bradycardia and, ultimately, cessation of cardiac contractions [6–8]. The reduction in heart rate and respiratory frequency in response to hypoxia normally serves to reduce the metabolic demand of the cardiac and respiratory muscles, and thus prolong survival [7].

Exaggeration of this protective response to hypoxia, however, could be detrimental. Sudden infant death syndrome (SIDS) is the leading cause of infant death in the post neonatal period [9–12]. Infants that succumb to SIDS typically experience a severe bradycardia that precedes or is accompanied by centrally mediated apnea [10,13]. Bradycardia is also the most prevalent and predictive event in infants monitored for apparent life threatening events [14]. Although the cause(s) of SIDS remains unknown, it has been speculated that an exaggeration of cardiorespiratory control, and in particular the parasympathetic control of cardiac function, may be involved [11,15–18]. Chronic fetal nicotine exposure by maternal smoking dramatically increases the risk of SIDS by 2–4 times [19,20].

2. Parasympathetic control of cardiac function

Heart rate is dominated by the activity of the cardioinhibitory parasympathetic nervous system. In conscious and anesthetized animals, there is a tonic level of parasympathetic nerve firing and little, if any, sympathetic activity at rest (humans [21], dogs [22], cats [23], rats [24,25]). During increases in arterial pressure, the initial reflex-induced slowing of the heart is caused primarily, if not exclusively, by increases in cardiac vagal nerve activity [22,25]. During decreases in arterial pressure, the baroreflex-induced tachycardia is caused mostly by decreases in parasympathetic in addition to increases in sympathetic nerve activity [18,22,26]. When both parasympathetic and sympathetic activities are present, parasympathetic activity generally dominates the control of heart rate. Increases in parasympathetic activity evoke a bradycardia that is more pronounced when there is a high level of sympathetic firing [27]. When there is a moderate or high level of parasympathetic activity, changes in sympathetic firing elicit negligible changes in heart rate [27].

3. Neurophysiology of cardiac vagal activity

The intrinsic firing properties and voltage gated currents in identified CVNs in the brainstem have recently been characterized [28–33]. These results demonstrate that in the absence of synaptic activity, CVNs in the nucleus ambiguus are normally silent. CVNs do not display any pacemaker-like activity such as repetitive or phasic depolarizations or action potentials. However, only a small depolarizing current (100 pA) is needed to evoke repetitive firing in CVNs, and this activity occurs with little delay and minimal spike frequency adaptation during any maintained depolarizing currents. The voltage gated currents and firing characteristics of CVNs enable them to follow fast synaptic drive closely as well as integrate long-lasting modulatory influences.

The activity of CVNs in the brainstem is controlled by the activation and modulation of three major synaptic inputs; glutamatergic, GABAergic and glycinergic [4,29,31–56]. Stimulation of the nucleus tractus solitarius (NTS) evokes a glutamatergic pathway that activates both NMDA and non-NMDA postsynaptic currents in CVNs [31,37]. This pathway may constitute the essential link between increases in blood pressure and afferent baroreceptor activity, which activates neurons in the NTS, and the reflex compensatory decrease in heart rate caused by increases in efferent cardioinhibitory cardiac vagal activity. Additionally, there is a GABAergic innervation of CVNs that can also be activated upon stimulation of the NTS [31,41]. Stimulation of afferents in the central end of a sectioned vagus nerve evokes both GABAergic and glutamatergic responses in CVNs [53]. Capsaicin, which inactivates C-fibers, increased the latency of the GABAergic response without changing the latency of the glutamatergic responses [53]. It is possible that this inhibitory GABAergic pathway evoked from vagal nerve or NTS stimulation is involved in patterning cardiac vagal activity which is bursting and synchronous with the cardiac cycle.

4. Mechanisms of respiratory sinus arrhythmia

While feedback from pulmonary stretch receptors, and direct respiratory-related changes in venous return and cardiac stretch can evoke respiratory-related changes in heart rate, the dominant source of respiratory sinus arrhythmia originates from the brainstem [57]. Respiratory sinus arrhythmia persists when the

lungs are stationary (caused by muscle paralysis or constant flow ventilation), and the respiratory modulation of heart rate remains synchronized with brainstem respiratory rhythms even if artificial ventilation of the lungs, and chemoreceptor activation, occurs at different intervals [18,58–61]. In both animals and humans, respiratory sinus arrhythmia is mediated via cardiac vagal activity. Respiratory sinus arrhythmia persists in animals upon sectioning sympathetic pathways, and in quadriplegic patients with spinal cord injury and sympathetic dysfunction [58–62]. Blockade of cardiac vagal activity abolishes respiratory sinus arrhythmia [63].

Cardiac vagal nerve activity has pronounced respiratory modulation. Cardiac vagal fibers fire most rapidly post inspiration and are often silent in inspiration [23,26,64]. Respiratory inputs do not seem to alter baroreceptor and chemoreceptor synapses at their first synapse in the NTS [18,26]. Rather, the little *in vivo* data that exist suggest that cardiorespiratory interactions occur within the nucleus ambiguus [2,26].

To identify the cellular mechanisms responsible for the inhibition of CVNs during inspiratory activity, GABAergic and glycinergic synaptic events were both isolated for examination. During inspiratory bursts, the frequency of both spontaneous GABAergic and glycinergic synaptic events in CVNs significantly increased [4]. Focal application of the nicotinic antagonist dihydro-beta-erythroidine (DHβE) in an α4β2 selective concentration (3μM) abolished the respiratory-evoked increase in GABAergic frequency. In contrast, the increase in glycinergic frequency during inspiration was not altered by nicotinic antagonists [4].

5. Cardiorespiratory responses to hypoxia and hypercapnia

Hypoxia and hypercapnia evoke profound cardiovascular and respiratory responses. Hypoxia initially elicits a transient increase, followed by a maintained decrease in respiratory frequency [5]. Respiration changes from the normal eupnic pattern of breathing to gasping in response to hypoxia and hypercapnia which increases the chance of autoresuscitation [5]. Gasping is characterized by a lower frequency of inspiratory bursts that are shorter in duration than eupnic inspiratory activity [65,66]. Gasping is an important component of autoresuscitation and is a highly effective gas exchange pattern [5].

6. Heart rate responses to hypoxia and hypercapnia

Both hypoxia and hypercapnia evoke a pronounced bradycardia that is mediated via increased parasympathetic activity. Studies in humans have shown that hypoxia-induced bradycardia can be blocked by atropine and are absent in heart transplant recipients [67–71]. The hypoxia-induced decrease in heart rate in experimental animals is prevented by prior application of atropine or vagotomy [72–77]. The changes in parasympathetic cardiac activity in response to hypoxia originate from the medulla since the discharge of cardiac efferent fibers in the central end of the transected vagus nerve is increased during hypoxia [78]. Similarly, hypercapnia evokes a bradycardia that is reduced by atropine [76,79]. Although peripheral chemoreceptors may also be involved, hypoxia-induced bradycardia persists after section of both the carotid sinus and aortic nerves, indicating that chemoreceptors within the central nervous system can activate pathways that increase the activity of CVNs [80–82]. The reduction in heart rate and respiratory frequency in response to hypoxia normally serves to reduce the metabolic demand of the cardiac and respiratory muscles, and thus prolong survival [83,84]. However, the bradycardia associated with sleep apnea can be detrimental and is likely mediated, in part, by increases in parasympathetic cardiac activity since atropine is partially effective in preventing the majority of arrhythmias during and after sleep apnea [85].

7. Fetal exposure to nicotine alters cardiorespiratory control and increases the risk of SIDS

Although the cause(s) for SIDS remains unknown it has been speculated that an abnormality of cardiorespiratory control, and in particular the parasympathetic control of cardiac function, may be involved [11,15–18]. Chronic fetal nicotine exposure by maternal smoking dramatically increases the risk of SIDS. Epidemiological studies indicate that smoking during pregnancy increases the risk of SIDS by 2–4 times [19,20]. Nicotine crosses the placental blood barrier and has been found in the blood and pericardial fluid of SIDS infants [86]. In infants who succumb to SIDS, a centrally mediated slowing of the heart, which precedes or accompanies apnea, is likely critically involved [11,17]. Bradycardia is also the most prevalent and predictive event in infants monitored for apparent life threatening events [14].

In animal models of SIDS, prenatal nicotine exposure augments the parasympathetic and diminishes the sympathetic control of heart rate [87]. Prenatal exposure to nicotine also exaggerated the cardiovascular responses to mild hypoxia. In control rats, hypoxia typically evokes an initial tachycardia followed by a slight decrease in heart rate, whereas in nicotine exposed animals, there was no tachycardia and heart rate declined rapidly and precipitously within

a few minutes (88). The changes in respiration were indistinguishable between the control and nicotine exposed animals, indicating that the nicotine exposed animals had an exaggerated centrally mediated increase in cardiac vagal activity in response to the respiratory stimulus (88).

The mechanisms responsible for the altered responses with prenatal nicotine exposure likely involve altered neurotransmission within the central nervous system. Numerous studies in other systems have demonstrated that chronic nicotine exposure causes an exaggerated response to subsequent acute nicotine exposures (89–91). Changes in the subtype, number and characteristics of the nicotinic receptors are likely involved. Chronic nicotine exposure has been shown to decrease the number of low affinity and increase the number and fraction of high affinity nicotinic receptors (92). The acetylcholine evoked ionic currents from these upregulated nicotinic receptors are augmented twofold or more, and are less sensitive to desensitization (92).

Since prenatal exposure to nicotine may be critical in the pathophysiology of SIDS and nicotinic receptors are essential for the inhibition of CVNs during inspiration, a recent study from this laboratory tested the hypothesis that prenatal nicotine alters hypoxia-induced changes in GABAergic and glycinergic inhibitory postsynaptic current (IPSC) frequency in CVNs (3). Hypoxia evoked a biphasic change in the frequency of both GABAergic and glycinergic IPSCs in CVNs, comprised of an initial increase, followed by a decrease in IPSC frequency. The initial inhibition of CVNs would result in a tachycardia due to a withdrawal of parasympathetic activity. The subsequent decrease in GABAergic IPSC frequency would elicit a bradycardia due to increased parasympathetic outflow to the heart.

Prenatal exposure to nicotine changed the GABAergic response to hypoxia from a biphasic response to a precipitous decrease in spontaneous GABAergic IPSC frequency. Prenatal nicotine exposure abolished the initial hypoxia-induced increase in spontaneous GABAergic IPSC frequency that occurred in unexposed animals and caused a greater and more rapid reduction in spontaneous GABAergic frequency during hypoxia (3). In addition, the enhanced increase in GABAergic IPSC frequency during inspiration caused by prenatal nicotine exposure was rapidly abolished during hypoxia (3). An exaggerated disinhibition of CVNs would induce a more rapid increase in parasympathetic outflow to the heart and a bradycardia *in vivo*.

In animals exposed to nicotine prenatally, the time course of the changes in spontaneous GABAergic IPSC frequency observed in this *in vitro* study also very closely mimic the heart rate changes observed in *in vivo* experiments using animals that have been exposed to nicotine prenatally (88). Compared to unexposed animals, animals prenatally exposed to nicotine respond to hypoxia with a greater decrease in heart rate. This impaired heart rate control reduces hypoxia tolerance in neonatal rats, and has been hypothesized to be the mechanism which accounts for the relationship between maternal smoking and SIDS (11,13,88). The enhanced hypoxia-induced withdrawal of GABAergic neurotransmission in animals exposed to nicotine prenatally provides one likely neurochemical mechanism for the substantial and potentially lethal exaggeration of the hypoxia-induced bradycardia observed in rats prenatally exposed to nicotine.

In addition, prenatal nicotine exposure alters the types of nicotinic receptors that facilitate excitatory inputs to CVNs (93). Nicotinic receptor activation of CVNs and facilitation of glutamatergic neurotransmission to CVNs is endogenously active in both unexposed animals and in animals exposed to nicotine in the prenatal period (93). Neostigmine (10 μM), an acetylcholinerase inhibitor, significantly increases the holding current, amplitude and frequency of miniature excitatory postsynaptic current (mEPSC) glutamatergic events in CVNs (93). In unexposed animals, the nicotine elicited facilitation of mEPSC frequency, but not mEPSC amplitude or inward current, is completely dependent on activation of α-7 subunit containing nicotinic receptors since the nicotine evoked increase in mEPSC frequency can be blocked by α-bungarotoxin. The nicotine mediated inward current and increase in mEPSC amplitude do not involve α4β2 nicotinic receptors since DHβE at a concentration of 3 μM, which selectively blocks α4β2 nicotinic receptors, had no effect (93,94).

Prenatal nicotine exposure significantly increases the endogenous activation of nicotinic receptors responsible for an inward current and augmentation of mEPSC frequency and amplitude in CVNs. In addition, prenatal nicotine exposure evoked both an exaggeration and change in nicotinic receptors responsible for these responses (93). In prenatal nicotine exposed animals, the increase in holding current was partially dependent on α-7 subunit containing nicotinic receptors, whereas in unexposed animals, α-bungarotoxin had no effect on the holding current responses. Furthermore, whereas in control animals, α-bungarotoxin abolished the increase in mEPSC frequency, in prenatal nicotine exposed animals, α-bungarotoxin only partially reduced the increase in mEPSC frequency (93). Therefore, prenatal nicotine exposure elicits the postsynaptic expression of α-7 subunit containing nicotinic receptors in CVNs, and nicotinic receptors other than α-7 nicotinic receptors are expressed at presynaptic glutamatergic terminals and can facilitate glutamatergic neurotransmission to CVNs. These results indicate that prenatal nicotine

exposure elicits an increase in the responses and alters the types of nicotinic receptors involved in the facilitation of glutamatergic neurotransmission to CVNs. As discussed above, CVNs are strongly modulated by respiratory inputs originating from the medulla, but the mechanisms by which central hypoxia and hypercapnia excite CVNs are unknown.

8. A single episode of hypoxia does not elicit any change in glutamatergic inputs but intermittent episodes incrementally recruits an excitatory glutamatergic pathway to CVNs

To characterize the respiratory-related synaptic responses in CVNs, we have developed an *in vitro* preparation that allows us to characterize synaptic inputs to CVNs while simultaneously recording endogenous rhythmic respiratory activity. In an initial operation, rats (2 days old) are anesthetized and the retrograde fluorescent tracer is applied to the terminals of CVNs surrounding the heart. After 1–3 days recovery, the animals are re-anesthetized and sacrificed by cervical dislocation. The brainstem is isolated and a transverse slice (600–800 μ thick) is obtained which generates spontaneous rhythmic respiratory activity. This preparation captures the pre-Botzinger complex, a region that is essential for the generation of the respiratory rhythm. Also contained within this preparation are the CVNs, identified by the retrograde fluorescent tracer, and the hypoglossal motor nucleus. Once the CVNs are identified, they are then imaged with differential interference contrast (DIC) optics, infrared illumination and cooled CCD cameras to gain better spatial resolution and to visually guide and position the patch pipette onto the surface of the identified neurons. The output of the hypoglossal neurons can be recorded as motor neuron population activity from rootlets of the XII nerve which are rhythmically active in inspiration and simultaneous with C4 spinal-phrenic nerve respiratory activity [95,96]. The inspiratory XII activity is also rectified, low pass filtered and integrated using an electronic filter (Paynter filter, time constant of 25 msec) to better define the duration and magnitude of inspiration.

Under control conditions, there is no respiratory modulation of glutamatergic neurotransmission to CVNs. This indicates that respiratory sinus arrhythmia, which is mainly due to decreased parasympathetic cardiac vagal activity during inspiration, is most likely caused by inspiratory evoked inhibition, rather than post-inspiratory or expiratory evoked excitation of CVNs. Similarly, single exposures to hypoxia, of either 5 or 15 minutes' duration, do not alter glutamatergic neurotransmission to CVNs.

However, intermittent episodes of hypoxia incrementally recruit excitatory glutamatergic neurotransmission to CVNs that occurs during respiratory bursts. During the second episode of hypoxia (Hypoxia 2), the frequency of glutamatergic neurotransmission to CVNs increased during the inspiratory bursts. The third exposure to hypoxia elicited a dramatic increase in EPSC frequency in CVNs during inspiratory activity, Hypoxia 2 and 3.

9. Prenatal exposure to nicotine exaggerates respiratory-related excitatory pathway to CVNs during both hypoxia and combined hypercapnia/hypoxia

The respiratory responses to hypoxia/hypercapnia in animals exposed to nicotine in the prenatal period closely mimicked and were not significantly different from the responses in unexposed animals. This is consistent with work from other investigators that have shown that the frequency and characteristics of the respiratory activity, and responses to hypoxia, were very similar if not indistinguishable between control and nicotine exposed animals [97]. Prenatal nicotine exposure did not alter the ventilatory response to hypoxia or hypercapnia in rats from 3 to 34 days old [98], and infants of smoking mothers did not have different ventilatory responses to hypoxia or hypercapnia, but these infants did have diminished arousal to hypoxia [99].

However, the responses in CVNs to hypoxia/hypercapnia are greatly exaggerated in prenatal nicotine exposed animals. A single episode of hypoxia/hypercapnia does not elicit an increase in excitatory neurotransmission to CVNs in unexposed animals, but in animals exposed to nicotine in the prenatal period, a single period of hypoxia/hypercapnia recruits an excitatory neurotransmission to CVNs. Similar results have been obtained in prenatal nicotine exposed animals with a single exposure to hypoxia.

These results strongly complement recent work that examined whether prenatal nicotine exposure alters inhibitory neurotransmission to CVNs during hypoxia. In unexposed animals, hypoxia evokes a biphasic change in the frequency of both inhibitory GABAergic and glycinergic synaptic events in CVNs, comprised of an initial increase followed by a decrease in IPSC frequency [3]. Prenatal exposure to nicotine changed the GABAergic response to hypoxia from a biphasic response to a precipitous decrease in spontaneous GABAergic IPSC frequency [3]. These results taken together would predict a much stronger excitation of CVNs during hypoxia and/or hypercapnia from animals exposed to nicotine in the prenatal period.

10. Nicotinic receptors responsible for facilitating this excitatory pathway

The mechanisms responsible for the altered responses with prenatal nicotine exposure likely involve altered glutamatergic neurotransmission within the central nervous system. Prenatal nicotine exposure has been shown previously to exaggerate the facilitation of glutamatergic neurotransmission to CVNs and also change the types of presynaptic and postsynaptic nicotinic receptors involved in exciting premotor CVNs [93]. Changes in the subtype, number and characteristics of the nicotinic receptors are likely involved. Chronic nicotine exposure has been shown to decrease the number of low affinity and increase the number and fraction of high affinity nicotinic receptors [92]. The acetylcholine evoked ionic currents from these upregulated nicotinic receptors are augmented twofold or more compared to control currents, and are less sensitive to desensitization [92]. In the presence of DHβE at a concentration which blocks all subtypes of nicotinic receptors (100 μM), hypoxia/hypercapnia did not increase glutamatergic neurotransmission to CVNs during inspiratory activity.

11. Tempol, a superoxide dismutase mimetic, abolishes recruitment of this excitatory neurotransmission in response to intermittent hypoxia

Episodic periods of hypoxia and hypercapnia evoke different cardiorespiratory responses than the responses to a single period of hypoxia or hypercapnia, despite equal total durations of exposure. For example, three or more 5-minute periods of hypoxia evokes respiratory long-term facilitation which is a long-lasting increase in ventilation or respiratory activity, whereas exposure to similar protocols of intermittent hypercapnia has been reported to elicit long-term depression [100,101]. One of the major differences between intermittent and continuous hypoxia is that the episodic reoxygenation with intermittent hypoxia increases generation of reactive oxygen species (ROS), especially oxygen free radicals [102].

ROS are generated by all mammalian cells as by-products of metabolism or apoptotic signals and by some cells in response to noxious stimuli. In general, production of ROS is associated with deleterious effects in pathophysiologic conditions, including inflammatory responses, apoptosis, or ischemia/reperfusion. For example, free radical production is associated with increased injury following intermittent fetal hypoxia–reoxygenation in fetuses of near-term pregnant rabbits [103]. Administration of antioxidants resulted in less brain edema and cell death [103]. In a growing number of systems, however, generation of ROS is useful and even required by physiologic systems. For example, recent evidence suggests that ROS are involved in signaling by angiotensin II in central autonomic networks [104], and may be involved in the pathogenesis of hypertension and the activation of the sympathetic nervous system [105–107]. Superoxide anions in the rostral ventrolateral medulla are increased in stroke-prone spontaneously hypertensive rats and may contribute to the neural mechanisms of hypertension in these animals [108].

To examine whether the generation of ROS is involved in the incremental recruitment of an excitatory pathway to CVNs during intermittent hypoxia, we included the cell-permeant superoxide dismutase mimetic 4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl (tempol, 1 mM) in the perfusate. Addition of tempol prevented the recruitment of excitatory glutamatergic neurotransmission to CVNs that occur during respiratory bursts with intermittent hypoxia in the absence of tempol.

12. One likely site of oxygen free radical production is the ventrolateral medulla

To localize the production of oxygen free radicals, we utilized a cell-permeant indicator, 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA), for ROS which is colorless and nonfluorescent until oxidized back to the fluorophore by ROS. The oxidant-sensing probe CM-H_2DCFDA is de-esterified within cells by endogenous esterases to the ionized free acid, 2′,7′-dichlorodihydrofluorescein. 2′,7′-dichlorodihydrofluorescein is nonfluorescent until intracellular oxidation by oxygen free radicals produces the fluorescent 2′,7′-dichlorodihydrofluorescein (H_2DCF). H_2DCF is a standard and commonly used dye for measuring the generation of ROS and has been used previously to localize and examine the production of ROS in *in vitro* neuronal slices [109–111].

Brainstem slices were incubated in control artificial cerebral spinal fluid containing CM-H_2DCFDA (10 μM) for 60 minutes to allow cellular esterases to hydrolyze the acetate group and render the dye responsive to oxidation. H_2DCF fluorescence was measured throughout intermittent hypoxia using the same protocols as in electrophysiological experiments and images were obtained every 10 seconds using excitation and emission wavelengths of 480 nm and 520 nm, respectively. Intermittent hypoxia incrementally increased H_2DCF fluorescence in individual neurons within the ventrolateral medulla. Tempol nearly completely blocked the increase in H_2DCF fluorescence.

13. Summary and future research

We have developed a novel brainstem preparation that spontaneously generates respiratory rhythms and responds to hypoxia and hypercapnia similarly to *in vivo* preparations in which we can examine the changes in synaptic activity in fluorescently identified CVNs during hypoxia, hypercapnia and combined hypoxia/hypercapnia. Our preliminary results indicate that while a single episode of hypoxia does not elicit any change in glutamatergic activity, intermittent episodes of hypoxia incrementally recruits an excitatory glutamatergic neurotransmission to CVNs that occurs during respiratory bursts. Prenatal nicotine exposure augments the excitatory pathway evoked by hypoxia and combined hypoxia/hypercapnia, and furthermore nicotinic receptors are involved in facilitating this excitatory pathway. The recruitment of this excitatory pathway to CVNs likely involves generation of ROS since tempol, a superoxide dismutase mimetic, abolishes the recruitment of this excitatory neurotransmission in response to hypoxia. Localization of the production of oxygen free radicals using probes that become fluorescent when oxidized by oxygen free radicals indicate that one site of oxygen free radical production are neurons in the ventrolateral medulla.

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